Nicotine increases GABAergic input on rat dorsal raphe serotonergic neurons through alpha7 nicotinic acetylcholine receptor

Departamento de Fisiología, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City, Mexico

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THE DORSAL RAPHE NUCLEUS (DRN) is located in the brain stem and contains the largest population of serotonergic (5-HT) neurons in the brain (Dahlström and Fuxe 1964). This nucleus provides 5-HT innervation to several targets including the forebrain and limbic structures (see Michelsen et al. 2008 for review) and is involved in several behavioral functions such as sleep-wake states, feeding, nociception, neuroendocrine regulation, learning and memory, and stress-induced responses (Hale et al. 2012; Jacobs and Azmitia 1992; Meneses 2013). In addition, dysregulation of this nucleus is associated with psychiatric disorders such as depression and anxiety (Lowry et al. 2008; Sharp and Cowen 2011). Clinical studies have demonstrated that transdermal nicotine improves mood in patients with major depression (Salin-Pascual and Drucker-Colin 1998) and reduces anxiety in both smokers and nonsmokers (Gilbert 1979; Kassel and Unrod 2000). Likewise, in rodents, nicotine reduces stress and anxiety induced by restraint behavior (Hsu et al. 2007).

Experimental studies have shown that nicotine increases the firing rate of the majority of DRN neurons (Li et al. 1998; Mihailescu et al. 2002) as well as 5-HT release in several brain areas such as prefrontal cortex (Ribeiro et al. 1993) and DRN itself (Mihailescu et al. 1998). Immunocytochemical studies have demonstrated the presence of α7 and α4β2 nicotinic acetylcholine receptor (nAChR) subtypes in 5-HT DRN neurons (Bittner et al. 2000; Commons 2008). Other studies have indicated that nicotine increases the firing activity of 5-HT neurons through direct stimulation of their somatodendritic α7 and α4β2 nAChRs (Galindo-Charles et al. 2008) and through presynaptic release of glutamate (Garduño et al. 2012) and norepinephrine (Li et al. 1998).

GABAergic inhibitory afferents to DRN originate in several brain areas such as lateral and posterior hypothalamus, lateral preoptic area, ventral pontine periaqueductal gray, substantia nigra, and ventral tegmental area (VTA), as well as interneurons from the DRN itself (Gervasoni et al. 2000). Serotonergic neurons are tonically inhibited by GABA through both GABAA and GABAB receptors (Bowery et al. 1987; Gervasoni et al. 2000). Supporting this idea, the administration of GABA_A antagonists, such as picrotoxin or bicuculline, increases action potential frequency in 5-HT DRN neurons (Gallager 1978; Gallager and Aghajanian 1976). It was suggested that the GABAergic input to DRN neurons increases during rapid eye movement (REM) sleep (Nitz and Siegel 1997), which explains the low activity of 5-HT DRN neurons registered during this period. However, there are few electrophysiological studies concerning the influence of nicotine on the GABAergic input to 5-HT DRN neurons, as well as the nicotinic receptor involved in this response. This is an interesting topic considering the above-mentioned stimulatory effect of nicotine on the firing rate of 5-HT DRN neurons. Thus the aim of this work was to determine the effect of nicotine on the GABAergic input to 5-HT DRN neurons and to identify the nAChR subtype involved in this effect.

METHODS

Slice preparation. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care Committee of the Universidad Nacional Autónoma de México. Experiments were performed in young (postnatal day 18–21) Wistar rats that were deeply anesthetized with isoflurane and then decapitated. Their brains were quickly removed and placed
into ice-cold (4°C) artificial cerebrospinal fluid (ACSF) consisting of (in mM) 125 NaCl, 3 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 1.2 CaCl₂, and 25 glucose, 300 mosM, pH = 7.3 by bubbling with 95% O₂-5% CO₂. Coronal slices (350 μm thick) containing the DRN were obtained with a Vibratome 1500 (Vibratome, St. Louis, MO) and allowed to rest in carbogen-bubbled ACSF at room temperature for at least 1 h before recording.

**Electrophysiological recordings.** Individual slices were transferred into a custom-made Plexiglas recording chamber and perfused with ACSF at a rate of 4–5 ml/min at 33°C maintained by an in-line solution heater (TC-324; Warner Instruments). DRN neurons were visualized with a videomicroscopy system (Olympus BX51WI) fitted with a ×60 water-immersion objective, differential interference contrast and infrared filter. The image from the microscope was enhanced with a CCD camera and displayed on a monitor. Whole cell current- and voltage-clamp recordings were performed with a Multiclamp 700A amplifier (Axon Instruments, Union City, CA) and monitored with a PC running Clampex 8 software (Axon Instruments). Signals were digitized by a Digidata 1320 series analog-to-digital converter at 10 kHz and stored online with pCLAMP8 software (Molecular Devices). Only one cell was recorded per brain slice. Micropipettes used for recordings were pulled from borosilicate glass tubes (WPI, Sarasota, FL) with a Flaming-Brown puller (Sutter Instrument, Novato, CA) (resistance 4–7 MΩ). The internal solution consisted of (in mM) 70 K-glucocan, 70 KCl, 5 NaCl, 1 MgCl₂, 0.02 EGTA, 10 HEPES, 2 Mg₂ATP, and 0.5 Na₂GTP, with bicytic 0.1%, pH = 7.3 with Trizma base, 280–300 mosM. Spontaneous inhibitory postynaptic currents (sIPSCs) were recorded at a holding potential of −70 mV, and 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX; 10 μM) and DL-2-amino-5-phosphonovaleric acid (APV; 50 μM) were used to block glutamatergic currents. Miniature inhibitory postynaptic currents (mIPSCs) were equally recorded according to the same protocol, but with tetrodotoxin (TTX; 1 μM) also added to the perfusion solution. Series resistance was monitored throughout the experiment; if it was unstable or exceeded four times the electrode resistance, the cell was discarded.

**Drug administration.** Several protocols of drug administration were used, as imposed by the purpose of the experiments. Recordings of at least 10 min were allowed for stabilization of sIPSC frequency, in which blockers of glutamate receptors (CNQX and APV) were present in the perfusion solution. Afterwards, bicuculline, nicotine, or nAChR agonists were added to the perfusion fluid and their effects were recorded during the administration (8–10 min) and during the period of washout (15 min or more). In the experiments using drugs susceptible to alter nicotine effects [nAChR antagonists, PNU-120596, TTX, blockers of calcium-induced calcium release (CICR) and of voltage-gated calcium channels (VGCCs)], one of these drugs was administered before nicotine and its effects on sIPSC frequency and amplitude were followed for at least 10 min. Nicotine was then added to the perfusion fluid for 10 min, and its effects on sIPSC frequency were recorded during the administration as well as 15 min after the washout of drugs.

**Immunohistochemistry.** Neurons were filled with bicocytin present in the internal solution during recordings. To identify whether recorded cells were 5-HT, we used an anti-5-HT antibody. After electrophysiological recording, slices were fixed on a Vibratome into 40-μm sections. Sections were incubated for 4–6 h in PBS solution containing 0.2 Triton X-100 and streptavidin conjugated to Cy3 (1 mg/ml; Zymed, South San Francisco, CA; diluted 1:100) 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 (ImmuNoStar, Hudson, WI; 1:2,000). After rinsing in PBS, sections were reincubated for 2–4 h with secondary antibodies conjugated to fluorescein (Vector Laboratories, Burlingame, CA; diluted 1:100). The reacted sections were first examined with an appropriate set of filters on an epifluorescence-equipped microscope. Afterwards, sections were mounted in an antiquenching medium (Vectorshield, Vector Laboratories) and examined under a confocal microscope (MRC 1024, Bio-Rad, Natford, UK) equipped with a krypton/argon laser. A two-line laser emitting at 550- and 500-nm wavelength was used for exciting Cy3 and fluorescein, respectively. Digitized images were transferred to a personal computer with image-capturing software (Confocal Assistant, T. C. Breleje).

**Drugs.** Drugs were dissolved in ACSF and administered by bath perfusion. The time required for obtaining equilibrated concentrations of the drugs in the recording chamber was ~3–4 min. TTX, APV, CNQX, (−)-bicuculline methiodide, methyllycaconitine (MLA), dihydro-β-erythroididine hydrobromide (DHβE), N-(5-chloro-2,4-dimethoxyphenyl)-N’-(5-methyl-3-isoxazolyl)-urea (PNU-120596), 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid ethyl (nitrindipine), and bicucullin were purchased from Sigma-Aldrich RBI (St. Louis, MO). Thapsigargin, ryanodine, (E)-N-methyl-4-(3-pyridinyl)-3-buten-1-amine oxalate (RJR-2403 oxalate), and N-(3R)-1-azabicyclo[2,2,2]oct-3-yl-4-chlorobenzamide (PNU-282987) were purchased from Tocris Bio-science (Ellisville, MO). o-Agatoxin-TK and o-conotoxin-GVIA were purchased from Pep tide Institute and Alomone Labs, respectively. All reagents were added from freshly prepared stock solution to the bath saline.

**Data analysis and statistics.** Data analysis was performed with Clampfit 8 software (Molecular Devices) and Mini Analysis software (Synaptosoft, Decatur, GA). The Mini Analysis software was used to detect sIPSCs and mIPSCs and to assess their frequency and ampli-

**RESULTS**

Identification of 5-HT DRN neurons used for recordings. A total of 83 cells located in the ventral part of the middle region of the DRN (bregma −7.80 to −8.00 mm), close to the midline, were obtained from 74 rats. The recorded cells were considered possibly serotonergic when presenting the electrophysiological characteristics established by Vandermaelen and Aghajanian (1983): a slow (4–10 Hz) firing rate upon application of depolarizing stimuli (Fig. 1A); long-lasting afterhyperpolarization (347 ± 22.7 ms, n = 83) and action potential (4.32 ± 0.163 ms, n = 83), and a discrete shoulder on the falling phase of the spike (Fig. 1A, inset). Figure 1B shows the firing frequency as a function of the intensity of injected current (I-F plot); note the low firing frequency (7.24 ± 0.33 Hz, n = 15) observed after application of the maximal depolarizing pulse (175 pA). To support the electrophysiological identification of 5-HT DRN neurons, immunohistochemical studies with anti-serotonin antibodies were performed post hoc (Fig. 1C). The recordings obtained from cells negative for 5-HT staining were discarded.
Spontaneous inhibitory postsynaptic currents recorded in 5-HT DRN neurons are GABAergic. sIPSCs were recorded in voltage-clamp mode, at a holding potential of −70 mV, with an internal solution with elevated chloride content (see METHODS) to enhance their resolution. Figure 2 shows representative recordings of sIPSCs without any drug (Fig. 2A, top), in the presence of glutamate antagonist CNQX (10 μM) and APV (50 μM) (Fig. 2A, middle) and after bicuculline administration (Fig. 2A, bottom). Note that after addition of bicuculline (a selective GABA_A receptor antagonist), all sIPSCs disappeared (Fig. 2B). Five experiments of this kind were performed in order to demonstrate that GABA acting on GABA_A receptors of 5-HT DRN neurons produced the sIPSCs recorded in our experiments. The frequency and amplitude of sIPSCs were 0.77 ± 0.10 Hz and 29.29 ± 1.58 pA, respectively (n = 83), whereas mIPSC frequency was 0.47 ± 0.17 Hz and amplitude 28.59 ± 2 pA (n = 14).

Nicotine increases sIPSC frequency in 5-HT DRN neurons. All experiments were performed in the presence of CNQX and APV (control conditions). We used a nicotine concentration of 1 μM because it is similar to that reached in the blood of a smoker after one cigarette (Henningfield et al. 1993). Nicotine was bath-administered in 18 identified 5-HT DRN neurons. In these conditions, nicotine significantly (Wilcoxon signed-rank test, Z = −3.180; P = 0.001) increased sIPSC frequency by 75% in 13 5-HT DRN neurons, decreased sIPSC frequency in 3 neurons, and had no effect in 2 other cells. Figure 3 shows direct recordings of GABAergic sIPSCs in a 5-HT neuron before (Fig. 3A, top), after nicotine administration (Fig. 3A, middle), and after nicotine washout (Fig. 3A, bottom). Nicotine administration reduced the interevent interval (Fig. 3B) but did not change the amplitude of GABAergic sIPSCs (Fig. 3C). The fact that sIPSC amplitude was not affected by nicotine suggests a presynaptic effect of the drug. In the presence of TTX, nicotine significantly increased mIPSC frequency by 45% (Wilcoxon signed-rank test, Z = −2.3666; P = 0.018; n = 7). The increases in IPSC frequency induced by nicotine and by bicuculline in the presence of TTX were not statistically significant (Mann-Whitney test, Z = −0.832, P = 0.43) (Fig. 3D).

Mecamylamine (MEC; 50 μM), a nonselective nAChR antagonist, administered alone, did not change sIPSC frequency (Wilcoxon signed-rank test, Z = −0.135; P = 0.814; n = 5; Fig. 3D). However, MEC blocked nicotine-induced increase in sIPSC frequency (n = 6), confirming a receptor-mediated effect by the drug. In addition, MLA (20 nM), a selective α7 nAChR antagonist, did not change sIPSC frequency when administered alone (Wilcoxon signed-rank test, Z = −0.73, P = 0.625; n = 4) but prevented the increase in sIPSC frequency caused by nicotine (Wilcoxon signed-rank test, Z = −0.338, P = 0.813, n = 7; Fig. 3D).

Nicotine-dependent increase in GABAergic sIPSC frequency is mediated by α7 nAChRs. To identify the nAChR subtype involved in nicotine’s effect, two selective nAChR agonists were used. The selective α4β2 nAChR agonist RJR-2403 (100 nM) did not change the GABAergic sIPSC frequency in 5-HT DRN neurons (n = 5; Fig. 4, A and B). In contrast, the administration of PNU-282987 (100 nM), a selective α7 nAChR agonist, significantly (Wilcoxon signed-rank test, Z = 2.201, P = 0.028; n = 6) increased GABAergic sIPSC frequency by 92% versus the control group (Fig. 4, C and D). PNU-282987 also significantly increased GABAergic mIPSC frequency as a function of injected current obtained from 15 identified 5-HT neurons.

Inset, an expanded action potential from the same neuron. B: discharge frequency as a function of injected current obtained from 15 identified 5-HT neurons. C: microphotographs showing the same recorded cell after biocytin staining (top), 5-HT immunoreactivity (middle), and merge (bottom).
frequency by 56% (Wilcoxon signed-rank test, $Z = -2.202$, $P = 0.028$; $n = 6$). The increases in IPSC frequency induced by PNU-282987 in the presence and absence of TTX were not statistically different (Mann-Whitney test, $Z = 1.121$; $P = 0.31$), suggesting that the increase in GABA release mediated by $\alpha 7$ nAChRs is independent of action potential generation by GABAergic neurons (Fig. 4E). In agreement with the previous results, this confirms that the effect of nicotine on GABA release is presynaptic. In summary, the results obtained in this experimental group show that the nicotine-induced increases in GABAergic sIPSC frequency of 5-HT DRN neurons are due to activation of a presynaptic $\alpha 7$ nAChR.

As another tool to corroborate that nicotine-induced increases in sIPSC frequency were due to activation of $\alpha 7$ nAChR, we used PNU-120596, a positive allosteric modulator specific for $\alpha 7$ nAChR (daCosta et al. 2011; Williams et al. 2011). Administration of PNU-120596 (10 $\mu$M) alone did not change either the amplitude or frequency of sIPSCs ($n = 3$; Fig. 5A, middle). When nicotine was administered after PNU-120596, a significant increase in GABAergic sIPSC frequency by 430% was observed (Wilcoxon signed-rank test, $Z = -1.604$; $P = 0.049$; $n = 3$) (Fig. 5, A, B, and D). PNU-120596 pretreatment also increased the amplitude of GABAergic sIPSCs obtained after nicotine administration by 187% (Fig. 5, A and C). The use of this positive allosteric modulator gave stronger evidence that the nAChR implicated in the effect of nicotine was the $\alpha 7$ type.

Blocking CICR but not VGCCs prevented nicotine-induced increases in sIPSC frequency. We also investigated whether $Ca^{2+}$ was involved in the effect of nicotine on sIPSCs. Among nAChRs, the $\alpha 7$ homomorphic subtype is the one with the highest $Ca^{2+}$ permeability (Alkondon et al. 1997; Fucile 2004). Calcium influx in axon terminals may induce the release of calcium from intracellular stores (mostly the endoplasmic reticulum), a phenomenon termed calcium-induced calcium release (CICR) (Berridge 1998). To determine whether CICR participates in nicotine-induced increase in GABAergic sIPSC frequency, two drugs were used: ryanodine (100 $\mu$M), a blocker of endoplasmic reticulum $Ca^{2+}$ channels, and thapsigargin (10 $\mu$M), a noncompetitive inhibitor of the sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA). Ryanodine or thapsigargin administered after CNQX and APV did not significantly alter sIPSC frequency [Wilcoxon signed-rank test for ryanodine: $Z = -0.249$ ($P = 0.87$, $n = 4$), thapsigargin: $Z = -0.365$ ($P = 0.87$, $n = 4$)] (Fig. 6, A, inset and B, inset). However, the nicotine-dependent increase in GABAergic sIPSC frequency of 5-HT DRN neurons was suppressed by pretreatment with either ryanodine (Fig. 6A) or thapsigargin (Fig. 6B). Therefore, $Ca^{2+}$ release from intracellular stores is necessary for nicotine-dependent increases in sIPSC frequency.

With the purpose of investigating whether the increase in GABAergic sIPSC frequency induced by nicotine was dependent on VGCCs, a mixture of selective blockers of VGCCs was administered to 5-HT DRN neurons before nicotine. The mixture consisted of $\omega$-agatoxin-TK (400 nM), $\omega$-conotoxin-GVIA (500 nM), and nitrendipine (10 $\mu$M), selective blockers of L-type, N-type, and P/Q-type VGCCs, respectively. Treatment of brain slices with this mixture of VGCC blockers significantly decreased sIPSC frequency by 27% (Wilcoxon signed-rank test: $Z = -1.826$, $P = 0.032$, $n = 4$; Fig. 6C, inset). Nevertheless, administration of VGCC blockers did not affect the increase in...
sIPSC frequency induced by nicotine (Wilcoxon signed-rank test, \(Z = -1.826, P = 0.038, n = 5\)) (Fig. 6C).

The effects of CICR and VGCC blockers on nicotine-dependent GABAergic sIPSCs, expressed as a percentage versus the control group, are presented in Fig. 6D. These results demonstrate that nicotine’s effect on sIPSC frequency is independent of VGCCs but is critically dependent on activation of CICR. A model summarizing the effect of nicotine on the input from GABAergic terminals to 5-HT DRN neurons is presented in Fig. 7.

**DISCUSSION**

The main findings of this study are that nicotine produces a \(\alpha_7\) nAChR-mediated presynaptic increase of the GABAergic input to 5-HT DRN neurons and that this effect is independent of action potential generation or opening of VGCCs but conditioned by CICR in GABAergic DRN terminals.

The results of the present paper indicate that nicotine-induced increase in GABAergic sIPSC frequency of the majority of 5-HT DRN neurons is produced by activation of \(\alpha_7\) nAChRs. Thus the nicotine-induced increase in GABAergic sIPSC frequency was \(J\) blocked by low concentrations of MLA, a selective antagonist of \(\alpha_7\) nAChRs; \(2\) mimicked by the selective agonist of \(\alpha_7\) nAChRs PNU-282987; and \(3\) greatly amplified by the positive allosteric modulator of \(\alpha_7\) nAChRs PNU-120596. The \(\alpha_{4}\beta_2\) nAChRs were not involved in nicotine-induced GABA release, since the selective agonist of \(\alpha_{4}\beta_2\) nAChRs, JRJ-2403, did not change the frequency of GABAergic sIPSCs.

The nicotine-induced GABA release in the DRN was achieved by a presynaptic mechanism, since in the presence of TTX both nicotine and PNU-282987 increased the frequency but not the amplitude of GABAergic IPSCs of 5-HT DRN neurons. These data are in agreement with studies indicating that nicotine induces GABA release in several brain areas with synaptic mechanisms. Thus the \(\alpha_7\) nAChR subtype was found responsible for the presynaptic nicotine-induced GABA release in the hippocampus (Kanno et al. 2005; Maggi et al. 2001; Radcliffe et al. 1999) and dorsal motor nucleus of the vagus (Bertolino et al. 1997), whereas the \(\alpha_{4}\beta_2\) nAChR subtype mediated the nicotine-induced presynaptic GABA release in the VTA (Corrigall et al. 2000), cortex, striatum, and thalamus (McClure-Begley et al. 2009), medial septum (Yang et al. 1996), periaqueductal gray (Nakamura and Jang 2010), prefrontal cortex (Aracri et al. 2010), and lateral spiriform nucleus (Zhu and Chiappinelli 2002).

In a previous study, Chang et al. (2011) reported that nicotine increased the GABAergic input to 5-HT DRN neurons in a reduced proportion (34%) of the cells tested. In contrast, in our study nicotine (1 \(\mu M\)) increased the frequency of GABAergic sIPSCs in \(\sim 72\%\) of identified 5-HT DRN neurons. This discrepancy may be attributed to the age dependence of nAChR expression: neonate rats (0–28 postnatal days of age) present higher densities of \[^{[3]}\H\]nicotine and \(\alpha_{[-1]}\H\)bungarotoxin binding sites in the brain stem than older animals (Zhang et al. 1998). We used rats of 18–21 postnatal days, whereas Chang et al. (2011) used rats of 60 postnatal days. In another recent study, Yang and Brown (2014) did not find a presynaptic effect of carbachol (a nonhydrolyzable analog of acetylcholine) on GABAergic

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**Fig. 3.** Nicotine increases GABAergic sIPSC frequency in the majority of 5-HT DRN neurons. A, top: control recording of sIPSCs in the presence of CNQX (10 \(\mu M\)) and APV (50 \(\mu M\)). Middle: in the same neuron, nicotine (1 \(\mu M\)) increases GABAergic sIPSC frequency. Bottom: after washout of nicotine, sIPSC frequency returned to basal values. B and C: cumulative probability distributions of frequency (\(B\)) and amplitude events (\(C\)) from the neuron in \(A\) showing that nicotine increases the frequency but not the amplitude of GABAergic sIPSCs. D: summary of the results showing the increases of sIPSC frequency with nicotine obtained in 72% of 5-HT DRN neurons ("responder cells"). Mecamylamine (MEC; 50 \(\mu M\)) and methyllycaconitine (MLA; 20 nM) did not alter sIPSC frequency in basal conditions but blocked the stimulatory effect of nicotine. Data are expressed as % of control group. *Significant difference vs. control (\(P < 0.05\)).
sIPSCs of 5-HT DRN neurons. This difference may be due to a much lower affinity of carbachol for \( \alpha_7 \) nAChRs compared with nicotine (Bolchi et al. 2013).

Presynaptic nicotinic actions are critically dependent on increase in intracellular Ca\(^{2+} \) levels. For example, the activation of \( \alpha_4 \beta_2 \) nAChRs produces an inward Ca\(^{2+} \) current, which induces membrane depolarization and the opening of VGCCs. The subsequent increase in intracellular Ca\(^{2+} \) concentration produces the release of Ca\(^{2+} \) from the endoplasmic reticulum (CICR) (Rathouz and Berg 1994; Vijayaraghavan et al. 1992). This last event increases intracellular Ca\(^{2+} \) concentration to the level required for neurotransmitter release (Dajas-Bailador et al. 2002; Dickinson et al. 2008; Garduño et al. 2012). The situation is different in the case of \( \alpha_7 \) nAChRs, which have the highest Ca\(^{2+} \)-permeability among all types of nAChRs (Alkondon et al. 1997). The activation of \( \alpha_7 \) nAChRs produces a much higher increase in intracellular Ca\(^{2+} \) compared with \( \alpha_4 \beta_2 \) nAChRs, and this increase is by itself sufficient to produce CICR without activation of VGCCs (Dickinson et al. 2007; Gray et al. 1996; Sharma et al. 2008; Sharma and Vijayaraghavan 2003). The mechanism by which CICR increases neurotransmitter release involves activation of Ca\(^{2+} \)-dependent kinases such as calcium/calmodulin-dependent protein kinase II (CaMKII) (Sharma et al. 2008) and protein kinase C (PKC) (Wu and Wu 2001).

Our results are consistent with a CICR-dependent effect of nicotine, since ryanodine, a blocker of endoplasmic reticulum calcium channels, or thapsigargin, a noncompetitive inhibitor of SERCA, blocked the nicotine-induced increase in GABAergic sIPSC frequency (see Fig. 7). In support for this idea, presynaptically located calcium stores have been found to regulate neurotransmitter release in different brain structures (Conti et al. 2004; Llano et al. 2000).

Our data indicate that VGCCs do not participate in nicotine-induced increase in GABA release since a mixture of selective blockers of VGCCs did not suppress the stimulatory effects of nicotine on sIPSC frequency. This experimental observation reinforces the idea that \( \alpha_4 \beta_2 \) nAChRs are not involved in nicotine-induced GABA release in the DRN.

**Physiological relevance.** Nicotine produces both inhibitory and excitatory effects on 5-HT DRN neurons. In brain slices, the excitatory effects observed in the majority of 5-HT DRN neurons are mediated by activation of somatodendritic \( \alpha_4 \beta_2 \) and \( \alpha_7 \) nAChRs of these neurons (Galindo-Charles et al. 2008). Also, nicotine produces excitatory effects by increasing glutamate and norepinephrine release through activation of presynaptic \( \alpha_4 \beta_2 \) and \( \alpha_7 \) nAChRs, respectively (Garduño et al. 2012; Li et al. 1998). On the other hand, nicotine also causes inhibition in a subpopulation of 5-HT DRN neurons (Li et al. 1998; Mihailescu et al. 2001, 2002). It is well known that this effect is mediated by nicotine-induced 5-HT release and subsequent stimulation of 5-HT\(_{1A} \) autoreceptors. This mechanism involves the activation of \( \alpha_4 \beta_2 \) nAChRs (Frias-Domínguez et al. 2013). Our study indicates that another way by which
Nicotine inhibits 5-HT DRN neurons is the increase in their GABAergic input. This idea is supported by a previous study from our laboratory showing that low concentrations of nicotine administered locally in the DRN increase both serotonin and GABA release in the DRN (Mihailescu et al. 2002). In that study, however, neither the subtype nor the neuronal location of nAChRs involved in GABA release was determined. While we found that nicotine increased the frequency of sIPSCs in 72% of 5-HT neurons, previous studies reported that nicotine increases the firing rate of 80% of DRN neurons (Li et al. 1998; Mihailescu et al. 1998, 2001, 2002), suggesting that the excitatory effects of nicotine prevail over the inhibitory effects. Comparing the results of the present study with those of Garduño et al. (2012), it was observed that the effect of nicotine on glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs) was of higher amplitude (88%) and lasted longer (>20 min) than the effect on GABAergic sIPSCs (the increase was of 75% and lasted for <15 min). Moreover, in a recent study (Soiza-Reilly et al. 2013), it was shown that the stimulation of GABAA receptors increases glutamate release from a subpopulation of glutamate terminals, while the activation of the GABAB subtype has the opposite effect.

An increase in GABA release in the DRN reduces the excitatory effects of nicotine on 5-HT DRN neurons due to stimulation of GABA_A and GABA_B receptors expressed by these cells (Gao et al. 1993; Serrats et al. 2003). This view is
also supported by a previous study performed in our laboratory, which indicated that the GABAA receptor antagonist bicuculline increases the excitatory effects of nicotine on 5-HT DRN neurons (Mihăilescu et al. 2002).

Fig. 6. Dependence of nicotine-induced increase in sIPSC frequency on Ca2+-induced Ca2+ release (CICR) but not on voltage-gated calcium channels (VGCCs). A and B: cumulative probability distributions of frequency events showing that ryanodine (100 μM; A) or thapsigargin (10 μM; B) blocks nicotine-induced increase in sIPSC frequency. Ryanodine or thapsigargin alone did not affect sIPSC frequency (insets). C: VGCC blockers [ω-agatoxin-TK (400 nM), ω-conotoxin-GVIA (500 nM), and nitrendipine (10 μM)] caused a decrease in sIPSC frequency per se (inset) but failed to suppress the nicotine effects on sIPSC frequency. D: summary of the results showing the effect on sIPSC frequency with Ca2+ drugs alone, nicotine alone, and nicotine with ryanodine, thapsigargin, or VGCC blockers. *Significant difference vs. control (P < 0.05).

Fig. 7. Model summarizing nicotinic effects on GABAergic terminals in the DRN. 1) Nicotine activates α7 nAChR on GABAergic terminals and induces Ca2+ entry through this receptor channel. 2) The increase in intracellular Ca2+ produces CICR from the endoplasmic reticulum (ER). 3) CICR evokes GABA release from the terminals. 4) GABA binds to GABAA receptors, which results in an increase of sIPSC frequency in 5-HT DRN neurons. Apparently, VGCC activation is not required for nicotine-induced GABA release. RyR, ryanodine receptors; SERCA, sarco(endo)plasmic reticulum Ca2+-ATPase.

The inhibitory effect of nicotine-induced GABA release on 5-HT neurons may have different intensities in various regions of the DRN. A recent study performed by Corteen et al. (2014) showed that the majority of 5-HT DRN neurons express GABAA receptors preferentially in their dendritic region. Stimulation of these receptors generates a tonic inhibition of 5-HT neurons. A small group of 5-HT DRN neurons located in the rostral regions of the DRN express GABAA receptors in both the somatic and dendritic neuronal areas. Stimulation of these receptors produces a stronger phasic inhibition of 5-HT DRN neurons. Therefore, the degree of inhibition of 5-HT DRN neurons may be influenced not only by the location and subtype of nAChRs on GABA terminals but also by the location of GABAA receptors on 5-HT cells. The existence of different degrees of inhibition of 5-HT neurons by GABA in various regions of the DRN may explain, at least in part, why systemic nicotine increases 5-HT release in some specific regions of the brain, whereas in others it produces decrease or no change of 5-HT extracellular levels (Ribeiro et al. 1993; Shearman et al. 2008; Singer et al. 2004).

The nicotine-induced presynaptic GABA release in the DRN also will inhibit the firing activity of GABAergic DRN neurons, which express both somatic and dendritic GABAA receptors (Corteen et al. 2014). The degree of this inhibition is...
difficult to predict, since GABAergic DRN interneurons receive glutamatergic input mainly from prefrontal cortex (Celada et al. 2001; Challis et al. 2014; Soiza-Reily et al. 2013) and also express nAChRs (Bitner and Nikkel 2002), which may also be stimulated directly or indirectly by nicotine.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


J Neurophysiol  doi:10.1152/jn.00223.2014 • www.jn.org
NICOTINE INCREASES GABAERGIC INPUT TO 5-HT DRN NEURONS


Wu WS, Wu LG. Protein kinase C increases the apparent affinity of the release machinery to Ca^{2+} by enhancing the release machinery downstream of the Ca^{2+} sensor. J Neurosci 21: 7928–7936, 2001.


