Nicotinic excitation of serotonergic projections from dorsal raphe to the nucleus accumbens

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TOBACCO IS one of the most commonly abused substances in the world and the leading cause of preventable illness in the United States (Centers for Disease Control and Prevention 2004). A large percentage of smokers attempt to quit each year, but remarkably few succeed. Withdrawal symptoms, including anxiety, irritability, restlessness, craving, and depression, contribute to the low success rate (Kenny and Markou 2001; Seth et al. 2002). Serotonin reuptake inhibitors (SSRIs) can diminish the anhedonic effects of nicotine withdrawal when administered with a blocker of 5-HT1A receptors (Harrison et al. 2001). While humans do not experience significantly improved smoking cessation in SSRI clinical trials, subjects in the treatment group report significantly lower withdrawal symptoms than control subjects (Hughes et al. 2007; Saules et al. 2004).

The rewarding effects of many drugs, including nicotine, are mediated through dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Di Chiara and Imperato 1988). Serotonergic projections from the dorsal raphe nucleus (DRN) to the NAc may contribute to addiction (Tork 1990) (Fig. 1A). In the case of nicotine, at least some of the behavioral reinforcement derives from the anxiolytic effects of the drug, which are mediated by serotonin (Cheeta et al. 2001). It has been hypothesized that a nicotine-induced alteration in the balance between serotonin and dopamine transmission in brain reward areas is important in establishing the addicted phenotype (Olausson et al. 2002). The cellular mechanisms that underlie nicotinic modulation of serotonin signaling and its ultimate role in reward and withdrawal are unclear.

Electrophysiology studies have revealed the influence of nicotine on serotonin release from the DRN. Serotonergic DRN cells express functional nicotinic acetylcholine receptors (nACHRs) and receive cholinergic input from the pedunculopontine tegmentum (Galindo-Charles et al. 2008). The majority of serotonergic DRN neurons increase their action potential (AP) firing in response to nicotine, and this leads to increased serotonin release (Li et al. 1998; Mihailescu et al. 2001, 2002). Here we have confirmed earlier findings demonstrating an anatomic connection between the mood and affect control centers of the DRN and the reward-associated NAc (Li et al. 1989). Physiological recordings from DRN neurons were conducted to assess the cellular mechanisms underlying nicotinic excitation of putative serotonergic neurons projecting to the NAc. Exploring the effects of nicotine on the serotonergic inputs to the NAc may provide insight into the link between nicotine and mood, which may influence both the addictive and withdrawal effects of the drug.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (>P60, weight 180–250 g at delivery; Harlan, Indianapolis, IN) were housed one or two per cage with a reverse light-dark cycle and free access to water and food. All animal experiments were approved by the University of Chicago Institutional Animal Care and Use Committee.
For recording, the slices were transferred to a chamber continuously perfused with aCSF without 1 mM ascorbic acid and bubbled with 95% O2-5% CO2. To label recorded neurons, in some experiments biocytin was included in the internal solution (0.1% wt/vol). All physiology experiments were performed at room temperature, on both backlabeled and randomly chosen unlabeled neurons that were distributed across all subdivisions of the DRN. Neurons were visualized under infrared illumination with an upright Zeiss Axioskop FS fixed-stage microscope (Carl Zeiss Microimaging, Thornwood, NY) equipped with a charge-coupled device (CCD) camera (Hamamatsu C2400, Bridgewater, NJ). Retrogradely labeled neurons were visualized with a fluorescent light source (Cy3 filter set). Infrared light was then used to identify the same neuron and guide the electrode placement. Whole cell patch-clamp recordings used microelectrodes (3–8 MΩ) pulled on a Flaming/Brown micropipette puller (model P-97, Sutter Instrument, Novato, CA). Data were acquired with an Axopatch 200B with a Digidata 1200 Interface and pCLAMP 8 software (Molecular Devices, Sunnyvale, CA).

To measure APs, recording electrodes were filled with potassium gluconate internal solution (in mM: 154 K-gluc, 1 KCl, 1 EGTA, 10 HEPES, 10 glucose, 5 ATP, 0.1 GTP, pH 7.4 with KOH). Slices were bathed with normal aCSF or aCSF with 12 μM phenylephrine. Both whole cell and on-cell recording techniques were used. On-cell recordings involved forming a “loose patch” on the cell membrane, with seal resistance close to 100 MΩ. Voltage fluctuations were recorded in current clamp with no current applied. Criteria for regular and frequent spontaneous AP firing were defined as baseline firing that was at least 0.05 Hz and an interspike interval that varied no more than 20-fold.

For the acetylcholine (ACh)-induced current measurements, rapid focal application of ACh was accomplished with a Picospritzer II (General Valve, Fairfield, NJ; 200-ms duration) and antagonists were applied by bath perfusion controlled with a BPS-4 valve system (ALA Scientific Instruments, Westbury, NY). One millimolar ACh, 1 μM prazosin, 100 μM mecamylamine (MEC), 10 mM methylylcysteine (MLA), and 1 μM dihydro-β-erythroidine (DHβE) solutions were prepared daily from frozen aliquots. All external aCSF included 1 μM tetrodotoxin (TTX, Alomone Laboratories, Jerusalem, Israel) and 1 μM atropine. The microelectrodes were filled with a cesium internal solution [in mM: 140 CsSO₃CH₃, 10 HEPES, 1 ethylene glycol-bis(2-aminooxy)ethane-N,N,N’,N’-tetraacetic acid (EGTA), 2 ATP, 0.1 GTP, 10 glucose, 5 tetraethylammonium-HCl, pH to 7.35 with CsOH].

For the synaptic modulation experiments measuring excitatory postsynaptic currents (EPSCs), 20 μM bicuculline was added to the aCSF to eliminate γ-aminobutyric acid (GABA)ergic transmission and a potassium gluconate internal solution was used. To measure inhibitory postsynaptic currents (IPSCs), 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX, Tocris, Ellisville, MO) was used to block AMPA-mediated currents and an internal solution with a higher chloride concentration was used (in mM: 78 K-gluc, 77 KCl in the K-glucenate internal solution). One micromolar nicotine was prepared fresh daily and applied to the slice via bath perfusion. All measurements of postsynaptic currents were performed in voltage clamp with a holding potential of ~70 mV. Cells were eliminated from these investigations if the input resistance or series resistance varied >15% during the course of the recordings.

Data analysis and statistics. Spontaneous EPSCs and IPSCs were analyzed with Mini-Analysis (Synaptosoft, Decatur, GA). Determination of “responsive” cells involved comparing the baseline frequency for a 1-min period immediately prior to nicotine application with a 1-min period centered on the peak nicotine effect by Student’s t-test. In the absence of a clear nicotine-induced change in frequency, the data were sampled from a 1-min window centered 1 min after the start of the nicotine application. This time point most commonly corresponds to the maximal effect of nicotine on synaptic transmission (Mansvelder et al. 2002; Mansvelder and McGhee 2000). The
change in synaptic transmission was quantified as the difference between the baseline and the average of 30 s of frequency data centered around the peak nicotine response.

All results are expressed as means ± SE. Graphs were produced with SigmaPlot version 10.0 (Systat Software, Richmond, CA).

AP frequency was analyzed with Mini-Analysis. Identification of responsive cells and response magnitudes used the same statistical methods described for assessing synaptic modulation. A χ²-test was used to compare the prevalence of nicotine responses between different populations of cells or between different experimental groups.

**Immunohistochemistry.** Tissue preparation for histology involved induction of deep anesthesia with isoflurane followed by perfusion with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) and 15% saturated picric acid (LabChem, Pittsburgh, PA) in 0.2 M phosphate buffer via the ascending aorta. Brains were removed and immersed in fixative solution (described above for perfusion) for 2 h and then cryoprotected in 30% sucrose for 72 h. Coronal sections (60 μm thick) were made with a cryostat from the NAc through the DRN. The NAc slices were immediately slide mounted for verification of injection sites with antifade mountant. For immunohistochemistry, DRN slices were washed, blocked with normal goat serum (NGS), permeabilized with 1% Triton X, and then exposed to a solution containing a tryptophan hydroxylase (TPH) antibody (1:400) (Chemicon, Temecula, CA), followed by an Alexa Fluor 488-conjugated secondary antibody (1:50) (Molecular Probes, Eugene, OR). Control experiments were conducted with secondary antibody alone, which did not yield cellular staining at this dilution. To visualize biocytin-filled cells after electrophysiological recording, sections were treated with Cy5-conjugated streptavidin (1:100) (Jackson Immuno, West Grove, PA). Images were obtained with Fluoview 200 software on an Olympus IX70 confocal microscope (Olympus America, Melville, NY) or with an Olympus IX81 inverted microscope using Metamorph software (Universal Imaging, Downingtown, PA).

**Drugs.** All reagents and chemicals were obtained from Sigma (St. Louis, MO), unless specified otherwise.

**RESULTS**

Fluoro-Red dye was injected bilaterally into the NAc (Fig. 1, B and C) and retrogradely transported to cell bodies located in the DRN (Fig. 1, D and E). Histology confirmed that injection cannula placement was in the NAc (Fig. 1C). Control studies performed by injecting dye dorsal to the NAc did not demonstrate any labeling of DRN neurons (data not shown).

Because the DRN is primarily a serotonergic nucleus, we investigated the percentage of backlabeled neurons that express the serotonin synthetic enzyme TPH. As expected, the majority of DRN neurons stained positively for TPH (Fig. 2A). In double-labeling experiments 74.5% of retrogradely labeled NAc projection neurons also expressed TPH (n = 1,378 backlabeled neurons counted in sections from 6 different rats; Fig. 2B).

To assess whether nAChRs contribute to excitability of DRN neurons, AP firing rate was measured with both whole cell and on-cell recording techniques. These experiments assessed nAChR effects only on neurons that displayed regular spontaneous AP firing (see MATERIALS AND METHODS for criteria). When recorded with normal aCSF, 4 of 15 neurons demonstrated a significant increase in firing rate in response to 1 μM nicotine to 190.66 ± 38.63% of baseline (Fig. 3). These data include seven cells that were backlabeled, and nicotine increased firing in two of these (250.3 ± 42.0% of baseline) and decreased firing in one (2.8% of baseline). There was no difference in the prevalence of either response to nicotine between backlabeled and randomly chosen neurons (P > 0.05, χ²-test).

Previous studies indicate that adrenergic input to the DRN affects baseline excitability of these neurons in vivo (Li et al. 1998). As these inputs are eliminated in our slices, the majority of neurons tested did not meet our criteria for regular spontaneous AP firing and were not tested with nicotine. To mimic

![Fig. 2.](http://jn.physiology.org/) The majority of cells in the DRN that project to the NAc are serotonergic. A: coronal section of the PAG stained for tryptophan hydroxylase (TPH; green), demonstrating TPH-positive cells clustered in the DRN at approximately bregma -8.6. B: overlay image of retrogradely labeled DRN neurons that project to the NAc (red) and TPH-positive cells (green) at approximately bregma -7.6. In the population of backlabeled DRN-NAc projection neurons, 74.5% were labeled for TPH (n = 1,378 backlabeled neurons from 6 rats).
endogenous adrenergic tone, and to increase the pool of testable cells, we repeated the above experiments in the presence of the α1-adrenergic agonist phenylephrine (12 μM), which resulted in a significant increase in the prevalence of spontaneously active neurons (phenylephrine: 14/16 neurons, aCSF: 5/46 neurons; $P < 0.0001$, χ²-test). In phenylephrine, nicotine induced an increase in firing rate in 5 of 14 cells to 194.37 ± 37.21% of baseline, a decrease in 2 cells (to 38.55 ± 29.85% of baseline), and no effect on the remaining neurons (Fig. 3, B and C). Of the eight backlabeled cells tested under these conditions, nicotine increased the firing rate in two cells (to 143.08 ± 8.39% of baseline) and decreased the firing in two cells. There was not a difference in the nicotine response prevalence in aCSF- versus phenylephrine-treated groups ($P > 0.05$, χ²-test).

To test mechanisms underlying the excitatory effects of nicotine on DRN neurons, we tested functional nAChR expression with focal application of 1 mM ACh. The average of the ACh-induced inward currents was 171.2 ± 36 pA in 60.7% (51/84) of the neurons tested. These included recordings from 17 backlabeled NAc-projecting DRN neurons, and 12 of these also responded. The prevalence of the ACh-responding DRN neurons was similar between randomly chosen (39/67) and backlabeled (12/17) neurons ($P = 0.4145$, χ²-test).

Next, we explored the pharmacology of the ACh effects on DRN neurons. The contribution of nAChR subtypes was tested by pretreating the slice with either the α3-selective antagonist MLA or the β2-nAChR antagonist DHβE. On average, MLA (10 nM) reduced the ACh current to 49.6 ± 8.9% of control ($n = 12$; Fig. 4, A and D). In the presence of DHβE (1 μM), the ACh current decreased to 56.4 ± 6.8% of control ($n = 14$; Fig. 4, B and D). The effects of both DHβE and MLA varied considerably between cells, suggesting that no one nAChR subtype dominates. These responses were entirely mediated by nAChRs, as the nonselective nAChR antagonist MEC (100 μM) blocked the responses completely (0 ± 0.9% of control, $n = 5$; Fig. 4, C and D). Consistent with this, the ACh response was completely inhibited when tested in the presence of both MLA and DHβE (not shown). Presynaptic nAChRs can modulate release of a number of different neurotransmitters including norepinephrine, which was shown to mediate nAChR-
induced excitatory effects in one study (Li et al., 1998). Under our recording conditions, pretreatment with the α1-adrenergic receptor antagonist prazosin (1 μM) had no effect on ACh-induced currents (98.8 ± 1.6% of control, n = 3; Fig. 4, C and D). Together, these data demonstrate that focal ACh application onto DRN neurons results in direct activation of nAChR-mediated excitatory inward currents, through activation of a mixture of α7- and β2-containing receptor subtypes.

Our lab has previously shown that nicotine enhances glutamate inputs to VTA dopamine neurons (Mansvelder and McGeeh 2000). To test for this phenomenon in the DRN, we recorded the effects of nicotine on the frequency of spontaneous EPSCs (sEPSCs). Inhibitory GABAergic transmission was blocked with 20 μM bicuculline in these experiments. Bath application of 1 μM nicotine increased sEPSC frequency in 7 of 19 neurons (192.47 ± 18.01% of baseline; Fig. 5) and had no effect in the other 12 neurons tested. Neither the prevalence nor the magnitude of this increase was significantly different between randomly chosen (4/10, 190.34 ± 19.01% of baseline) and backlabeled (3/9, 195.3 ± 39.26% of baseline) cells (P > 0.05, χ²-test, Student’s t-test, respectively). Under these conditions, the excitatory synaptic inputs to these neurons were glutamatergic, as all synaptic currents were eliminated when the preparation was exposed to 10 μM DNQX (data not shown).

Nicotine also modulates inhibitory GABAergic synaptic transmission in other areas of the midbrain (Mansvelder et al. 2002), so we investigated whether DRN neurons have similar effects. Spontaneous GABAergic IPSCs (sIPSCs) were recorded from DRN neurons by using an elevated intracellular chloride concentration to enhance their resolution. DNQX (10 μM) was included in the aCSF to inhibit AMPA receptor-mediated excitatory currents. A small percentage of DRN neurons (2 of 12) responded to 1 μM nicotine with an increase in sIPSC frequency (137.13 ± 9.02% of prenicotine baseline; Fig. 6). Furthermore, in 2 of 12 DRN neurons 1 μM nicotine decreased the sIPSC frequency (56.07 ± 3.93% of prenicotine baseline; Fig. 6B).

**DISCUSSION**

We explored the contribution of nAChRs to the excitability of DRN neurons, including a subset of DRN neurons that project to the NAc. We demonstrate that nicotine can increase the firing rate of DRN neurons and that this increased excit-
ability is mediated directly, via nAChRs expressed on the DRN neurons themselves, and indirectly by modulation of synaptic inputs to these neurons. In the subset of backlabeled DRN neurons that project to the NAc, we found similar physiological effects of nicotine. These findings support our hypothesis that nicotine may affect serotonergic signaling to the NAc.

Previous anatomic studies demonstrated the presence of α7-, α7-, and β2-nAChR subunits in the DRN (Bittner and Nikkel 2002; Bittner et al. 2000; Cucchiaro et al. 2005; Cucchiaro and Commons 2003). The nAChR α7-subunit was found on large-diameter DRN neurons (15–25 μm in diameter) that colocalized with TPH and small-diameter neurons (5–10 μm) that were GABAergic (Bittner and Nikkel 2002). Our investigation of the neurochemical makeup of DRN neurons that project to the NAc indicates that almost 75% were TPH positive. This indicates a strong serotonergic projection from DRN to the NAc, where serotonin may modulate reward-related signaling. Taken together, these data further support the influence of nicotine on serotonin signaling.

Our results are consistent with previous studies demonstrating nicotine-induced increases in DRN neuron firing rate and serotonin release in vitro (Mihaielae et al. 2001, 2002). In those experiments, nicotine increased excitability in 70% of DRN neurons, with a maximal effect at 2.15 μM. In our experiments, the slightly lower prevalence and magnitude of the excitatory effects may be due to a lower concentration of nicotine tested (1 μM), which is close to the arterial blood concentrations achieved after smoking (Henningfield et al. 1993).

Interestingly, in vivo systemic nicotine was reported to decrease the firing rate in the majority of DRN neurons and increased firing in only a small subset (Engberg et al. 2000). However, nicotine was without effect when micro-iontophoresed locally into the DRN. This does not agree with our results, but it is important to note that serotonergic neurons are strongly influenced by the animal’s arousal state (sleep-waking) (Ranade and Mainen 2009). Thus it is possible that the effect of nicotine on DRN neuron firing rate in vivo was influenced by anesthesia. Other groups have demonstrated nicotine-induced increases in firing rates in vivo in awake as well as anesthetized animals (Evrard and Changeux 2008; Huang et al. 2010; Schilstrom et al. 2003).

To assess the direct excitation of DRN neurons by nicotine, we tested nAChR expression with focal application of a saturating concentration of ACh in the presence of blockers of synaptic transmission. The nAChR responses had a mixed pharmacology, suggesting expression of α7- and β2-containing nAChR subtypes. In addition, there was variability in the antagonist sensitivity of the nAChR responses between individual neurons, suggesting considerable diversity of nAChR expression within the DRN. These findings are consistent with recent reports of nAChR expression in the DRN (Cucchiaro et al. 2005; Cucchiaro and Commons 2003; Galindo-Charles et al. 2008).

One study reports that the excitatory effects of nicotinic agonists in DRN neurons are dependent upon presynaptic modulation of norepinephrine release (Li et al. 1998). The recording conditions used by that group facilitated resolution of the indirect nAChR modulation of monoamine transmission. In contrast, our on-cell recording method provided assessment of firing rate, but not nicotine-induced changes in membrane potential. In our whole cell voltage-clamp recordings from DRN neurons that project to the NAc the average input resistance was 273 ± 27 MΩ, indicating that the 2- to 5-mV changes in membrane potential reported by Li et al. (1998) are mediated by currents that are at or below the limit of detection in voltage clamp. The slow time course of metabotropic receptor-mediated changes in holding current can also confound resolution of those currents. The lack of evidence for direct activation of nAChRs in that study could be attributed to differences in rat age or strain, or a slower drug application system, which could desensitize postsynaptic nAChRs. However, their data did show a nicotine-induced enhancement of serotonin release in DRN, which indirectly supports nAChR expression on serotonin neurons.

Nicotine modulates the release of various neurotransmitters via presynaptic nAChRs (Mansvelder et al. 2002), and we sought to extend previous studies by examining the influence of nicotine on glutamatergic and GABAergic input to DRN-NAc projection neurons. We observed that nAChR activation increased excitatory glutamatergic input, while the effects on inhibitory GABAergic input were minimal. These data taken together support a net excitatory role for nAChRs in the DRN through presynaptic modulation, as well as direct excitation.

The somatodendritic localization of nAChRs on DRN neurons does not guarantee that they mediate fast synaptic transmission (McGehee and Role 1996). However, the DRN is one of the few brain loci where synaptic currents mediated by nAChRs have been demonstrated by stimulating brain stem cholinergic nuclei (Galindo-Charles et al. 2008). Thus, in addition to the acute effects of exogenous nicotine, fast cholinergic synaptic transmission contributes to the excitability of serotonergic DRN neurons that project to the NAc.

Thus, in the context of previous work, our data suggest that acute exposure to nicotine excites DRN neurons by direct depolarization and indirect synaptic modulation. These effects were seen with high prevalence in DRN neurons backlabeled from NAc, and the majority of these projection neurons express serotonin. Therefore, we conclude that these effects likely contribute to nicotine-induced increases in serotonin release in the NAc.

This relationship between nicotine and serotonin release may have important implications in nicotine addiction, as serotonin modulates excitability of many brain areas, including those that are involved in reward and mood. Abundant serotonin receptor expression has been demonstrated in the NAc (Clemett et al. 2000) and activation of these receptors can enhance DA release in vivo (Benloucif et al. 1993). Serotonin receptor antagonists administered locally into the NAc inhibit cocaine-induced increases in locomotion (McMahon and Cunningham 2001). Furthermore, activating NAc serotonin receptors modulates the facilitation of reward by systemic cocaine in an intracranial self-stimulation paradigm (Katsidoni et al. 2011). Finally, a recent human PET scan study demonstrated lower levels of certain serotonin receptors in the NAc of patients with major depressive disorder compared with control subjects (Murrough et al. 2011), suggesting a contribution of dysfunctional serotonin signaling to that condition. Together these data support the idea that NAc serotonin contributes to mood and reward-associated behavior.

Given the excitatory effects of nicotine on DRN neurons, serotonin likely contributes to the rewarding effects of nico-
tine. In support of that idea, behavioral sensitization to repeated nicotine exposure was enhanced when inhibitory 5-HT_{1A} autoreceptors were blocked or desensitized, and this correlated with increased release of serotonin (Lanteri et al. 2009). Intracranial self-stimulation studies indicate that chronic nicotine lowers the brain reward thresholds, suggesting increased reward, whereas withdrawal from nicotine has the opposite effect (Harrison and Markou 2001). That study also showed reversal of withdrawal by SSRIs combined with a 5-HT_{1A} receptor antagonist, suggesting that decreased serotonergic tone is a key aspect of nicotine withdrawal.

In summary, we have shown that the serotonergic neurons arising in the DRN and projecting to the NAc express nAChRs. Furthermore, acutely administered nicotine increases the excitatory input to these neurons and ultimately increases their firing rate. The fact that nicotine increases DRN serotonergic output may help explain some of its influence on mood and affect, which ultimately contributes to the high relapse rate in smokers who are attempting to quit. Withdrawal from chronic systemic nicotine increases anxiety, which is relieved by nicotine in the DRN (Cheeta et al. 2001; Irvine et al. 2001). This anxiogenic effect of withdrawal is thought to be caused by a persistent increase in excitability of DRN neurons and increased serotonergic tone. Therefore, relief from nicotine withdrawal may involve changes in serotonergic tone selectively in brain regions mediating reward, such as the NAc.

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DISCLOSURES

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

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