Presynaptic $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptors Increase Glutamate Release and Serotonin Neuron Excitability in the Dorsal Raphe Nucleus

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Several behavioral effects of nicotine are mediated by changes in serotonin (5-HT) release in brain areas that receive serotonergic afferents from the dorsal raphe nucleus (DRN). In vitro experiments have demonstrated that nicotine increases the firing activity in the majority of DRN 5-HT neurons and that DRN contains nicotinic acetylcholine receptors (nAChRs) located at both somata and presynaptic elements. One of the most common presynaptic effects of nicotine is to increase glutamate release. Although DRN receives profuse glutamatergic afferents, the effect of nicotine on glutamate release in the DRN has not been studied in detail. Using whole-cell recording techniques, we investigated the effects of nicotine on the glutamatergic input to 5-HT DRN neurons in rat midbrain slices. Low nicotine concentrations, in the presence of bicuculline and tetrodotoxin (TTX), increased the frequency but did not change the amplitude of glutamate-induced EPSCs, recorded from identified 5-HT neurons. Nicotine-induced increase of glutamatergic EPSC frequency persisted 10–20 min after drug withdrawal. This nicotinic effect was mimicked by exogenous administration of acetylcholine (ACh) or inhibition of ACh metabolism. In addition, the nicotine-induced increase in EPSC frequency was abolished by blockade of $\alpha 4\beta 2$ nAChRs, voltage-gated calcium channels, or intracellular calcium signaling but not by $\alpha 7$ nAChR antagonists. These data suggest that both nicotine and endogenous ACh can increase glutamate release through activation of presynaptic $\alpha 4\beta 2$ but not $\alpha 7$ nAChRs in the DRN. The effect involves long-term changes in synaptic function, and it is dependent on voltage-gated calcium channels and presynaptic calcium stores.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels with a pentameric structure, composed of α and non- α (β) subunits. The existence of nine types of α subunits and three types of β subunits indicates a high structural and functional diversity of neuronal nAChRs, a conclusion supported by experimental studies (Wu and Lukas, 2011). However, it is known that homologous $\alpha 7$ and heterologous $\alpha 4\beta 2$ nAChRs are more commonly expressed in the CNS (Whiting et al., 1987; Flores et al., 1992; Séguéla et al., 1993). Several studies have demonstrated that neuronal nAChRs have a preferential presynaptic location and act to increase the release of a large number of neurotransmitters (Wonnacott, 1997; Sher et al., 2004; Jensen et al., 2005).

Cholinergic nicotinic signaling is involved in a variety of processes, including anxiety, depression, arousal, memory,

and attention among others (Hogg et al., 2003; Picciotto, 2003). Conversely, behavioral studies have suggested that certain effects of nicotine are mediated by increases in serotonin (5-HT) release in cortical and subcortical areas (Seth et al., 2002). Therefore, understanding the actions of nicotine on the dorsal raphe nucleus (DRN) and particularly its effects on 5-HT neurons is of broad scientific and clinical significance.

Immunocytochemical studies have demonstrated that both 5-HT and non-5-HT DRN neurons express postsynaptic nAChRs of $\alpha 4\beta 2$ and $\alpha 7$ subtypes (Bitner et al., 2000; Bitner and Nikkel, 2002; Commons, 2008). Also, immunolabeling for the $\alpha 4$ receptor subunit has been detected in axon terminals in the DRN (Commons, 2008). Data obtained in our laboratory revealed that postsynaptic nAChRs of 5-HT and non-5-HT DRN neurons are functional (Galindo-Charles et al., 2008) and that nicotine increases the firing frequency of ~80% of 5-HT DRN neurons (Mihailescu et al., 1998, 2002). Accordingly, a recent study has demonstrated the presence of functional somatic and presynaptic nAChRs in DRN neurons that project to the nucleus accumbens (Chang et al., 2011). Nicotine stimulatory effect on 5-HT DRN neurons has been explained previously through presynaptic release of noradrenaline from afferents originating in locus coeruleus (Li et al., 1998). However, DRN also receives glutamatergic afferents from cortical and subcortical areas and contains a significant number of glutamatergic interneurons (Soiza-Reilly and Commons, 2011). In addition, 5-HT DRN neurons express both

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ionotropic and metabotropic postsynaptic glutamatergic receptors (Celada et al., 2001; Kawashima et al., 2005). One of the most common presynaptic effects of nicotine is to increase glutamate release (McGehee et al., 1995). Taking into account these data, we hypothesized that nicotine increases glutamate release in the DRN, which explains in part the nicotinic stimulatory effects on the firing rate of 5-HT DRN neurons reported previously (Mihailescu et al., 1998, 2002; Chang et al., 2011).

The results of the present study demonstrate that both nicotine and endogenous acetylcholine (ACh) increase the release of glutamate in the DRN through activation of presynaptic $\alpha 4\beta 2$ but not $\alpha 7$ nAChRs. This effect involves long-term changes in DRN glutamatergic synapses by a mechanism dependent on voltage-gated calcium channels (VGCCs) and Ca^{2+} -induced Ca^{2+} release (CICR) from presynaptic calcium stores but independent of action potentials (APs).

Materials and Methods

Preparation of slices. All procedures were performed in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care Committee of the National Autonomous University of Mexico. The experiments were performed on brain slices obtained from postnatal days 21–25 male Wistar rats. Animals were deeply anesthetized with isoflurane and decapitated. Their brains were quickly removed and placed into ice-cold (4°C) bath (external) saline containing the following: 125 mM NaCl, 3 mM KCl, 25 mM NaHCO_3 , 1.25 mM Na_2HPO_4 , 1 mM MgCl_2 , 1.2 mM CaCl_2 , and 25 mM glucose, 300 mOsm/L, pH 7.3 by bubbling with 95% O_2 and 5% CO_2 . Coronal midbrain slices containing DRN (350 μm thick) were cut using a vibratome (Vibratome 1500) and stored in oxygenated bath saline at room temperature for at least 1 h before recording.

Whole-cell recordings. Individual slices were transferred into a custom-made Plexiglas recording chamber and superfused with saline at a rate of 4–5 ml/min at 33°C. Whole-cell current- and voltage-clamp recordings were performed with a Multiclamp 700A amplifier (Molecular Devices) and monitored with a personal computer running Clampex 8 software (Molecular Devices). The micropipettes used for recordings were pulled from borosilicate glass tubes (WPI) with a Flaming-Brown puller (Sutter Instruments). The internal solution consisted of the following: 140 mM K-gluconate, 5 mM NaCl, 1 mM MgCl_2 , 0.02 mM EGTA, 10 mM HEPES, 2 mM $\text{Mg}_2\text{-ATP}$, 0.5 Na_2GTP , and 0.1% biocytin, pH 7.2–7.3 with Trizma base, 280–300 mOsm/L. In some experiments, we added 1–2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA; 10 mM) in the same internal solution but with 130 mM K-gluconate. The direct current impedance of the electrodes varied between 4 and 7 M Ω . Experimental data were stored in a personal computer using a Digidata 1322A analog-to-digital converter (Molecular Devices), at a sampling rate of 5 kHz. Individual neurons were visualized using an infrared video-microscopy system (BX51WI; Olympus Instruments) endowed with a 60 $\times$ water-immersion objective. Neurons selected for recording were located close to the midline area of the DRN, identified as the translucent area between the medial longitudinal fasciculus and the aqueduct. Each recorded cell was identified based on its electrophysiological characteristics by generating a stimulus–response curve.

Recording of EPSCs. Spontaneous EPSCs (sEPSCs) were recorded continuously in whole-cell voltage-clamp mode at a holding potential of -70 mV. All the experiments were made in the presence of bicuculline (10 μM) to block GABA $_A$ receptors. Nicotine or ACh were applied in the bath solution during 5 min to attain concentration equilibrium. Thereafter, the drugs were washed out, and a recording period of at least 30 min was taken for analysis. Access resistance was monitored by using depolarizing step pulses of 5 mV and 50 ms duration. When access resistance varied $>15\%$, the experiment was discarded. In some experiments, concentric bipolar tungsten electrodes (FHC) (12 μm pencil tip) were placed just outside the boundaries of the DRN and ventral to the aqueduct. Field stimulation was made while recording from 5-HT DRN neurons. In these experiments, low stimulus intensities (subthreshold stimuli) were

Figure 1. Electrophysiological characteristics of the DRN serotonergic neurons. **A**, Current traces in response to hyperpolarizing and depolarizing current steps recorded from a 5-HT neuron. Inset, An expanded AP from the same neuron. **B**, Plots of discharge frequency as a function of injected current obtained from 13 identified 5-HT neurons are shown. Filled circles represent the instantaneous frequency obtained with the reciprocal of the first interspike interval. Open circles represent the frequency obtained from the last interspike interval. **C**, Microphotographs illustrating the recorded cell in **A**, after biocytin staining (top), 5-HT immunoreactivity (middle), and merge (bottom).

used to avoid the activation of action currents. Paired subthreshold stimuli were delivered at 0.1 Hz with an interstimulus interval of 50 ms. Stimuli were applied before and after nicotine and in the presence of bicuculline. An isolation unit (Digitimer) was used to adjust the stimulus parameters during the experiments.

Immunocytochemistry. Neurons were filled with biocytin during recordings. A combination of intracellular labeling and 5-HT immunocytochemistry was used to determine the serotonergic nature of the recorded neuron. Slices containing injected neurons were fixed overnight with 4% paraformaldehyde and 1% picric acid in 0.1 M PBS, pH 7.4. The slices were then infiltrated with 30% sucrose and cut on a vibratome into 40 μm sections. The

sections were incubated 4–6 h in PBS solution containing 0.2% Triton X-100 and streptavidin conjugated to Cy3 (1 mg/ml; diluted 1:100; Zymed) to label the recorded neuron. Sections were rinsed in PBS and incubated for 18–24 h at 4°C with primary rabbit anti-5-HT antisera (diluted 1:2000; ImmunoStar). After rinsing in PBS, sections were reincubated for 2 h with secondary antibodies conjugated to fluorescein (diluted 1:100; Vector Laboratories). The reacted sections were first examined with an appropriate set of filters on an epifluorescence-equipped microscope. Afterward, the sections were mounted in an anti-quenching media (Vectashield; Vector Laboratories) and examined under a confocal microscope (MRC 1024; Bio-Rad) equipped with a krypton/argon laser. A two-lines laser emitting at 550 and 500 nm wavelength was used for exciting Cy3 and fluorescein, respectively. Digitized images were transferred to a personal computer by using the image-capturing software (Confocal Assistant, T. C. Brejle, Minneapolis, MN). Omission of primary antisera resulted in no detectable signal (data not shown).

Drugs. Drugs were dissolved into the bath saline from daily-made stock solutions and administered using a gravity-driven superfusion system. The time required for obtaining equilibrated concentrations of the drugs in the recording chamber was 3–4 min. TTX, DL-2-amino-5-phosphonovaleric acid, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), (–)bicuculline methiodide, methyllycaconitine (MLA), dihydro- β -erythroidine hydrobromide (DH β E), atropine, BAPTA, BAPTA-AM, cadmium chloride (CdCl₂), and biocytin were purchased from Sigma/RBI. Thapsigargin, cyclopiazonic acid (CPA), and ryanodine were purchased from Tocris Bioscience. ω -Agatoxin-TK was obtained from Peptide Institute, and ω -conotoxin-GVIA was obtained from Alomone Labs. All reagents were added from freshly prepared stock solution to the bath saline.

Data analyses. Offline analysis of the data was performed using Clampfit 8 (Molecular Devices), Mini Analysis (Synaptosoft), and graphing and statistical software packages (Origin version 6, Microcal; and Systat). Data are expressed as means $\pm$ SEM. Statistical comparisons between samples were made by using the Student's *t* test (*p* < 0.05 was taken as significant).

Results

5-HT neurons were identified based on their electrophysiological properties. Figure 1*A* shows APs elicited by increasing depolarizing current steps recorded from an identified 5-HT neuron (Fig. 1*C*). As previously reported (Penington et al., 1991; Bayliss et al., 1997; Galindo-Charles et al., 2008) when positive direct current is injected, 5-HT neurons exhibit repetitive firing with adaptation of the firing frequency. 5-HT neurons also exhibit prolonged afterhyperpolarizing potentials, long AP durations ($\sim$ 4 ms at the base), and an inflection or “shoulder” during AP descending phase (Vandermaelen and Aghajanian, 1983; Beck et al., 2004; Marinelli et al., 2004; Galindo-Charles et al., 2008) (Fig. 1*A*, inset). Figure 1*B* illustrates the firing frequency as a function of injected current (*I*–*F* plot) calculated at the initial and final interspike intervals. At the steady state, 5-HT neurons maximal firing rate was 6.7 ± 0.7 Hz, whereas

Figure 2. Low concentrations of nicotine enhance glutamatergic synaptic transmission. *A*, sEPSCs recorded by using whole-cell patch-clamp technique (voltage-clamp mode) in the presence of bicuculline (10 μ M). The traces show 10 s taken in control conditions (left), after nicotine (300 nM) added to the bath solution (middle), and after 20 min wash (right). A region of the middle trace (dashed box) is shown below at a slower sweep time. Two of the synaptic events are indicated by asterisks. *B*, Time-frequency histogram (10 s bin) for the same cell in *A*, showing that nicotine increased the frequency but did not change the amplitude of sEPSCs (inset). *C*, Summary of the results from five neurons showing the effect on sEPSCs frequency in the presence of nicotine ($*p < 0.05$). *D*, Microphotographs illustrating the recorded cell in *A* after biocytin staining (left), 5-HT immunoreactivity (middle), and merge (right).

non-5HT neurons reach frequencies >25 Hz (Galindo-Charles et al., 2008). All the data were obtained from neurons identified as 5-HT positive by immunocytochemistry (Fig. 1*C*), bringing additional support to electrophysiological identification. This study was focused on the effects of nicotine on 5-HT DRN neurons. Therefore, when a pattern of electrophysiological activity characteristic of non-5-HT neurons was detected, the respective cell was discarded.

Nicotine increased the frequency of sEPSCs recorded from 5-HT DRN neurons

Under voltage-clamp conditions, low concentrations of nicotine were applied to slices containing the DRN. sEPSCs were recorded from 5-HT neurons at a holding potential of -70 mV (Fig. 2*A*). All the experiments were made in the presence of bicuculline (10 μ M). Nicotine (300 nM) increased the frequency but did not change the amplitude of sEPSCs (Fig. 2*A,B*). The effect of nicotine persisted for $\sim$ 15 min after drug withdrawal, and it was seen in five of seven 5-HT-positive cells tested (Fig. 2*D*). sEPSCs fre-

Figure 3. Nicotinic effects are mimicked by endogenous ACh. **A**, Traces showing sEPSCs recorded from a 5-HT-positive neuron in control, in the presence of nicotine, 10 min after nicotine washout, and 10 min after adding CNQX (10 μ M). **B**, **C**, Cumulative probability distributions of frequencies and amplitudes for the same neuron in **A**, in control (dark gray traces), nicotine (black traces), and 10 min wash (light gray traces). **D**, Average normalized time–frequency histogram from 16 5-HT neurons tested with nicotine (1 μ M). Inset, Normalized time–frequency histogram from five 5-HT neurons tested with eserine (10 μ M). **E**, Summary of the results showing the effect on sEPSC frequency with nicotine alone, nicotine in the presence of TTX, ACh, and eserine. All experimental groups were compared against control (before drug application). The last three bars represent the wash time after nicotine alone. All the experiments were done in the presence of bicuculline (10 μ M). ACh experiments were done in the presence of bicuculline and atropine (10 μ M) (* p < 0.05, ** p < 0.01).

quency increased $40 \pm 8\%$ (p < 0.05, n = 5) with respect to the baseline (Fig. 2C).

To have more consistent and robust responses, we used 1 μ M nicotine in subsequent experiments. At this concentration, nicotine also increased sEPSCs frequency (Fig. 3A,B), and the effect was observed in 16 of 18 5-HT-positive neurons tested. Blocking the AMPA/kainic acid receptors with 10 μ M CNQX abolished the sEPSCs completely, demonstrating their glutamatergic nature. Nicotine did not change sEPSCs amplitude (Fig. 3C), suggesting a presynaptic action mechanism. The averaged time–frequency histogram of 16 cells is shown in the Figure 3D. sEPSC frequency was increased $88 \pm 20\%$ (Fig. 3E) with respect to the baseline (p < 0.01, n = 16), and the effect extended 20 min beyond the drug administration period (Fig. 3D,E). ACh (1 mM) had similar effects to nicotine (Fig. 3E): sEPSC frequency increased $66 \pm 10\%$ by ACh in 10 of 14 5-HT-positive neurons (p < 0.01, n = 10).

To investigate whether endogenous ACh was able to mimic the effect of nicotine and external ACh, we applied eserine, an acetylcholinesterase inhibitor. Eserine (10 μ M) also increased the sEPSCs frequency in five of seven 5-HT-positive cells (Fig. 3D, inset, E). sEPSC frequency was increased $40 \pm 28\%$ with

respect to the baseline (p < 0.05). In these experiments, sEPSC frequency returned to baseline much faster than with nicotine, suggesting that acetylcholinesterase is regulating a constant cholinergic tone. Eserine effect lasted ~ 8 min after drug washout (Fig. 3D, inset). The AP dependence of nicotinic effect was assessed by adding TTX (500 nM) to the perfusion solution. In the presence of TTX, nicotine (1 μ M) still enhanced the frequency of sEPSCs in 11 of 12 5-HT cells tested (Fig. 3E). sEPSC frequency was increased $80 \pm 10\%$ with respect to the baseline (p < 0.01, n = 11). This increase was not statistically different from the one produced by nicotine alone.

The enhancement of glutamate-induced sEPSCs is mediated by $\alpha 4\beta 2$ nAChRs

Immunocytochemical studies have revealed the presence of $\alpha 7$ and $\alpha 4\beta 2$ nAChRs within the DRN (Bitner et al., 2000; Bitner and Nikkel, 2002). In an attempt to identify the nAChR subtype mediating the effect of nicotine, we tested the selective nAChR antagonists MLA and DH β E. Blocking the $\alpha 7$ nAChRs with MLA (100 nM) did not prevent the increase of glutamate sEPSC frequency induced by nicotine (1 μ M) (Fig. 4A,B). Nicotine increased sEPSC frequency but did not change the amplitude (data not shown) in 10 of 11 5-HT-positive cells. sEPSC frequency was increased $82 \pm 27\%$ with respect to the baseline (p < 0.005, n = 10) in the presence of MLA. In contrast, the application of the selective $\alpha 4\beta 2$ nAChR antagonist DH β E (100 nM) completely blocked the effect of nicotine in all the 5-HT-positive cells tested (Fig. 4C,D). The time–frequency histogram (Fig. 4D) illustrates the lack of effect of nicotine in the presence of DH β E. However, DH β E by itself decreased the sEPSC frequency compared with the control (2.6 ± 0.7 Hz in control; 1.4 ± 0.3 Hz in DH β E; n = 5; p < 0.05; data not shown), which supports the presence of a cholinergic tone within the DRN.

To further investigate the identity of the nAChR subtype involved in nicotine-induced glutamate release, we used $\alpha 7$ and $\alpha 4\beta 2$ nAChR-selective agonists. The selective $\alpha 4\beta 2$ nAChR agonist RJR-2403 [(*E*)-*N*-methyl-4-pyridin-3-ylbut-3-en-1-amine] (100 nM) mimicked the effect of nicotine and ACh; it increased the glutamate sEPSCs frequency in five out of six 5-HT cells. However, the effect disappeared 5 min after removing the drug from the bath. sEPSC frequency was increased $56 \pm 17\%$ with respect to the baseline (Fig. 5A,B; p < 0.05, n = 5). Conversely, the selective $\alpha 7$ nAChR agonist PNU-282987 (*N*-[(3*R*)-1-azabicyclo[2.2.2]octan-3-yl]-4-chlorobenzamide hydrochloride) (100 nM) did not change the glutamate sEPSC frequency in any of the five 5-HT-positive cells tested (Fig. 5C,D). These data sug-

gest that glutamate release in the DRN is presynaptically modulated through high-affinity $\alpha 4\beta 2$ nAChRs.

Buffering presynaptic intracellular calcium abolished the effect of nicotine

Recently, it has been shown that DRN neurons possess functional $\alpha 7$ and $\alpha 4\beta 2$ nAChRs (Galindo-Charles et al., 2008). Therefore, we could not discard a possible contribution of postsynaptic nAChRs linked to the nicotinic effect observed in this study. To test this possibility, in a group of five 5-HT-positive cells, we buffered the postsynaptic Ca^{2+} by loading the patch pipette with an internal solution containing BAPTA (10 mM). Intracellular BAPTA had no effect on either spontaneous synaptic activity or sEPSC frequency enhancement induced by nicotine. sEPSCs frequency was increased $102 \pm 2\%$ with respect to the baseline (Fig. 6*Aa*; $p < 0.05$). On the contrary, nicotine failed to increase the sEPSC frequency when slices were perfused for 30 min with the membrane-permeable Ca^{2+} chelator BAPTA-AM (100 μM) in all the five cells tested (Fig. 6*Ab*). As expected, BAPTA-AM itself decreased the frequency of spontaneous synaptic activity. The sEPSC frequency decreased by $36 \pm 0.8\%$ after BAPTA-AM with respect to control (data not shown). A summary of these data is shown in Figure 6*Ac*.

In a set of experiments, we used paired pulses (50 ms interval, 100 μs duration) applied through a bipolar concentric electrode (12 μm tip diameter) placed in the boundaries of the DRN, just below the aqueduct as illustrated in Figure 6*Ba*. In six of eight 5-HT-positive cells, nicotine increased the amplitude of the first response (Fig. 6*Bb*, top, *Bc*, top) and decreased the number of failures to the first stimulus (Fig. 6*Bb*, bottom). Nicotine also decreased the paired-pulse ratio (Fig. 6*Bc*, bottom). Paired-pulse ratio was 0.94 ± 0.09 and 0.58 ± 0.05 in control and nicotine, respectively ($p < 0.001$, $n = 6$). These results reinforce the idea that nicotine-induced increase in glutamate sEPSC frequency depends on presynaptic nAChRs.

Blocking the VGCCs prevented nicotine-induced synaptic potentiation

It is well known that nAChRs are permeable to Ca^{2+} (Tsuneki et al., 2000; Fucile, 2004). Also, it has been shown that VGCCs are associated with nicotinic effects mediated through $\beta 2$ -subunit containing nAChRs (Tsuneki et al., 2000; Shoop et al., 2001; Dajas-Bailador et al., 2002; Dajas-Bailador and Wonnacott, 2004). Therefore, an increase of presynaptic Ca^{2+} via $\alpha 4\beta 2$ could lead to the opening of VGCCs and facilitate glutamate release. To assess this possibility, we tested the effect of nicotine in the presence of CdCl_2 (100 μM). Surprisingly, nicotine still increased the glutamate sEPSC frequency in the presence of cadmium in all six 5-HT-positive cells tested (Fig. 7*A*, *D*). Moreover, the nicotine-induced enhancement of sEPSC frequency in the presence of cadmium was $351 \pm 30\%$ ($p < 0.01$, $n = 6$) (Fig. 7*D*), a value that is

Figure 4. Nicotinic effects are performed through $\beta 2$ -containing nAChRs. **A**, Traces showing sEPSCs recorded from a 5-HT-positive neuron in the presence of MLA (100 nM, top), MLA plus nicotine (1 μM , middle), and nicotine washout (bottom). **B**, Time–frequency histograms from 10 identified 5-HT neurons tested with nicotine (1 μM) in the presence of MLA. The inset shows the normalized sEPSC frequency. **C**, Traces showing sEPSCs recorded from a 5-HT-positive neuron in the presence of DH β E (100 nM, top), DH β E plus nicotine (1 μM , middle), and nicotine washout (bottom). **D**, Time–frequency histogram from 12 identified 5-HT neurons tested with nicotine (1 μM) in the presence of DH β E. The inset shows the normalized sEPSC frequency. In all the experiments, nicotine was applied after 10 min pretreatment with DH β E or MLA.

much higher when compared with the effect of nicotine alone (see Fig. 3*E*). This effect could be the result of a potentiation of nAChRs produced by cadmium (Hsiao et al., 2001). Cadmium could elevate Ca^{2+} concentrations at glutamate terminals by enhancing Ca^{2+} influx through nAChRs. In these conditions, nicotine might be able to increase glutamate release even if VGCCs were blocked. Therefore, we decided to use selective Ca^{2+} channel blockers to see whether the effect of nicotine was dependent on VGCCs. A mixture containing ω -agatoxin-TK (400 nM), ω -conotoxin-GVIA (500 nM), and nifedipine (10 μM) was applied during 15 min in the bath solution to block P/Q-, N-, and L-type Ca^{2+} channels, respectively. In these conditions, the administration of nicotine (1 μM) failed to produce significant sEPSC frequency changes (Fig. 7*B*, *D*). sEPSC frequency was 0.8 ± 0.2 and 0.68 ± 0.15 Hz in the presence of the VGCC blockers and VGCC blockers plus nicotine, respectively. These values were not statistically significant ($p > 0.05$, $n = 6$; Fig. 7*D*).

Depleting intracellular calcium stores abolished nicotine-induced sEPSCs

To explore whether CICR contributes to nicotine-induced synaptic potentiation, we used thapsigargin and CPA, two blockers of the sarcoplasmic/endoplasmic reticulum calcium ATPase pump (SERCA). Thapsigargin (10 μM) completely abolished the effect of nicotine in five of the 5-HT-positive cells tested. The

Figure 5. Nicotinic effects are mimicked by the $\alpha 4\beta 2$ nAChR-selective agonist RJR-2403. **A**, Traces showing sEPSCs recorded from a 5-HT-positive neuron in control (top), in the presence of RJR-2403 (100 nM, middle), and RJR-2403 washout (bottom). **B**, Time–frequency histogram from five identified 5-HT neurons tested with RJR-2403. The inset shows the normalized sEPSC frequency. **C**, Traces showing sEPSCs recorded from another 5-HT-positive neuron in control (top), in the presence of the selective $\alpha 7$ nAChR agonist PNU-282987 (100 nM, middle), and PNU-282987 washout (bottom). **D**, Time–frequency histograms from five identified 5-HT neurons tested with PNU-282987. The inset shows the normalized sEPSC frequency.

time–frequency histogram in Figure 7C illustrates the lack of effect of nicotine in the presence of thapsigargin. sEPSC frequency was 0.7 ± 0.3 and 0.58 ± 0.2 Hz in the presence of thapsigargin alone and thapsigargin plus nicotine, respectively. These values were not statistically significant ($p > 0.05$, $n = 5$). CPA (10 μ M) also blocked the nicotine-induced enhancement of sEPSC frequency. Frequency values were 1.9 ± 0.3 and 1.76 ± 0.35 Hz in the presence of CPA alone and CPA plus nicotine, respectively ($p > 0.05$, $n = 5$). Additional evidence for CICR involvement was obtained by applying nicotine on neurons pretreated (15 min) with ryanodine (100 μ M) to block stored calcium. sEPSC frequency was 0.56 ± 0.3 and 0.4 ± 0.24 Hz in the presence of ryanodine alone and ryanodine plus nicotine, respectively ($p > 0.05$, $n = 4$). Figure 7D shows a summary of the effect of nicotine in the presence of CdCl_2 , Ca^{2+} channel blockers, thapsigargin, CPA, or ryanodine, on the normalized sEPSCs ($p < 0.01$). These results indicate that CICR is necessary for nicotine-induced glutamate release in the DRN.

Finally, Figure 8 shows a model summarizing the mechanisms by which nicotine induces a long-term increase in glutamate release in the DRN. The DRN receives cholinergic inputs originating in the pedunculopontine (PPT) and laterodorsal tegmental (LDT) nuclei (Woolf and Butcher, 1989). This model compares the physiological conditions in which only the spontaneous release of ACh is

present (Fig. 8A) with the condition when nicotine is also present (Fig. 8B).

Discussion

The main finding of this study was that the activation of $\alpha 4\beta 2$ nAChRs by nicotine or ACh provokes a long-lasting enhancement of the glutamatergic input to 5-HT DRN neurons. In addition, our data indicate that CICR from presynaptic stores of glutamatergic terminals is necessary for nicotine-evoked glutamate release.

According to our results, both nicotine and exogenous ACh increased the frequency of glutamate-dependent sEPSCs recorded from identified 5-HT DRN neurons. This effect was mediated by high-affinity $\alpha 4\beta 2$ nAChRs because it was manifest at low nicotine concentrations (300 nM) (Zoli et al., 1998; Lambe et al., 2003), blocked by low concentrations of DH β E (100 nM) (Zoli et al., 1998), and unaffected by MLA, a selective $\alpha 7$ nAChR blocker. Furthermore, the $\alpha 4\beta 2$ nAChR-selective agonist RJR-2403 mimicked the effect of nicotine. Although RJR-2403 exerts less desensitization on $\alpha 4\beta 2$ receptors (Papke et al., 2000), our results show a more rapid washout of RJR-2403-induced effects compared with nicotine. This could be explained by the low concentrations of RJR-2403 (100 nM) used in this study. Conversely, at micromolar concentrations, RJR-2403 can lose its selectivity for neuronal $\alpha 4\beta 2$ nAChRs (Yang et al., 2009).

It should be mentioned that nicotine-induced glutamate release is more frequently mediated by $\alpha 7$ nAChRs than other nAChRs subtypes (McGehee et al., 1995; Girod and Role, 2001; Maggi et al., 2004; Sharma et al., 2008). However, in a few brain areas, non- $\alpha 7$ nAChRs have also been involved in glutamate release modulation (Rousseau et al., 2005; Couey et al., 2007), and it has been reported that $\alpha 4\beta 2$ nAChRs facilitate glutamate release in thalamocortical afferents (Gioanni et al., 1999; Lambe et al., 2003).

Our data indicate that, in midbrain slices, endogenous ACh also increases the glutamatergic input to 5-HT DRN neurons because the administration of eserine, an inhibitor of acetylcholinesterase, significantly increased the frequency of glutamatergic sEPSCs in 5-HT DRN neurons. In support of this finding, it has been demonstrated that DRN neurons receive a cholinergic input from the LDT and PPT nuclei (Woolf and Butcher, 1989) and that these cholinergic connections are functional in midbrain coronal slices (Galindo-Charles et al., 2008).

According to our data, the magnitude of the effect of 1 μ M nicotine on glutamate release is comparable with that of 1 mM exogenous ACh (see Fig. 3E). This could be explained by the high affinity of nicotine for $\alpha 4\beta 2$ nAChRs (Alkondon and Albuquerque, 1995; Buisson et al., 1996) and also by the fast degradation of ACh by acetylcholinesterase.

In the present study, a single nicotine administration induced glutamate release effects within the DRN that persisted 10–20

Figure 6. Nicotinic action mechanism is presynaptic. **Aa**, Traces showing sEPSCs recorded from a 5-HT-positive neuron with BAPTA (10 mM) in the recording pipette (top) and after adding nicotine in the bath solution (bottom). **Ab**, Traces showing sEPSCs recorded from a 5-HT-positive neuron with BAPTA-AM (10 mM) applied in the bath solution (top) and after adding nicotine (bottom). **Ac**, Bar histogram shows normalized sEPSC frequency. **B**, Paired-pulse protocol is illustrated in **Ba**. A concentric stimulation electrode (S) with a 10 μ m tip diameter was placed in the proximities of the DRN, ventral to the aqueduct. Excitatory evoked currents were recorded from 5-HT neurons within the DRN in the presence of bicuculline (10 μ M). Evoked current traces of a single neuron in response to paired stimuli (S1 and S2) are shown in **Bb** (top). Gray traces are the responses in control conditions. Black traces are the responses in the presence of nicotine (1 μ M). Temporal course of the first stimulus amplitude (S1) from the cell in **Bb** (top) is shown in **Bb** (bottom). Dark gray circles represent the control, black circles represent nicotine, and light gray circles represent nicotine washout. The circles at zero current represent synaptic failures. **Bc**, Bar histograms show the change in the normalized S1 amplitude induced by nicotine (top; *** p < 0.001, n = 6) and the change in the paired-pulse ratio induced by nicotine (bottom; *** p < 0.001, n = 6).

min after nicotine withdrawal. In fact, nicotinic effects on glutamate transmission lasted longer and had a higher prevalence than reported previously by Chang et al. (2011). These discrepancies could be attributed to animal age or strain or the fact that they performed all physiology experiments at room temperature, whereas our experiments were done at 33°C. Recently, it was reported that the response of nAChRs to their agonists depends on temperature (Jindrichova et al., 2012).

Nicotine-evoked glutamate release in the DRN is achieved through presynaptic mechanisms because nicotine increased the frequency but not the amplitude of glutamate sEPSCs. This assertion is supported by the results obtained in this study using the paired-pulse paradigm; nicotine increased the amplitude of the response to the first stimulus and decreased the paired-pulse ratio, effects that are suggestive of a presynaptic effect (Zucker and Regehr, 2002). Furthermore, nicotine-induced glutamate release requires the increase of calcium levels in glutamatergic axon terminals because, in our experiments, buffering intracellular calcium with the membrane-permeable compound BAPTA-AM

decreased both the basal and nicotine-evoked glutamate release. The possibility that a Ca^{2+} -dependent retrograde messenger, released by the 5-HT neurons, could be responsible of the glutamate sEPSC enhancement was ruled out because intracellular postsynaptic BAPTA did not change the effect of nicotine.

Conversely, nicotine-induced increase of glutamate release persisted in the presence of TTX, which indicates that firing activity of glutamatergic neurons is not required for this effect.

Previous studies have demonstrated that nicotine excitatory actions on neurotransmitter release exerted through β 2 subunit-containing nAChRs are entirely dependent on the activation of VGCCs, which induces an additional increase in intracellular calcium levels and CICR (Tsuneki et al., 2000; Shoop et al., 2001; Dajas-Bailador et al., 2002; Dickinson et al., 2008). In contrast, Ca^{2+} influx through presynaptic α 7 nAChRs is sufficient to induce CICR and neurotransmitter release without the intervention of VGCCs (Gray et al., 1996; Sharma and Vijayaraghavan, 2003; Sharma et al., 2008). Accordingly, our data indicate that blocking N-, P/Q-, and L-type Ca^{2+} channels by using selective

Figure 7. Nicotinic effects depend on VGCCs and intracellular CICR. **A**, Time–frequency histogram shows the effect of nicotine on the sEPSC frequency in the presence of CdCl_2 (gray bar). **B**, Time–frequency histogram shows the lack of effect of nicotine on the sEPSC frequency in the presence of a mixture containing the Ca^{2+} channel blockers ω -agatoxin-TK, ω -conotoxin-GVIA, and nifedipine (gray bar). **C**, Time–frequency histogram shows the lack of effect of nicotine on the sEPSC frequency in the presence of the SERCA blocker thapsigargin (gray bar). **D**, Bar graph shows the effect of nicotine on the sEPSC frequency in slices pretreated with CdCl_2 , Ca^{2+} channel blockers, thapsigargin, CPA, or ryanodine (** $p < 0.01$). Gray bar represent the control (before nicotine application).

antagonists completely suppresses nicotine-induced glutamate release. Furthermore, depleting the intracellular Ca^{2+} stores with thapsigargin or CPA or perfusing the cells with ryanodine also abolished the effects of nicotine. These results suggest that, in the DRN, the activation of β_2 -containing nAChRs, located at glutamate terminals, produces changes in synaptic efficacy through a mechanism involving VGCC activation and CICR. Similar conclusions have been reached in several studies involving other brain areas (Léna and Changeux, 1997; Mansvelder et al., 2002; Lambe et al., 2003). In support of this idea, presynaptically located calcium stores have been found to regulate neurotransmitter release in different brain structures (Llano et al., 2000; Conti et al., 2004).

Unexpectedly, the VGCCs broad-spectrum blocker CdCl_2 failed to block the effect of nicotine. Instead, the nicotine-induced increase of sEPSC frequency was potentiated in the presence of CdCl_2 . One possible explanation for this effect is that, like other divalent cations, cadmium potentiates neuronal nAChRs (Hsiao et al., 2001). If this is the case, an increased influx of Ca^{2+} through $\alpha_4\beta_2$ nAChRs, induced by cadmium, might be sufficient to cause CICR, leading to a potentiation of nicotine-induced glutamate release without the intervention of VGCCs.

Although nicotine produces fast desensitization of β_2 -containing nAChRs (Wooltorton et al., 2003), it provoked a persistent increase of glutamate sEPSCs. According to our model of nicotine-induced glutamate release potentiation (Fig. 8), nicotine activates more nAChRs and is degraded much slower than ACh. As a consequence, nicotine will induce a higher Ca^{2+} influx, resulting in CICR, activation of Ca^{2+} -dependent kinases (Dickinson et al., 2008; Sharma et al., 2008), and enhancement of synaptic efficacy at gluta-

mate terminals. Once the changes in the synaptic release efficacy have occurred, they will extend beyond nAChR desensitization. Therefore, we speculate here that nicotine will induce a higher release of glutamate in smokers than endogenous ACh in non-smokers.

DRNs receive glutamatergic projections from cortical and subcortical areas, as well as from local interneurons. The results of our experiments suggest that nicotine-dependent glutamate release in this nucleus might be associated with an increased excitability of 5-HT DRN neurons. Previously, we found that all 5-HT identified neurons tested with local application of ACh exhibited either $\alpha_4\beta_2$ or α_7 nicotinic currents (Galindo-Charles et al., 2008). In addition, in this work, we observed that nicotine produced an increase of glutamate EPSCs in most 5-HT neurons. Therefore, we assume that presynaptic and postsynaptic effects of nicotine coexist in the majority of 5-HT neurons. Postsynaptic effects are short lasting because of nAChRs desensitization, whereas presynaptic effects are long lasting because of glutamate release potentiation. These combined presynaptic and postsynaptic effects of nicotine would increase firing frequency of DRN neurons (Mihailescu et al., 2001, 2002; Chang et al., 2011), which in turn would change the levels of 5-HT in the brain.

Contrary to our findings, systemic nicotine administration in rats transiently inhibited 5-HT DRN neurons (Engberg et al., 2000). Because 5-HT neuron firing strongly depends on the arousal state, it is possible that the inhibitory effect of nicotine *in vivo* was influenced by anesthesia.

Glutamate neurotransmission within the DRN has been implicated in the pathophysiology of affective disorders, such as depression (Paris and Cunningham, 1994; Grahn et al., 2000; Paul and Skolnick, 2003) and stress (Amat et al., 2005; Kirby et al., 2007). Indeed, in humans and animal models, glutamate receptor antagonists have been reported to be effective antidepressants (Trullas and Skolnick, 1990; Yilmaz et al., 2002). These effects could rely on the ability of glutamatergic neurotransmission to change the circuits regulating the serotonergic tone within the DRN and other brain regions. For example, it has been proposed that an increase of DRN serotonergic output to the nucleus accumbens could partially explain the rewarding and addictive effects of nicotine (Chang et al., 2011).

Conversely, low concentrations of nicotine applied into the DRN of rats consistently induces anxiolytic effects (Cheeta et al., 2001). Interestingly, the systemic administration of ABT-418 (3-methyl-5-[(2S)-1-methylpyrrolidin-2-yl]-1,2-oxazole), an agonist of $\alpha_4\beta_2$ nAChRs, mimics the anxiolytic effects of nicotine and attenuates the anxiogenic response evoked by nicotine withdrawal (Brioni et al., 1994). Because $\alpha_4\beta_2$ nAChR agonists increase glutamatergic input to 5-HT DRN neurons, we hypothesize that the effects of these agonists on mood and nicotine withdrawal symptoms are at least partly

Figure 8. Model summarizing nicotinic effects on glutamate terminals in the DRN. **A**, In physiological conditions, the cholinergic tone is regulating the excitatory glutamatergic input to the DRN 5-HT neurons through the activation of $\alpha 4\beta 2$ nAChRs, located at glutamate terminals. Released ACh is quickly metabolized by the enzyme acetylcholinesterase. As a result, the Ca^{2+} influx into the glutamate terminals and glutamate release will be maintained at low levels. **B**, When nicotine is present (for example, in smokers), more $\alpha 4\beta 2$ nAChRs will be activated, because nicotine cannot be degraded in the synaptic cleft. This will enhance Ca^{2+} entry, followed by depolarization of glutamate terminals and activation of VGCCs. This, in turn, will increase even more intracellular calcium and produce CICR from the endoplasmic reticulum (ER) through the activation of ryanodine receptors (RyR). This last event generates a long-term potentiation of glutamate release.

dependent on a glutamate-induced increase in activity of 5-HT DRN neurons.

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