

Nicotinic Activation of Laterodorsal Tegmental Neurons: Implications for Addiction to Nicotine

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Identifying the neurological mechanisms underlying nicotine reinforcement is a healthcare imperative, if society is to effectively combat tobacco addiction. The majority of studies of the neurobiology of addiction have focused on dopamine (DA)-containing neurons of the ventral tegmental area (VTA). However, recent data suggest that neurons of the laterodorsal tegmental (LDT) nucleus, which sends cholinergic, GABAergic, and glutamatergic-containing projections to DA-containing neurons of the VTA, are critical to gating normal functioning of this nucleus. The actions of nicotine on LDT neurons are unknown. We addressed this issue by examining the effects of nicotine on identified cholinergic and non-cholinergic LDT neurons using whole-cell patch clamp and Ca^{2+} -imaging methods in brain slices from mice (P12–P45). Nicotine applied by puffer pipette or bath superfusion elicited membrane depolarization that often induced firing and TTX-resistant inward currents. Nicotine also enhanced sensitivity to injected current; and, baseline changes in intracellular calcium were elicited in the dendrites of some cholinergic LDT cells. In addition, activity-dependent calcium transients were increased, suggesting that nicotine exposure sufficient to induce firing may lead to enhancement of levels of intracellular calcium. Nicotine also had strong actions on glutamate and GABA-releasing presynaptic terminals, as it greatly increased the frequency of miniature EPSCs and IPSCs to both cholinergic and non-cholinergic neurons. Utilization of nicotinic acetylcholine receptors (nAChR) subunit antagonists revealed that presynaptic, inhibitory terminals on cholinergic neurons were activated by receptors containing $\alpha 7$, $\beta 2$, and non- $\alpha 7$ subunits, whereas, presynaptic glutamatergic terminals were activated by nAChRs that comprised non- $\alpha 7$ subunits. We also found that direct nicotinic actions on cholinergic LDT neurons were mediated by receptors containing $\alpha 7$, $\beta 2$, and non- $\alpha 7$ subunits. These findings led us to suggest that nicotine exposure from smoking will enhance both the excitability and synaptic modulation of cholinergic and non-cholinergic LDT neurons, and increase their signature neurotransmitter outflow to target regions, including the VTA. This may reinforce the direct actions of this drug within reward circuitry and contribute to encoding stimulus saliency.

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INTRODUCTION

Nicotine is the addictive ingredient that drives increasing numbers of people to tobacco dependence. Understanding the mechanisms by which nicotine acts on the brain is fundamental for the development of effective therapies for nicotine addiction, and may also have implications for understanding the neural systems underlying addiction to other drugs of abuse. Investigations of the neurobiology of addiction to nicotine have so far largely focused on the mechanisms that underlie its reinforcing effects within the mesolimbic dopaminergic pathway, which encompasses the ventral tegmental area (VTA; Corrigall *et al*, 1994; Laviolette

and van der Kooy, 2003; Nisell *et al*, 1994) along with its projections to the nucleus accumbens (NAcc; Di Chiara and Imperato, 1988; Koob, 1996; Nestler, 2001; Redgrave *et al*, 1999) and the prefrontal cortex (Arnsten, 1997; Brozoski *et al*, 1979; Carr and Sesack, 2000a; Goldman-Rakic, 1995). This pathway, traditionally viewed as reward circuitry, responds to salient stimuli, and a major factor contributing to tobacco addiction is nicotine-induced modulation of dopamine (DA) release within this pathway (Dani and Heinemann, 1996; Di Chiara and Imperato, 1988; Koob, 1992; Nestler, 1992; Nisell *et al*, 1994; Picciotto, 1998; Picciotto *et al*, 1998; Pidoplichko *et al*, 1997). Although activation of nicotinic acetylcholine receptors (nAChRs) located in the VTA is critical for nicotine addiction (Calabresi *et al*, 1989; Clarke, 1993; Maskos *et al*, 2005; Picciotto *et al*, 1998; Pidoplichko *et al*, 1997; Pons *et al*, 2008; Wada *et al*, 1989), synaptic mechanisms both upstream and downstream of DA release from the VTA likely contribute to the etiology of nicotine addiction

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(Maskos, 2008; Mena-Segovia *et al*, 2008; Williams and Adinoff, 2008).

One likely important upstream regulator of the VTA is the pontine laterodorsal tegmental nucleus (LDT), which is a major source of the endogenous cholinergic input to the nAChRs and muscarinic receptors within the established VTA reward circuitry (Forster *et al*, 2002b; Mansvelder *et al*, 2002). Recent studies have shown that substantial direct input from cholinergic afferents targets DA-containing cells of the mesoaccumbal pathway (Omelchenko and Sesack, 2006), and that the morphology and synaptology of cholinergic axons within the VTA are similar to those observed for the projections arising from the LDT (Omelchenko and Sesack, 2005). Although terminals that contain acetylcholine (ACh) make up a substantial portion of the LDT-derived innervation to the VTA, data suggest that GABA and glutamate afferents to DA neurons also arise from the LDT (Omelchenko and Sesack, 2006). Neurochemical studies indicate that these projections are functional, as stimulation of the LDT results in DA efflux in the NAcc through cholinergic and glutamatergic mechanisms (Forster and Blaha, 2000). Moreover, a normally functioning LDT is required for the burst firing of dopaminergic VTA neurons (Lodge and Grace, 2006), the functionally relevant firing pattern that signals stimulus salience (Berridge and Robinson, 1998; Grace, 1991; Schultz, 1998). Finally, the LDT is involved in development of behavioral sensitization to amphetamine in rats, a finding which suggests the occurrence of a drug-induced, neuronal plasticity in the LDT (Nelson *et al*, 2007). On the basis of these and other studies (Alderson *et al*, 2005; Forster and Blaha, 2000; Forster *et al*, 2002a,b), the LDT is implicated in the neurobiology of addiction, in addition to its apparent role in arousal (Hobson and Pace-Schott, 2002; Jones, 2004; Pace-Schott and Hobson, 2002).

However, the electrophysiological responses of cells within this nucleus to drugs of abuse had not been previously described. As mRNAs for nAChR subunits are present in LDT neurons (Azam *et al*, 2003), we investigated the direct and indirect actions of nicotine on membrane potential, intracellular calcium levels, and synaptic activity in cholinergic and non-cholinergic LDT cells in mouse brain slices. We found that nicotine has excitatory actions in the majority of cells within the LDT, and that these actions varied depending on cell type and on anatomical location of the cell. As it has been shown that exogenous stimulation of this pathway stimulates DA release from VTA neurons (Forster and Blaha, 2000), we hypothesize that nicotine exposure from smoking will enhance both the excitability and synaptic modulation of cholinergic LDT neurons, and thereby increase cholinergic outflow to their target regions, including the VTA. These, and the actions on non-cholinergic LDT neurons could, therefore, have a role in the neuronal underpinnings of nicotine addiction.

MATERIALS AND METHODS

Slice Preparation and Artificial Cerebrospinal Fluid

All procedures complied with the NIH guidelines for ethical use of animals. In addition, all procedures were reviewed

and approved by the Institutional Animal Care and Use Committee of New York Medical College.

Brain slices were prepared from 12- to 45-day-old male and female C57BL/6NTac mice (Taconic, New York), as previously described (Kohlmeier and Leonard, 2006). Animals were decapitated after induction of deep anesthesia using isofluorane. A block of the brain containing the LDT was rapidly removed and incubated in ice-cold ACSF, which contained (in mM) 124 NaCl, 5 KCl, 1.2 NaH₂PO₄, 2.7 CaCl₂, 1.2 MgSO₄, 26 NaHCO₃, and 10 dextrose (295–305 mOsm), and was oxygenated by bubbling with carbogen (95% O₂, 5% CO₂). The brainstem was then blocked in a coronal plane and sectioned at 250 μ m on a Leica vibrotome (VT1000S). Slices containing the LDT were incubated at 35°C for 15 min and then stored at room temperature. Recordings were obtained from slices submerged in a recording chamber, which was perfused at 1–3 ml/min with the continuously oxygenated ACSF at room temperature.

Drugs

Nicotine bitartrate (Sigma, St Louis, MO, USA) was prepared with minimal light exposure on the day of the experiment to final concentration. Nicotine was applied by two different methods: by the bath at 1–10 μ M for 1–5 min, or by a 'puff' with controlled pressure pulses (10 mM, 35–1000 ms, 137.9 kPa; Picospritzer II, General Valve) from a patch pipette positioned just above the slice, but downstream from the cell from which recordings were being obtained. 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP, 50 μ M, Sigma), a specific nAChR agonist (Phelan and Gallagher, 1992) was applied by a puff (100 ms, 137.9 kPa). In those cases wherein calcium imaging was being conducted, to minimize any artifact due to moving of the dendrite, a lower puff pressure of 34.4 kPa was used. ACh was applied using the latter method (1 mM, 100–300 ms, 137.9 kPa) thus allowing very local application of drug with a quick wash in and wash out time.

Atropine, an irreversible muscarinic receptor antagonist, was obtained from Sigma and applied at a final concentration of 5 μ M. Atropine occasionally has actions at nAChRs (Zwart and Vijverberg, 1997); therefore, the lowest possible concentration of this antagonist should be used to try to limit blocking actions on muscarinic antagonism. In a separate series of experiments, we found that 0.5 μ M failed to completely block ACh-mediated outward currents in cholinergic LDT neurons (2.4–78.4% of outward current remaining, $n = 7$ of 7), putatively mediated by activation of muscarinic receptors (Luebke *et al*, 1993; Leonard and Llinas, 1994). We therefore bath applied 5 μ M atropine to assure that we completely blocked muscarinic responses, which is consistent with reports in these and other cells (Kohlmeier and Leonard, 2006; Zhang and Warren, 2002). Methyllycaconitine citrate (MLA, 10 nM, Sigma), a selective antagonist of $\alpha 7$ -subunit-containing nAChRs (Alkondon *et al*, 1992; McGehee and Role, 1995; Seguela *et al*, 1993), mecamylamine (MEC, 1 μ M, Sigma), a selective antagonist of non- $\alpha 7$ -subunit-containing nAChRs (McGehee and Role, 1995), and dihydro- β -erythroidine hydrobromide (DH β E, 500 nM, Sigma), another non- $\alpha 7$ antagonist, which is selective for $\beta 2$ -containing nAChRs (Alkondon and Albuquerque, 1993; Luetje *et al*, 1990), were dissolved in

ACSF at a stock concentration and dissolved to the final concentration in ACSF on the day of the experiment. TTX (500 nM, Alamone) was dissolved in the superfused ACSF to block voltage-gated sodium channels. The ionotropic receptor antagonists, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 15 μ M, Sigma), D(-)-2-amino-5-phosphopentanoic acid (APV, 50 μ M, Sigma), bicuculline (10 μ M, Sigma) or SR-95531 (Gabazine, 20 μ M, Sigma), and strychnine (2.5 μ M, Sigma), were added to ACSF in some recordings (Burlet *et al*, 2002). For low calcium solution recordings, the $[Ca^{2+}]$ of the ACSF was buffered to $<20 \mu$ M by the addition of 2.7 mM EGTA (calculated with Patcher's Power Tools XOP for Igor Pro (Wavemetrics, Lake Oswego, OR)).

Whole-Cell Electrophysiological Recording and Calcium Imaging

Micropipettes (2–4 M Ω) used for patch clamp recordings (Borosilicate, Catalog number 8050, AM systems) were pulled on a horizontal puller (Sutter Instruments, P-2000). In those cases in which calcium imaging was not being conducted, pipettes were filled with a recording solution containing (in mM) 144 K-gluconate, 0.2 EGTA, 3 MgCl₂, 10 HEPES, 0.3 NaGTP, and 4 Na₂ATP (310 mOsm), or, in those cases in which calcium imaging was being conducted, pipettes contained the potassium salt of Fura-2 (*bis*-fura 2, 50 μ M, Molecular Probes), which was dissolved in the patch pipette solution of (in mM) 144 K-gluconate, 3 MgCl₂, 10 HEPES, 0.3 NaGTP, and 4 Na₂ATP. Recordings conducted to examine IPSC activity were performed using a high chloride recording solution to optimize detection of inhibitory events by increasing the drive for, and reversing the polarity of, chloride-mediated currents (in mM) 144 KCl, 0.2 EGTA, 3 MgCl₂, 10 HEPES, 0.3 NaGTP, and 4 Na₂ATP. In all cases, biotinylated Alexa-594 (25 μ M) was also included in the patch solution so that recovered cells could be histochemically identified after recordings.

Neurons were visualized for whole-cell recordings at $\times 160$ magnification using visible light, differential interference contrast optics, using a Nuvicon tube camera (Dage VE-1000) mounted on a fixed-stage microscope (Olympus BX50WI). Cells for recording were chosen within the nucleus of the LDT, which were identified using a $\times 4$ objective; however, locations of recorded neurons within the LDT were verified after the tissue had been processed for cell identification.

Seals of the pipette to the cellular membrane exceeding 1 G Ω in resistance were obtained under visual control using an Axopatch 200B amplifier (Axon Instruments) operated in voltage-clamp mode and filtered at 2 or 5 KHz using a four-pole Bessel filter at the amplifier output and sampled at 4, 10, or 20 KHz. After establishing the whole-cell recording configuration, cells were filled with both the calcium indicator (when present) and biotinylated Alexa-594 by either passive diffusion or brief, hyperpolarizing pulses. Data were not collected until at least 20 min had passed after breakthrough to allow dye equilibration. Neurons were imaged through a $\times 40$ objective using a cooled, CCD camera equipped with a back illuminated EEV 57 frame transfer chip with an imaging area of 512×512 pixels (field size = 160 μ m per side; MicroMax, Roper Scientific).

bis-Fura 2 and Alexa-594 were excited at 380 and 594 nm, respectively, with light from a 75 W Xenon lamp that was shuttered to reduce light exposure to the tissue in-between data acquisition. Recordings were either conducted in voltage clamp or 'I-clamp fast' current-clamp mode, after appropriate compensation for the pipette capacitance; quality of the cells was assayed by monitoring the holding current and the input resistance, as determined by the voltage or current response to a brief, negative-going step. Recordings were uncorrected for liquid junction potentials, which were calculated to be ~ 12 mV. Current and voltage traces were digitized and command pulses were generated by custom designed software (TIWB; Inoue *et al*, 2001) executed by a Mac OS/G4 computer, which controlled an ITC-18 interface (Instrutech). The TIWB software also controlled the camera and shutter, which allowed precise synchronization between electrophysiological and optical signals. The camera was read out through a 1-MHz, 14-bit A/D converter. Images were binned (4×4) on the chip and acquired in two different modes: (1) 'discontinuous', a low-speed imaging mode used to monitor changes in calcium associated with depolarizations or inward currents during which an image was collected every 4 s or, (2) 'continuous', a high-speed imaging mode in which an image was collected every 50 ms, a rate fast enough to monitor changes in calcium-accompanying rapid alterations of the membrane potential. Changes in fluorescence (dF/F) were quantified by the average pixel values within regions of interest (ROI) that were positioned on background- and baseline-subtracted fluorescent images. The background was determined from a ROI that was positioned at a location remote from the filled cell or its processes. Baseline fluorescence was determined as the fluorescence measured in the first few frames of each sequence before stimulation. As dF/F traces recorded from dendrites have a low signal to noise ratio, each data point in this analysis represents an average of 10 frames. *bis*-Fura 2 fluorescence decreases when calcium increases and is bound by the dye; however, for purposes of clarity, dF/F responses have been inverted in figures such that positive dF/F traces indicate rises in calcium.

Electrophysiological Analysis

Current and voltage waveforms were analyzed and figures were prepared using Igor Pro Software. Holding currents were calculated from 1-s averages of the holding current at -60 mV. Differences between means were compared using a two-sample, two-tailed *t*-test corrected for multiple comparisons (when necessary) and repeated-measures ANOVA. Detection of PSCs and some analyses were done using Mini Analysis software (Synaptosoft, Decatur, GA). To compare the effects of nicotine on the number and amplitudes of PSCs, epochs of at least 30 s were used before application of nicotine (control) and after effects of nicotine (drug). Cumulative distributions of EPSCs or IPSCs were then compared for significant differences between the control and nicotine condition in individual cells using Kolmogorov–Smirnov (K-S test) statistics. Mean increases (% elevation over control) are reported for the population of cells that showed individual significance using the K–S statistic. As only effects on increasing PSC frequency were observed, a paired, one-tailed *t*-test was used to quantify changes in this

parameter for histograms. A two-tailed, paired *t*-test was used to quantify changes in PSC amplitude for the data reported in the histograms. Statistical significance was determined by $p < 0.05$. All numerical results are reported as mean \pm SEM.

Histochemistry

Cholinergic LDT neurons express high levels of neuronal nitric oxide synthase (nNOS) and presence of this enzyme can serve to identify cholinergic neurons in the LDT (Bredt *et al*, 1991; Vincent and Kimura, 1992; Vincent *et al*, 1983), and, although by no means a homogeneous nucleus (Wang and Morales, 2009), presence of the cholinergic neurons has been proposed to delineate the nucleus (for example, see Leonard *et al*, 1995). During recordings, neurons were filled with Alexa-594, which passively diffused into the cell. After the electrophysiological recordings, the tissue was fixed in 4% paraformaldehyde at 4°C, and then stored in 0.01 M phosphate-buffered saline and 30% sucrose for cryoprotection. Slices were then resectioned at 40 μ m and incubated during gentle shaking at 4°C for 24 h in darkness with a nNOS antibody (Sigma, Catalog number N7280, rabbit polyclonal antibody, 1:400) followed by incubation with the FITC-conjugated secondary antibody (nNOS A11008, goat anti-rabbit antibody, Molecular Probes). Further histochemical processing was not necessary to visualize the recorded Alexa-594-filled cell, which was viewed under a Texas-red filter-cube set.

RESULTS

Identification of Cholinergic vs Non-Cholinergic Neurons

All data in this report were obtained from neurons located within a region delineated by the cholinergic neurons in the LDT. As in previous studies (Kohlmeier and Leonard, 2006; Kohlmeier *et al*, 2008), cholinergic neurons from which recordings were obtained were those identified as both nNOS+ (Figure 1a1) and Alexa-594+ (Figure 1a2) under fluorescence optics (Figure 1a, arrowhead). Non-cholinergic neurons from which recordings were obtained were identified as Alexa-594+ and nNOS−.

Nicotine Directly Depolarizes Cholinergic and Non-Cholinergic LDT Neurons

Bath application (1 min) of 10 μ M nicotine resulted in a depolarization in cholinergic cells (6.8 ± 1.5 mV, $n = 10$ of 10) and non-cholinergic neurons (6.9 ± 1.5 mV, $n = 8$ of 8) when recorded in current-clamp mode. Depolarization was accompanied by a decrease in input resistance that was significant in both cell types (nNOS+ cells, $13.5 \pm 6.6\%$, $n = 7$; nNOS− cells, $26.4 \pm 4.8\%$, $n = 7$). Repeat actions of nicotine could be elicited if at least 15 min elapsed between applications. Often, bath application of nicotine produced sufficient depolarization to induce action potentials ($n = 10$ of 18). Nicotine never induced a membrane hyperpolarization.

Although delivery of nicotine through the bath may more accurately mimic the exposure profile of a smoker's brain

by slowly ramping up and remaining constant for some time at the final drug concentration (Henningfield *et al*, 1993), the relatively gradual exposure to an increasing nicotine concentration may result in desensitization of the receptors, thus blunting the potential maximal response elicited by the agonist. In addition, nicotine is known to have presynaptic actions in other nuclei, in which this has been studied. To facilitate the detection of maximal responses and to limit responses to postsynaptic receptor activation, we examined the actions of nicotine on membrane potential in the presence of blockers of GABA and ionotropic, glutamatergic receptors while using a more rapid introduction of nicotine into the slice. Accordingly, 10 mM of nicotine was puffed onto cells (40–60 ms) through a picospritzer in the presence of blockade of the NMDA, AMPA, glycine, and GABA_A receptors with inclusion in the ACSF of APV, DNQX, strychnine, and bicuculline, respectively. Nicotine, applied in this manner, induced depolarization in cholinergic (14.7 ± 2.2 mV, $n = 21$ of 21; Figure 1b) and non-cholinergic neurons (9.7 ± 4.4 mV, $n = 2$ of 2). In some cases, depolarization was sufficient to induce action potentials (nNOS+ cells, $n = 15$ of 21, in all cells recorded, $n = 16$ of 23; Figure 1b1). The nicotine-induced depolarization after puffer application was never reduced by TTX (21.8 ± 5.3 mV, $n = 9$; unpaired *t*-test, $p > 0.05$; Figure 1b and c); however, action potentials were abolished when TTX was included in the bath even in those cases in which similar levels of depolarization of the membrane were achieved by nicotine ($n = 9$; Figure 1b2). Taken together, these data suggest that a brief, local application of nicotine causes depolarization by the activation of nicotinic receptors on the postsynaptic cell and that spikes elicited are mediated by activation of voltage-activated sodium channels, rather than voltage-operated calcium channels, which have been shown to be activated in these cells by other excitatory neuroactive substances (Kohlmeier *et al*, 2008).

Nicotine Enhances Cellular Excitability in LDT Neurons

In addition to a direct depolarizing effect, nicotine may also alter excitability of LDT neurons. To test this, we examined whether the sensitivity to injected current was altered by nicotine in these neurons. Cholinergic LDT neurons contain A-type outwardly rectifying potassium conductances (Kamondi *et al*, 1992; Leonard and Llinás 1990; Sanchez *et al*, 1998), which nicotine has been shown to inhibit (Hamon *et al*, 1997; Wang *et al*, 2000), resulting in heightening the excitability of the cell through an NMDA-involved mechanism, as shown in cultured hippocampus neurons (Kim *et al*, 2007). To avoid this possible complication, we tested the ability of nicotine to alter the sensitivity to injected current by looking at firing rates when the cell was stepped to a more depolarized potential by the injection of current in the presence of blockade of excitatory and inhibitory transmission using APV, DNQX, strychnine, and bicuculline. Using the same current step, and using DC current to bring the drug-treated cell back to baseline membrane potential to avoid contamination by voltage-activated conductances and the heightening of excitability induced by depolarization, we found that a slightly higher, but significant firing rate was achieved in

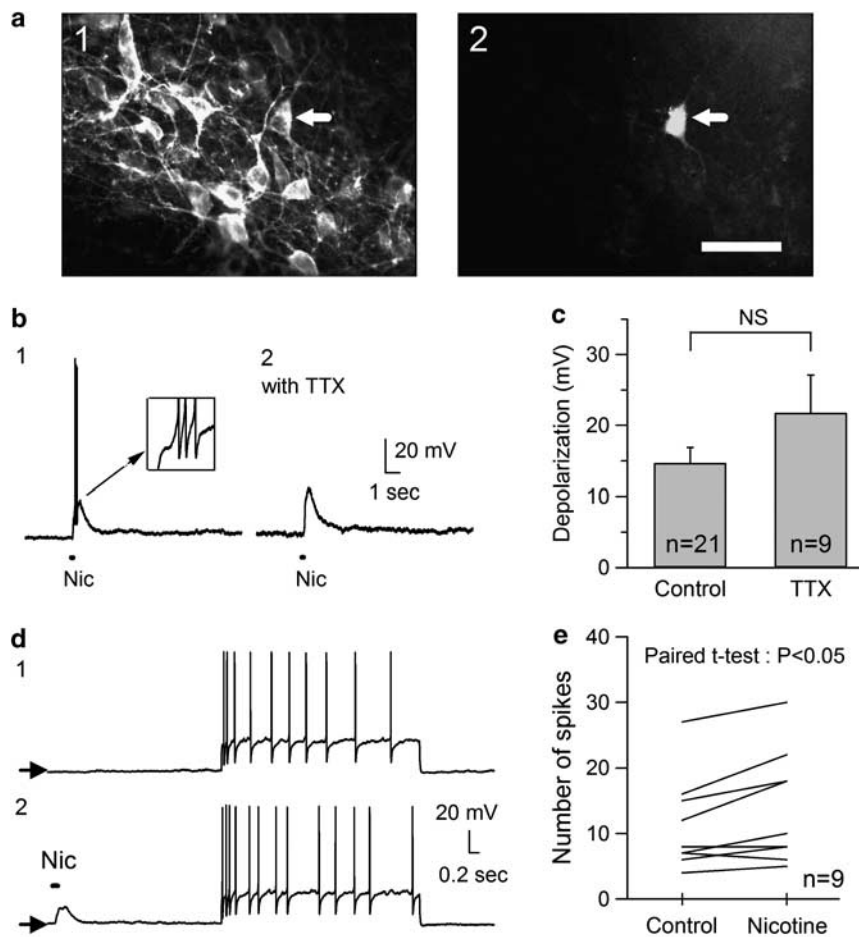


Figure 1 Nicotine excites cholinergic LDT neurons and enhances sensitivity to injected current. (a) Cells from which electrophysiological recordings were obtained in this study were determined to be cholinergic based on presence of nNOS+. Fluorescent images from a field of the LDT from one cell from which data are presented in this report (arrow). Comparison of the field in the right panel (Alexa-594, a2) and the field in the left panel (nNOS, FITC, a1) indicates that this recorded cell is nNOS+ and, therefore, cholinergic. Scale bar, 50 μ m. (b1) Puff application of nicotine (10 μ M, 40 ms) elicited depolarization in the majority of LDT neurons, which was often sufficient to elicit action potentials when cells were recorded in current-clamp mode. Inset depicts recording at a higher gain detailing individual action potentials arising from the nicotine-induced depolarization. (b2) In the presence of TTX to block action potential generation, depolarization persisted, but spikes were abolished. (c) Summary of nicotine-induced depolarization in the presence and absence of block of sodium-dependent action potentials across a population of cells. TTX did not block induction of depolarization by nicotine. (d) Response of one cell before (1) and after (2) nicotine puff application after the injection of a fixed step of positive current. The membrane potential of the cell was brought back to control levels (−65 mV at arrow) so as to remove the influence of depolarization on the firing response. (e) Paired plots of the number of spikes in control and nicotine conditions for each cell, highlighting that nicotine induced an increase in the number of spikes elicited by an identical current pulse in the majority of cells tested.

the presence of nicotine (22.5% increase in firing frequency, $n=9$; nNOS+ cells; paired t -test, $p<0.05$) (Figure 1d and e). In other cell types, nicotine has been shown to lower the threshold for firing, which may be one mechanism altering the nicotine-induced sensitivity to the injected current. However, the threshold for firing was not significantly altered during nicotine exposure in LDT neurons (control, -40.7 ± 0.8 mV; nicotine, -41.1 ± 1.1 mV; $n=9$; $p>0.05$). These data indicate that nicotine does have specific actions on cellular excitability of LDT neurons. We conclude that the mechanism of nicotine-induced enhancement is not dependent on activation of the NMDA receptor, nor on induction of EPSP or IPSC activity in the postsynaptic cell and did not depend on alteration of the threshold of action potential induction, as has been the one reported action of nicotine in other cell types (Kawai *et al*, 2007; Mansvelder *et al*, 2002).

Nicotine Induces an Inward Current in LDT Neurons

In voltage-clamp mode in the presence of bicuculline, APV, DNQX, strychnine, and TTX, puffer-applied nicotine induced an inward current in all recorded cells (nNOS+ cells, 551.5 ± 113.6 pA, $n=16$; nNOS− cells, 598.1 ± 224.2 pA, $n=6$; Figure 2a). Amplitudes of nicotine-induced inward currents were not statistically different between nNOS+ and nNOS− cells ($p>0.05$). An inward current was also induced by the specific nAChR agonist, DMPP. Puffer-applied DMPP (50 μ M; 100 ms; same recording conditions as above) elicited inward currents in all cholinergic LDT neurons recorded (33.6 ± 3.45 pA, $n=9$ of 9, $p<0.05$; Figure 2b). As nicotine-induced inward currents persisted in the presence of low calcium solution (1.5 mM Ca, 2.7 mM EGTA, $n=5$), these data indicate that nicotine-induced depolarization/inward currents resulted from a postsynaptic

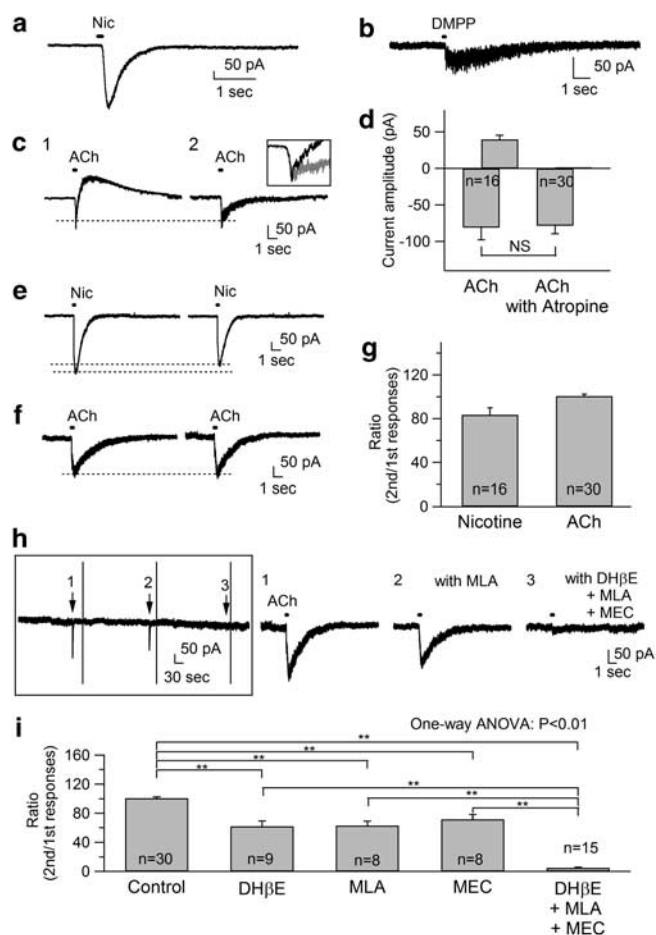


Figure 2 Nicotine and ACh induce inward currents mediated by nicotinic receptors containing the $\beta 2$ and $\alpha 7$ subunits. (a) Nicotine application (40 ms, puff) induces an inward current in nNOS+ cells that persists in presence of blockers of excitatory and inhibitory synaptic transmission (bicuculline, APV, strychnine, and DNQX with TTX). (b) DMPP in the presence of blockers of fast synaptic transmission also induces an inward current. (c1) ACh induces a fast inward current, followed by an outward current in nNOS+ cells; however, when atropine is added to the bath to block muscarinic receptors, only an inward current is elicited, which is repeatable after a wash out of agonist for 10 min (c2). (c2, inset) High gain detail of inward currents in absence (black) and presence of atropine (gray). (d) Summary detailing the current responses to application of nicotine and ACh in the absence and presence of atropine. The inward current induced by ACh in the presence of atropine did not differ significantly from that induced in absence of the muscarinic antagonist. (e) Second applications of nicotine induced an inward current that was on average 80% of control levels, whereas, responses to second applications of ACh were fully repeatable (f). (g) Summary detailing the ratio of the amplitude of inward current induced by the first application of nicotine or ACh with atropine and inward current elicited by the second application. (h) High and low gain (inset) current responses to ACh (\downarrow) in the absence and presence of MLA. A cocktail of three nAChR antagonists with atropine nearly abolished the nicotinic response of these cells to ACh. (i) Summary data indicating that DH β E, MLA, and MEC were all effective at reducing ACh-stimulated inward currents by approximately 30–40% (ANOVA, $p < 0.01$). These data indicate that cholinergic LDT neurons contain $\beta 2$ and $\alpha 7$, as well as non- $\alpha 7$ subunits, $^{**}P < 0.01$.

action rather than from stimulation of nicotinic receptors on presynaptic terminals.

We also found that bath application of 10 μ M nicotine in conjunction with TTX and blockers of excitatory and inhibitory transmission, also resulted in inward currents

in LDT neurons (15.9 ± 6.8 pA, $n = 12$, data not shown). The nicotine-induced inward current was not significantly different between cell phenotypes (nNOS+ cells, 12.2 ± 4.7 pA, $n = 5$; nNOS– cells, 22.7 ± 19.6 pA, $n = 4$; $p > 0.05$). Nicotine-induced inward currents exhibited a reversal potential of -21.6 ± 3.2 mV in nNOS+ cells ($n = 6$) and of -24.6 ± 2.5 mV in nNOS– cells ($n = 4$) consistent, in both cell types, with the opening of cationic channels permeable to Na^+ , K^+ , and/or Ca^{2+} , as has been reported for nAChRs.

ACh Induces an Inward Current in LDT Neurons

The action of ACh, the endogenous ligand for nAChRs, was also examined. We and others have shown that cholinergic stimulation of LDT neurons results in activation of muscarinic receptors, which activates an inwardly rectifying potassium conductance resulting in outward currents (Leonard and Llinas, 1994; Luebke *et al*, 1993; Kohlmeier and Leonard 2006). ACh (Puff; 1 mM) induced a rapid inward current followed by an outward current in nNOS+ cells (Figure 2c). To prevent ACh-induced outward current, we used atropine, which blocks muscarinic receptors. As expected, in the presence of 5 μ M atropine, ACh elicited only an inward current in the same cholinergic cells in which, in the absence of atropine, an outward current was seen (78.3 ± 10.9 pA, $n = 30$; Figure 2c and d). The amplitude of the ACh-induced inward current was not statistically different from that elicited by ACh when atropine was absent (80.9 ± 16.8 pA, $n = 16$; unpaired t -test, $p > 0.05$, Figure 2d), suggesting that atropine did not block ACh-mediated responses at nAChRs. Taken together, along with our DMPP and nicotine findings that these two nAChR agonists elicited only inward currents, we conclude (1) that ACh-induced outward currents resulted from stimulation of muscarinic receptors known to be present on these cells and (2) because the amplitude of ACh-induced inward current was not significantly different between atropine and control conditions, that atropine was not blocking ACh activation of nAChRs at the concentration we used. Given the caveat that equipotent concentrations may not have been used, the amplitudes of ACh-induced inward currents were not compared with those elicited by nicotine. Comparison of inward currents elicited by subsequent puffs of ACh or nicotine revealed attenuation in inward current induced by second applications of nicotine across a population of cells (second responses to nicotine were $83.4 \pm 6.3\%$ of the first response, $n = 16$), although individual cells could show second responses that were nearly or more than 100% of the first ($n = 7$ of 16; Figure 2e and g). ACh responses to second applications were not significantly different than initial responses (second responses to ACh were $100.6 \pm 1.7\%$ of the first response, $n = 30$; Figure 2f and g), indicating that ACh evokes little receptor desensitization or use-dependent actions at the nicotinic postsynaptic receptor (Figure 2g).

Subunit-Specific Antagonists Attenuate Nicotinic Currents in Cholinergic LDT Neurons

Cholinergic neurons within the LDT have been reported to express mRNA for $\alpha 7$ and $\beta 2$ subunits; mRNA for the $\alpha 4$ subunit was shown to be present in non-cholinergic

neurons (Azam *et al*, 2003). However, functional evidence regarding the nicotinic subunits comprising nAChRs in the LDT is lacking. Therefore, the subunits of the nAChRs mediating inward currents on postsynaptic cells were explored pharmacologically within the LDT. Given that nicotine effects were not consistently repeatable on induction of inward currents, as has also been shown in other cells types (Giniatullin *et al*, 2000), but ACh actions on induction of inward currents were repeatable, we used ACh for this series of studies. Antagonists were determined to be effective if the second application of ACh in the presence of antagonist resulted in inward currents reduced by two standard deviations (18%) of those elicited by the first application of agonist alone.

Using these criteria in ACSF containing TTX, bicuculline, APV, strychnine, and DNQX, DH β E, a β 2 nAChR subunit antagonist, at a concentration shown by others to fully antagonize responses mediated by β 2 nAChR subunits (500 nM; Mansvelder *et al*, 2002), significantly reduced the nicotine-induced inward currents in nNOS+ cells by $37.9 \pm 7.5\%$ (ANOVA, $p < 0.05$; $n = 9$). This suggests that in cholinergic neurons, not only do nAChRs containing the β 2 nAChR subunit contribute to the current response but other subtypes are also probably involved. Consistent with this interpretation, MLA, at a concentration specific for antagonism of the α 7 receptor, also significantly reduced postsynaptic responses by $37.1 \pm 6.1\%$ (ANOVA, $p < 0.05$; $n = 8$; Figure 2h2 and i). Responses resistant to either MLA or DH β E could be mediated by nAChRs devoid of the β 2 or α 7 subunit, and we found that MEC at a concentration specific for antagonism of non- α 7 subunits reduced the postsynaptic inward current by $28.4 \pm 6.9\%$ (ANOVA, $p < 0.01$; $n = 8$). A cocktail of DH β E, MEC, and MLA reduced postsynaptic receptor-induced currents by $95.0 \pm 1.1\%$ (ANOVA, $p < 0.01$; $n = 15$; Figure 2h3 and i). These data are summarized in Figure 2h and show that β 2 and α 7 subunits are present in nAChRs located on cholinergic neurons, providing pharmacological evidence that receptors containing these subunits are functionally expressed. In addition, our results provide the first functional data that activation of nAChRs in LDT neurons results in induction of inward currents mediated by the β 2 and α 7 subunits and that subunits other than the β 2 or α 7 also comprise nAChRs on these cells.

Nicotine Induces Changes in Intracellular Calcium

The calcium permeability of nAChRs depends on the composition of their subunits. Homopentameric receptors containing the α 7 subunit exhibit a high relative permeability to calcium (Bertrand *et al*, 1993; Seguela *et al*, 1993), whereas, nAChRs comprised of the α 4 β 2 subunits are less permeable to calcium. The presence of α 7 subunit in nAChRs on cholinergic neurons led us to ask whether nicotinic activation of these cells results in increases in intracellular calcium ($[Ca^{2+}]_i$). Accordingly, we assayed nicotine-mediated changes in $[Ca^{2+}]_i$ by monitoring changes in baseline and activity-dependent $[Ca^{2+}]_i$ using a calcium-sensitive dye, *bis*-Fura 2. After the cell was filled passively with *bis*-fura 2 through the recording electrode, a ROI overlying the soma or dendrite(s) was selected and changes in fluorescence was monitored within this area by

collection of images of the ROI at every 50 ms. We found that nicotine failed to significantly increase baseline calcium levels in somata of LDT cells ($n = 18$). These data did not rule out calcium-induced changes in the dendrites, and interestingly, we found that in about half of the cells recorded, all of which were identified as nNOS+, nicotine induced a significant increase in the amplitude of baseline calcium in dendrites (all cells: $n = 10$ of 18; nNOS+ identified: $n = 10$ of 10, 1.6% rise in dF/F in identified cholinergic neurons, $p < 0.05$; Figure 3b1). This effect was repeatable (nNOS+, $n = 6$ of 6, Figure 3b2). These rises were significantly attenuated by application of MLA to the

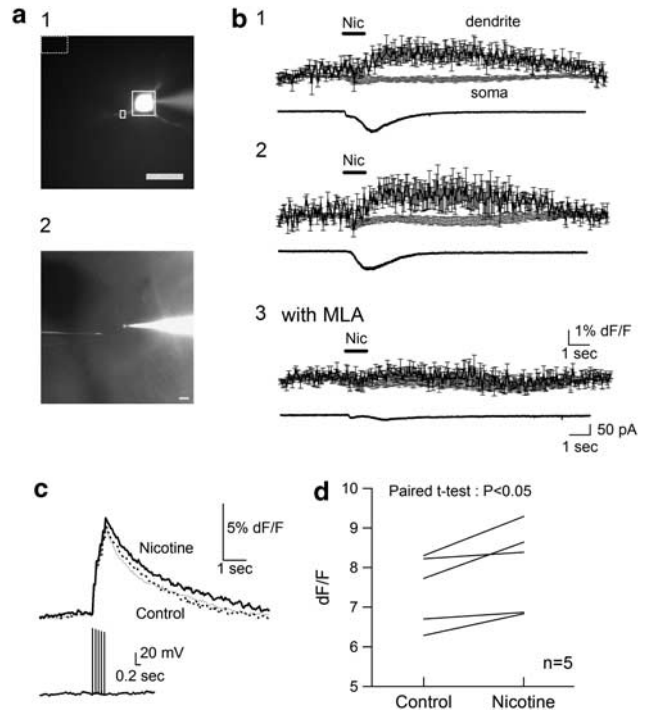


Figure 3 Nicotine induces rises in baseline calcium in dendrites and alters activity-dependent calcium measured at the soma. (a1) High gain ($\times 40$) image under 380 nm fluorescence showing a *bis*-fura 2-filled cell with ROI overlaid on a dendrite and the soma to indicate regions from which changes in dF/F were monitored. Low gain ($\times 4$) fluorescent image of excitation of the slice using light of appropriate wavelength (594 nm) to allow visualization of the marker dye, Alexa-594 (a2). The region of the LDT from which recordings were obtained can be seen. Scale bar, 40 μ m (a1). Scale bar, 100 μ m (a2). (b1) Puff application of nicotine induced a change in dF/F in some of the dendrites of cholinergic LDT neurons ($n = 10$ of 18), but failed to elicit a similar change in the somas of these cells ($n = 18$). Nicotine-induced inward currents are shown below somatic and dendritic dF/F traces in this nNOS+ -identified cell. (b2) Effects of nicotine were repeatable, and inhibited by pre-incubation of the tissue in MLA, an inhibitor of α 7-subunit-containing nAChRs (b3, $n = 7$ of 7). All baseline calcium recordings were carried out in the presence of blockade of excitatory glutamate and inhibitory GABAergic transmission, as well as TTX to block sodium-dependent action potentials. (c) The enhanced response in dF/F elicited in one cell induced by firing of five spikes (8 Hz) after the application of nicotine show that nicotine can induce changes in activity-dependent calcium at the soma. This effect of nicotine on calcium was recovered (dotted line). Response of the membrane potential to this protocol is also shown. (d) Paired plots showing the control dF/F and that following nicotine application for each cell, indicating that nicotine significantly increased dF/F induced by action potentials in all cells tested ($n = 5$, paired t -test, $p < 0.05$), suggesting that activity-dependent calcium was enhanced by nicotine.

slice before nicotine exposure ($n = 7$ of 7; Figure 3b3). These data indicate that there are $\alpha 7$ -subunit-containing nAChRs in the dendrites of cholinergic neurons and that activation of these receptors stimulates increases in dendritic $[Ca^{2+}]_i$. Several sources of Ca^{2+} might have contributed to these increases, including influx through $\alpha 7$ -subunit-containing nAChRs, activation of voltage-gated Ca^{2+} channels, and Ca^{2+} -evoked Ca^{2+} release.

In a second series of experiments, we monitored nicotine-induced changes in spike-evoked calcium levels. Positive current pulses sufficient to trigger five action potentials were injected through the pipette into the soma (5 Hz) and changes in fluorescence resulting from these spikes were monitored before and after bath application of nicotine (5 min). In the majority of neurons, when glutamatergic and GABAergic input were blocked by inclusion of bicuculline, strychnine, APV, and DNQX in the bath, nicotine significantly enhanced the amplitude of activity-dependent $[Ca^{2+}]_i$ changes in cholinergic cells ($108.0 \pm 0.2\%$, $n = 5$; Figure 3c and d). In addition, nicotine significantly and reversibly enhanced the amplitude of dF/F measured during the decay phase by $138.7 \pm 10.4\%$ (66% of the peak during the decay, $p < 0.05$, $n = 5$; Figure 3c), an effect which was recovered. Taken together, these data indicate that, although nicotine does not alter baseline calcium levels at the soma, if firing occurs, resultant somatic calcium levels will be enhanced and remain higher for a longer period of time than in the absence of nicotine, which could have important implications for nicotine-induced, activity-dependent, synaptic plasticity.

Nicotine Activates Presynaptic Glutamatergic Neurons

Nicotine also induced robust excitatory synaptic activity that was sensitive to blockers of fast, glutamatergic transmission (Figure 4a). Second applications of nicotine failed to induce an increase in synaptic activity in which first applications had induced a significant enhancement of EPSC events. Given that repeat application of nicotine induced inward currents, although sometimes these currents were attenuated, this suggests differences in desensitization kinetics between mechanisms involved in nicotine-induced inward current and synaptic activity. In a striking difference in effect of agonists, puffer application of ACh sufficient to activate inward currents did not induce synaptic activity in naïve cells, whereas in the same cells, subsequent application of nicotine in the presence of atropine induced synaptic activity ($n = 3$; Figure 4c). Several possible explanations for this lack of effect exist, including rapid hydrolysis of ACh or differences in activation or desensitization between ACh and nicotine acting at differing combinations of subunits comprising the nAChRs (Albuquerque *et al*, 1997; Alkondon and Albuquerque, 2006; Alkondon *et al*, 1997b; Bradaia and Trouslard, 2002; Fenster *et al*, 1997; Gopalakrishnan *et al*, 1995; Virginio *et al*, 2002), mediating inward currents and synaptic activity, although nicotine and ACh have been shown in other cell types to have equivalent actions on induction of PSCs (Mansvelder *et al*, 2002; Sharma *et al*, 2008). More rapid catabolization of ACh may explain this difference in cholinergic LDT neurons, as, although ACh applied in the presence of 1 μ M eserine with or without atropine did not

result in differences in inward current (ACh, 80.9 ± 16.7 pA, $n = 16$; ACh in atropine, 72.2 ± 18.3 pA, $n = 7$; ACh in eserine with atropine, 85.6 ± 17.4 pA, $n = 6$; unpaired *t*-test; $p > 0.05$), an increase in synaptic activity was sometimes seen during acetylcholinesterase blockade ($n = 6$). Our data suggest that more rapid breakdown of ACh may, in part, account for our observed differences in agonist effect and raise the possibility that presynaptic sites of nAChRs are protected from exogenous ACh. Regardless, in order to focus on nicotine, the drug of abuse, and to avoid the use of atropine, only first applications of nicotine were used for the quantification of spontaneous synaptic activity induced by activation of nAChRs.

In the presence of bicuculline and strychnine to block inhibitory input, in the majority of cells, puffer application of nicotine induced a significant increase in the frequency of spontaneous EPSCs (sEPSCs; K-S test on individual cells, $p < 0.05$, $n = 13$ of 14). The mean frequency increase was $438.7 \pm 118.8\%$ ($n = 13$, Figure 4a). Mean amplitude was significantly increased in 8 of 14 cells (K-S test, $p < 0.05$, $n = 8$ of 14, Figure 4a) by an average of $118.4 \pm 3.2\%$. In all cells identified as nNOS+, application of nicotine induced a significant increase in sEPSC frequency (K-S test, $p < 0.05$, $n = 11$ of 11). The average change in mean frequency in these cells was $456.5 \pm 138.5\%$, and mean amplitude was increased in 6 of 11 cells (K-S test, $p < 0.05$, $n = 6$ of 11) by $118.5 \pm 4.2\%$. In one of the two nNOS- cells recovered and identified, nicotine induced a significant increase in the frequency of sEPSCs (156.4%; K-S test, $p < 0.05$, $n = 1$ of 2), as well as an increase in sEPSC amplitude (122.5%; K-S test, $p < 0.05$, $n = 1$ of 2).

We also examined the actions of nicotine on miniature EPSCs (mEPSCs). In the presence of TTX, nicotine still produced large changes in the distribution of mEPSC frequency in almost all cells examined (K-S test, $p < 0.05$, $n = 28$ of 29; Figure 4b). The average change in mean frequency was $407.2 \pm 45.0\%$. Nicotine significantly increased the amplitude of mEPSCs in 15 of these cells (K-S test, $p < 0.05$, $n = 15$ of 29) by $137.8 \pm 10.1\%$. In 93.8% of the cells identified as nNOS+, nicotine significantly increased the frequency of mEPSCs (K-S test, $p < 0.05$, $n = 15$ of 16). The average increase in mean sEPSC frequency was $488.5 \pm 70.9\%$. In 43.8% of the cells, the amplitude distribution was significantly different (K-S test, $p < 0.05$, $n = 7$ of 16) with an average increase in mean amplitude of $153.1 \pm 19.8\%$. In all of the nNOS- cells recorded, nicotine induced a significant increase in mEPSC frequency of $268.4 \pm 41.6\%$ (K-S test, $p < 0.05$, $n = 10$ of 10) and in the majority of the cells, nicotine significantly increased their amplitude (K-S test, $p < 0.05$, $n = 6$ of 10) by $122.0 \pm 7.0\%$. These actions were not significantly different from the actions of nicotine on these parameters in cells recorded without TTX in the bath (unpaired *t*-test, $p > 0.05$; Figure 4d). The increase in nicotine-induced frequency of mEPSCs in nNOS+ cells was significantly greater than the increase induced in nNOS- cells (unpaired *t*-test, $p < 0.05$; Figure 4e), suggesting the possibility that exposure to nicotine would result in a greater glutamatergic excitation of cholinergic neurons by presynaptic excitatory terminals than that induced in non-cholinergic cells. In low calcium solution, nicotine failed to induce mEPSC activity ($n = 4$ of 4). The success of low calcium solution, coupled with the

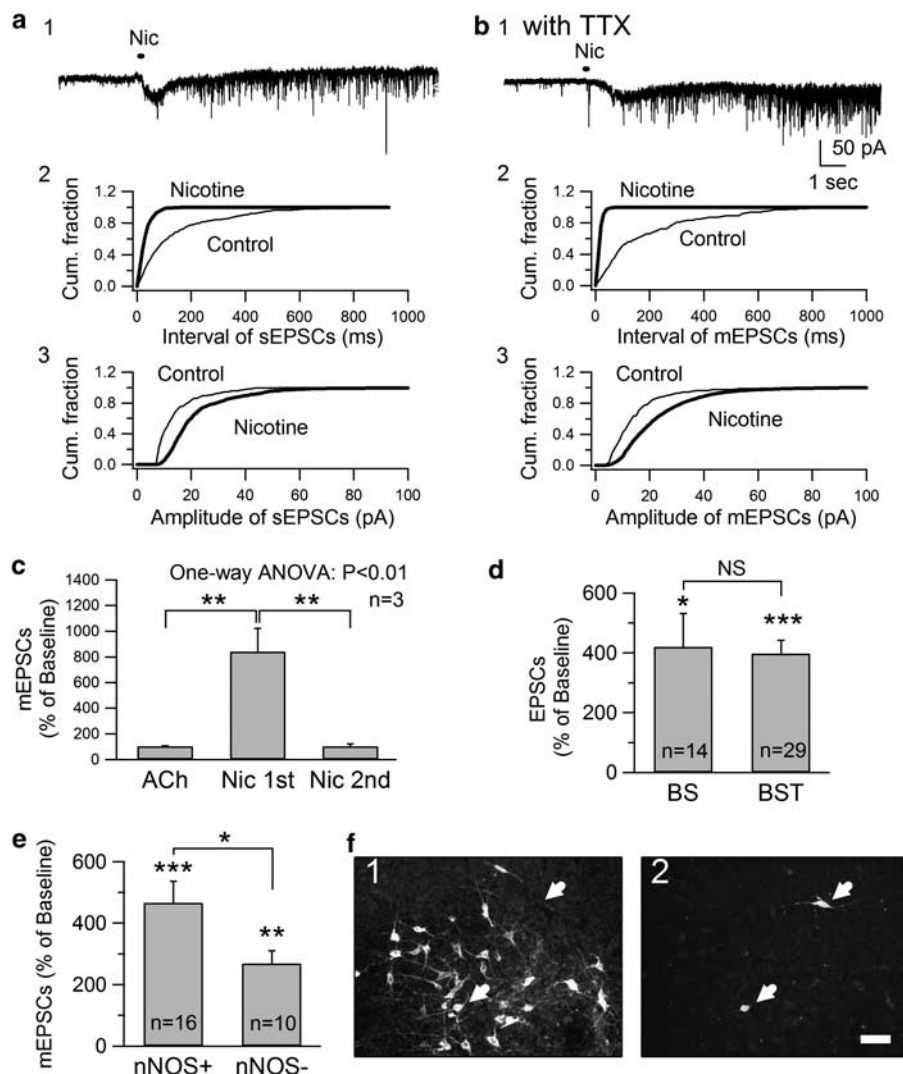


Figure 4 Nicotine induces excitatory synaptic activity through stimulation of nAChRs located on glutamatergic terminals. (a1) Nicotine application induced inward current and EPSPs in this nNOS+ cell when recorded in voltage-clamp mode with inclusion of bicuculline and strychnine (BS) in the ACSF to block IPSCs. (a2, 3) Cumulative fraction from the cell shown in a1 indicates that the frequency (2) and amplitude (3) of sEPSCs increased after nicotine application. These actions persisted in TTX to block sodium-dependent action potentials, as shown in b1 for the same cell as in a1. (b2, 3) Cumulative fraction from the same cell in b1 showing a significant increase in frequency (2) and amplitude (3) of mEPSCs after nicotine exposure in TTX (K-S test, $p < 0.05$). (c) Summary data showing that ACh failed to induce synaptic activity in the same cells in which nicotine subsequently induced robust EPSC activity. However, elicitation of this effect by second applications of nicotine was not effective. (d) Summary data in the population of cells showing that inclusion of TTX (BST) had no effect on the nicotine-induced enhancement of EPSCs. (e) Summary data showing that nicotine induced a significantly higher increase in frequency of EPSCs in nNOS+ cells as compared with the increase induced in nNOS- cells. (f) Immunofluorescence showing identification of a representative nNOS+ cell (left arrow) and an nNOS- cell (right arrow) from which data in the EPSC study were collected, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

failure of TTX, in abolishment of the nicotine-induced changes in frequency of EPSPs indicate that nicotine is acting on presynaptic, glutamatergic terminals.

To verify that nicotine had similar excitatory actions on synaptic transmission when delivered in a manner more consistent with that experienced by the brain of a smoker, nicotine was bath applied. Bath-applied nicotine ($10 \mu\text{M}$) in presence of bicuculline and strychnine induced a significant increase in frequency of sEPSCs in 66% of the cells recorded (K-S test, $p < 0.05$, $n = 8$ of 12) with an average change of $334.9 \pm 85.0\%$; and in 42% of these cells, amplitude was significantly increased (K-S test, $p < 0.05$; $n = 5$ of 12) by $113.3 \pm 7.0\%$. In the majority of cells identified as nNOS+ (Figure 4f1), nicotine applied through the bath induced a

significant increase in mean sEPSC frequency (K-S test, $p < 0.05$, $n = 4$ of 5) of $403.0 \pm 153.9\%$, but failed to induce a significant increase in amplitude in the majority of cells ($p > 0.05$, $n = 4$ of 5) with an average amplitude change of $98.5 \pm 6.2\%$. In 50% of the cells identified as nNOS- cells (Figure 4f2), similarly applied nicotine induced a significant increase in sEPSC frequency (K-S test, $p < 0.05$, $n = 2$ of 4) with an average change of $196.3 \pm 90.5\%$, and failed to significantly increase the amplitude in the majority of cells ($p > 0.05$, $n = 3$ of 4) with an average change of $85.6 \pm 8.0\%$. These data support the conclusion that synaptic activity resulting from bath-applied nicotine results from activation of presynaptic glutamatergic neurons. In two nNOS+ cells, nicotine also significantly increased the mEPSC frequency

(K-S test, $p < 0.05$), but failed to affect the amplitude distribution, indicating that bath delivery of nicotine also activates presynaptic glutamatergic terminals on cholinergic neurons.

Subunit Composition of nAChRs on Glutamatergic Terminals

As second applications of nicotine failed to induce EPSC activity, we were not able to compare the effects of nAChR subunits' antagonists on nicotine-induced increases in EPSC frequency in the same cells. Therefore, comparisons were done across two separate cell populations. The increase in mean frequency of mEPSCs in nNOS+ cells was not significantly affected by inclusion of MLA (10 nM; $95.5 \pm 17.4\%$ of control, $n = 16$, $p > 0.05$; Figure 5a3 and b) or DH β E (500 nM; $135.8 \pm 27.6\%$ of control, $n = 14$, $p > 0.05$; Figure 5a2 and b) in the recording solution. However, MEC, an antagonist of the non- $\alpha 7$ nAChR subunit, blocked the nicotine-induced increase in mEPSC frequency ($20.7 \pm 4.8\%$

of control, $n = 10$, $p < 0.01$; Figure 5a4 and b). Taken together, this pharmacological profile suggests that the $\beta 2$ and $\alpha 7$ subunits are not major components of nAChRs located on glutamatergic terminals presynaptic to cholinergic LDT neurons, but rather that non- $\alpha 7$ -containing nAChRs mediate the increase in glutamatergic EPSCs on LDT neurons.

Nicotine Activates Presynaptic GABA-Containing Neurons

Puffer application of nicotine also induced inhibitory synaptic activity that was bicuculline and strychnine-sensitive. Nicotine-induced actions on inhibitory synaptic activity were quantified in recordings with high chloride pipettes to reverse the chloride gradient, and with APV and DNQX to block excitatory synaptic transmission. Under these conditions, in the vast majority of cells recorded (87.5%), nicotine induced a significant increase in spontaneous IPSC (sIPSC) frequency (K-S test, $p < 0.05$, $n = 21$ of 24) with an average change in mean frequency of $280.0 \pm 28.5\%$ from control; and, only a minority of these cells showed a significant increase in their amplitude (K-S test, $n = 7$ of 24, $p < 0.05$) of $131.3 \pm 13.5\%$. In 94% of the nNOS+ cells recovered, nicotine significantly increased the frequency of IPSCs (K-S test, $p < 0.05$, $n = 15$ of 16; Figure 6a) with an average change of $292.9 \pm 30.7\%$. In 75% of the identified nNOS- cells, nicotine also induced a significant increase in frequency of IPSCs (K-S test, $p < 0.05$, $n = 3$ of 4), with an average change of $308.0 \pm 131.1\%$ in these cells. The change in amplitude of spontaneous IPSCs increased significantly in 4 of 16 nNOS+ cells (K-S test, $p < 0.05$, $n = 14$ of 16) by an average of $148.0 \pm 20.4\%$ and in half of the identified nNOS- cells (K-S test, $p < 0.05$, $n = 2$ of 4) with an average change of $107.1 \pm 4.7\%$.

To determine whether these changes could be accounted for by actions at presynaptic terminals, the effect of nicotine on mIPSCs was examined. In the majority of cells recorded with TTX in the ACSF, nicotine significantly increased mIPSCs (K-S test, $p < 0.05$, $n = 17$ of 21) by an average of $747.9 \pm 212.7\%$; mIPSC amplitude was also significantly enhanced in 47.6% of the cells (K-S test, $p < 0.05$, $n = 10$ of 21) with an average change of $168.5 \pm 14.6\%$. In 71% of the nNOS+ cells identified, and in all of the nNOS- cells, nicotine significantly increased the frequency of mIPSCs (K-S test, $p < 0.05$; nNOS+: $n = 10$ of 14; nNOS-: $n = 5$ of 5, Figure 6b) by an average of $1092.2 \pm 321.0\%$ and $290.3 \pm 82.1\%$, respectively. The amplitude of nicotine-induced mIPSCs was not significantly different in the majority of nNOS- cells (K-S test, $p > 0.05$, $n = 4$ of 5); however, in 50% of the nNOS+ cells recorded ($n = 7$ of 14), the amplitude of mIPSCs did increase significantly after nicotine administration (K-S test, $p < 0.05$, $n = 7$ of 14) by an average of $186.0 \pm 16.9\%$. The difference in frequency of mIPSCs induced by nicotine was not significantly different between nNOS+ cells and nNOS- cells ($p > 0.05$; Figure 6c). Although the change in IPSC frequency in the population of nNOS+ cells seemed to be greater when TTX was included in the ACSF from that evoked by nicotine in its absence, the increase in nicotine-induced synaptic activity was not significantly different between the two

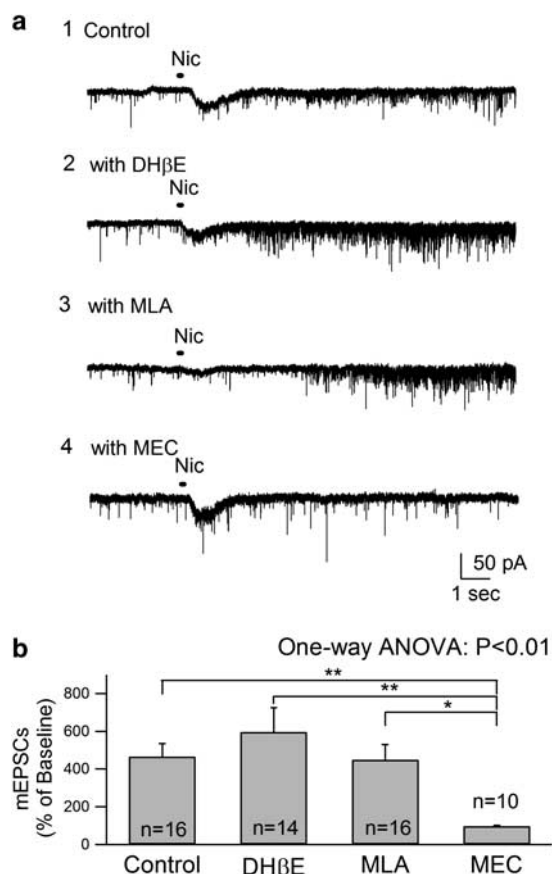


Figure 5 The enhancement in cholinergic neurons of mEPSC frequency by nicotine is mediated by non- $\alpha 7$ -subunit-containing nAChRs. (a) Nicotinic enhancement of mEPSCs recorded in cholinergic LDT neurons was not affected by application of 500 nM DH β E (a2) or 10 nM MLA (a3), but was reduced by application of 1 μ M MEC (a4). Bicuculline, strychnine, and TTX were included in the recording ACSF to block inhibitory synaptic transmission and voltage-gated sodium channels. (b) Summary data detailing the inhibition of nicotine-induced increases in mEPSC frequency by the three different nAChR antagonists. Only the actions of MEC were significantly different, * $P < 0.05$, ** $P < 0.01$.

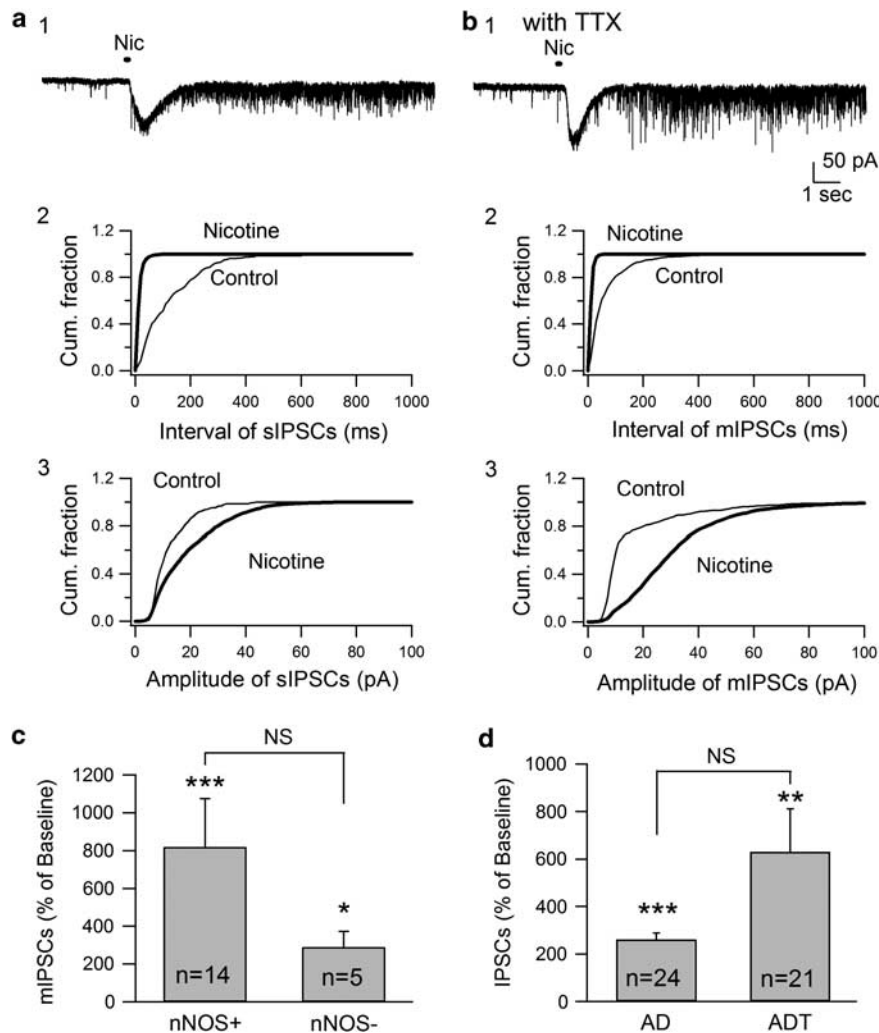


Figure 6 Frequency of IPSCs is enhanced by nicotine in both nNOS + cells and nNOS– cells. (a1) Puffer application of nicotine-induced IPSCs that were not inhibited by presence of TTX in the ACSF (b1). ACSF also contained APV and DNQX (designated 'AD' or 'ADT' with inclusion of TTX) to block excitatory synaptic transmission, and recordings were conducted with high chloride pipettes to reverse the chloride gradient, facilitating detection of IPSC events as inward currents. (a2) Cumulative distribution from the cell shown in a1, indicating that the interval of IPSC events decreased significantly (K–S test, $p < 0.05$), thereby indicating an enhancement of frequency of sIPSCs induced by nicotine application acting at presynaptic terminals. (a3) In addition, the cumulative distribution of this cell shows that the amplitude of sEPSCs also increased significantly (K–S test, $p < 0.05$). (b2, 3) Cumulative distributions of the cell shown in b1, recorded in TTX, in which the frequency of mIPSCs and the amplitude of mIPSCs were enhanced by nicotine (K–S test, $p < 0.05$). (c) mIPSC frequency was significantly increased by nicotine in both populations of nNOS + cells and nNOS– cells (one-tailed, paired t -test; $p < 0.05$). (d) Summary data with the population of cells indicating that TTX did not affect the increase in nicotine-induced increase in IPSC frequency. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

conditions in cholinergic neurons, probably because of the variability in nicotine-enhanced frequency changes in these cells in the TTX condition ($p > 0.05$; Figure 6d). Combined with the increase in amplitude of mIPSCs, these data suggest that some nicotine-stimulated inhibition of GABAergic cells presynaptic to nNOS+ cells occurs and that this inhibition is blocked when action potentials are inhibited, thereby allowing a barrage of inhibition to be directed to cholinergic cells that may summate in time, or in spatial point of occurrence. However, this possibility was not investigated further. In the presence of low calcium ACSF, nicotine failed to induce mIPSCs ($n = 4$, data not shown). Given that nicotine-induced inhibitory synaptic currents require extracellular calcium, but are not dependent on generation of action potentials, we conclude that nicotine excites GABA and/or glycinergic terminals imping-

ing on cholinergic neurons as well as inhibitory terminals directed to non-cholinergic cells.

The maximum frequency change in IPSPs when nicotine was bath-applied ($10 \mu\text{M}$) was significantly greater than that induced when nicotine was puffed onto cells. With APV and DNQX in the bath to block excitatory amino acid transmission, the frequency of sIPSCs increased in all cells (K–S test, $p < 0.05$, $n = 4$ of 4) by $1399.8 \pm 722.8\%$. One cell was identified as nNOS+ and increased in frequency by 3097.9% ; one cell was identified as nNOS– and increased by 2103.8% , suggesting that this phenomenon extended to both cholinergic and non-cholinergic cells. When TTX was included in the ACSF, in the majority of recorded cells, nicotine increased the frequency of mIPSPs (K–S test, $p < 0.05$, $n = 8$ of 11) by an average of $1008.4 \pm 343.6\%$. In the majority of nNOS+ cells tested, nicotine increased the

frequency of mIPSCs (K-S test, $p < 0.05$, $n = 4$ of 5) by $1502.6 \pm 572.2\%$ and in 66% of the nNOS $^-$ cells tested, miniature inhibitory events increased (K-S test, $p < 0.05$, $n = 2$ of 3) by an average of $335.9 \pm 91.2\%$. The data obtained using either bath or puffer applications suggest that nicotine can induce GABA release from terminals located presynaptic to cholinergic and non-cholinergic LDT cells.

Subunit Composition of nAChRs on Gabaergic Terminals

The increase in mIPSCs induced by nicotine in nNOS $^+$ cells was significantly attenuated by inhibition of the $\alpha 7$ subunit with MLA (10 nM) ($75.4 \pm 11.9\%$ reduction from control, $n = 12$, $p < 0.05$; Figure 7a1, a3 and b). DH β E (500 nM) also significantly reduced the nicotine-induced increase in mIPSC frequency ($80.4 \pm 18.7\%$ reduction from control, $n = 10$, $p < 0.05$; Figure 7a2 and b). MEC (1 μ M), an antagonist of non- $\alpha 7$ -subunit-containing receptors, abolished the nicotine-induced increase in mIPSC frequency ($98.0 \pm 1.3\%$ reduction from control, $n = 11$, $p < 0.01$; Figure

7a4 and b). These data indicate that nAChRs on GABAergic terminals presynaptic to cholinergic cells contain $\alpha 7$, $\beta 2$, and non- $\alpha 7$ subunits, and our data taken together with the effects of nAChRs antagonists on mEPSCs, suggest that the subunit composition of nAChRs on GABA-containing presynaptic terminals and those on glutamate-containing presynaptic terminals within the LDT is different.

DISCUSSION

In this study, we show that nicotine has profound direct and indirect actions on both cholinergic and non-cholinergic LDT neurons. In almost all LDT neurons examined, regardless of presence or absence of nNOS, nicotine elicited membrane depolarization with a concurrent decrease in input resistance. Often, membrane potential changes were sufficient to induce sodium channel-dependent action potentials. Interestingly, nicotine activation of nAChRs resulted in calcium rises in dendrites of cholinergic LDT neurons, whereas similar rises were not observed in the somata. However, nicotine potentiated intracellular calcium changes at the soma arising from action potentials and increased cellular excitability, as shown by higher firing rates produced by constant current injection. Nicotine also induced a TTX-resistant increase in the frequency of excitatory glutamatergic and inhibitory GABAergic synaptic activity in nNOS $^+$ and nNOS $^-$ cells. Utilization of nAChR subunit antagonists provides the first functional evidence for the presence of $\beta 2$, $\alpha 7$, and non- $\alpha 7$ subunits on cholinergic neurons, and revealed a differential distribution of subunits comprising nAChRs located on glutamatergic and GABAergic terminals presynaptic to cholinergic LDT neurons. Furthermore, antagonist-specific actions on calcium elevations induced by nicotine in dendrites of nNOS $^+$ neurons indicated that dendritic nAChRs in these cells contain $\alpha 7$ subunits. Taken together, our data suggest that exposure of the LDT to nicotine would excite all cell types in this nucleus, although likely to different degrees. Moreover, owing to the differences in nAChR subunit distribution within different cell types, nicotine would be expected to differentially regulate neuronal function within the heterogeneous LDT nucleus.

Although to our knowledge, this report represents the first study of the electrophysiological actions of nicotine on mouse LDT neurons, a selective nicotinic receptor agonist (DMPP) has been shown to have effects on presumed cholinergic neurons of the rat pedunculopontine tegmental (PPT) nucleus. The PPT is in close anatomical proximity to the LDT and includes another large collection of mesopontine cholinergic neurons. PPT cells recorded from animals between ages P12 and 17, and identified as cholinergic based on the presence of A-type current, responded to bath-applied DMPP with depolarization (Good *et al*, 2007), which agrees with our findings in the mouse. However, by age 21 days, in a striking response reversal, DMPP was reported to elicit membrane hyperpolarization (Good *et al*, 2007). In our study, we never observed a hyperpolarization in nNOS $^+$ or nNOS $^-$ neurons in response to nicotine even though we recorded data from animals up to 45 days of age and used two methods of agonist application. Furthermore, DMPP did not elicit outward currents in LDT cells recorded from

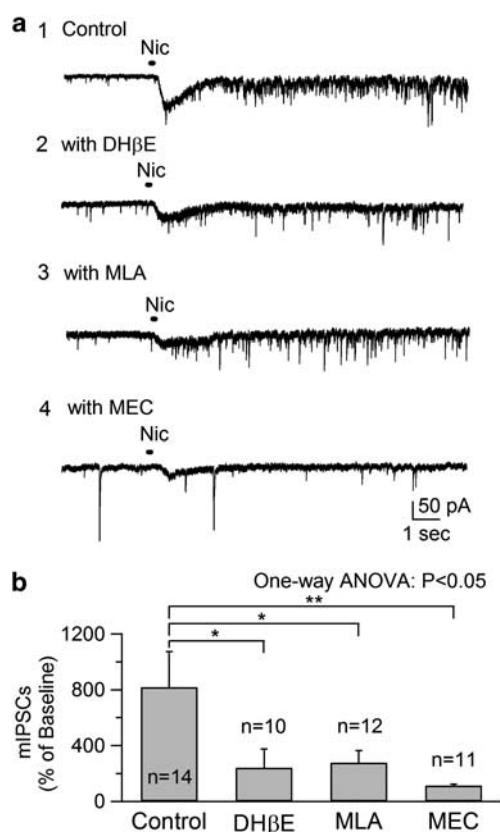


Figure 7 Nicotine-induced IPSCs in cholinergic neurons are mediated by nAChRs containing $\alpha 7$, $\beta 2$, as well as non- $\alpha 7$ subunits. (a) Response of cholinergic cells to nicotine applied in control TTX conditions (a1), or in the presence of nAChR antagonists DH β E (a2), MLA (a3), or MEC (a4). (b) Summary data showing the effectiveness of all three nAChR antagonists in reducing the nicotine-induced increase in mIPSC frequency elicited in cholinergic neurons. All three antagonists were effective at reducing the nicotine-induced increase in mIPSC frequency in these cells, indicating that nAChRs on GABA-containing terminals impinging on cholinergic neurons are comprised of $\alpha 7$, $\beta 2$, and non- $\alpha 7$ -containing subunits, * $P < 0.05$, ** $P < 0.01$.

animals between the ages of 21 days and 23 days. Thus, it seems unlikely that we overlooked a hyperpolarizing response to nicotine in LDT neurons. With the caveat that different species, route of administration, recording methods, and slice temperatures were used in the two studies, it is possible that these different responses are authentic and should be further investigated. Such a difference might reflect the disparate functions predicted to be subserved by the LDT and PPT based on their differential innervation of reward circuitry (Omelchenko and Sesack, 2005, 2006; Geisler and Zahm, 2005) and the different addiction-related behavioral outcomes of lesioning these two nuclei (Alderson *et al*, 2008; Laviolette *et al*, 2000; Forster *et al*, 2002a; Winn, 2008).

Previous studies have shown a nicotinic receptor-mediated, presynaptic enhancement of fast, ionotropic glutamatergic transmission (Girod *et al*, 2000; Gray *et al*, 1996; McGehee *et al*, 1995). Consistent with these reports, we found in the current study that nicotine enhanced the frequency of APV and DNQX-sensitive EPSCs in cholinergic and non-cholinergic LDT neurons. This seemed to be due to the action of nicotine in promoting Ca^{2+} -influx into glutamate-releasing presynaptic terminals, as the increase in mEPSC frequency was insensitive to TTX and was attenuated by lowering extracellular Ca^{2+} , as described for other neurons (Fisher *et al*, 1998; Lu *et al*, 1999; Radcliffe *et al*, 1999). Interestingly, nicotine induced a greater increase in mean frequency of EPSCs in cholinergic neurons than in non-cholinergic neurons. The cell-specific degree of nicotinic activation of EPSCs represents a potentially important functional difference. Although immunohistochemical data suggested that ACh-containing LDT neurons may colocalize GABA and glutamate (Charara *et al*, 1996; Omelchenko and Sesack, 2006), recent *in situ* evidence contradicts this conclusion (Wang and Morales, 2009). Very few cholinergic LDT neurons were found to contain mRNA transcripts encoding proteins for the vesicular transports of glutamate or for the synthesis of GABA, suggesting that glutamate and GABA are unlikely to be coreleased with ACh from LDT neurons (Wang and Morales, 2009). Furthermore, the cholinergic neurons seemed not to be the major neuronal phenotype within the LDT with vGluT2- or GAD-containing neurons being equal to or twice as prevalent, respectively. Accordingly, the pool of non-cholinergic neurons in our study were likely to be, in part, comprised of GABAergic and glutamatergic cells. Our data, indicating that nicotine elicits a relatively larger excitatory glutamatergic drive onto cholinergic as compared with non-cholinergic cells, taken in combination with the *in situ* data, indicating segregation of neurotransmitter phenotype across LDT cells, raises the possibility of relatively enhanced release of ACh over glutamate and GABA at LDT projection targets after identical nicotinic exposure.

GABAergic transmission has also been shown to be enhanced by nicotine in many different cell types (Alkondon *et al*, 1997a, 2000; Fisher *et al*, 1998; Lena and Changeux, 1997; Lena *et al*, 1993; Radcliffe *et al*, 1999). Nicotine induced a TTX-resistant, low calcium solution-sensitive increase in frequency of strychnine- and bicuculline-sensitive IPSCs directed to nNOS+ and nNOS- LDT neurons. The increase in frequency of IPSCs was not significantly different between cholinergic and non-choli-

nergic cells. These data suggest that nAChRs are present on GABA-containing terminals presynaptic to cholinergic and non-cholinergic LDT neurons and indicate that after nicotine exposure, inhibition would be comparably augmented in both cholinergic and non-cholinergic LDT neurons. Our data showing that in the presence of blockers of excitatory transmission, bath application induced significantly more sIPSCs than did puff application, whereas elicitation of EPSCs was similar regardless of method of drug application, suggest that bath-applied nicotine excited nAChRs located at more distal sites than did the more locally applied puff and, accordingly, that there may be a more local presence of nAChRs on glutamate-containing terminals impinging on the recorded cells. Accordingly, our findings suggest a difference between distribution of nAChRs on GABA-containing and on glutamate neurons that terminate on LDT neurons.

Nicotinic receptors are pentameric, ligand-gated ion channels composed of different combinations of α (2–10) and/or β (2–4) subunits (Gotti *et al*, 1997; Jones *et al*, 1999; McGehee and Role, 1995; Role and Berg, 1996; Salamone and Zhou, 2000). Nicotinic receptors can be loosely divided into two classes based on their subunit composition. The first class is homopentameric, composed of the $\alpha 7$ subunit and highly calcium permeable (Fucile, 2004). The second class is heteropentameric formed by both α and β subunits and shows low calcium permeability (Fucile, 2004). Azam *et al* (2003) found expression of $\beta 2$ and $\alpha 7$ mRNAs in almost all cholinergic neurons of the LDT, which suggested that $\alpha 7$ -subunit-containing nAChRs may mediate nicotinic actions in cholinergic neurons with or without the involvement of the $\beta 2$ subunit. Non-cholinergic neurons expressed mRNA for the $\alpha 4$ nAChR subunit, suggesting the possibility of nAChRs containing $\alpha 4\beta 2$ subunits in these cells (Azam *et al*, 2003). Our data using nAChR antagonists indicate that inward currents induced by nicotine in cholinergic neurons were mediated by nAChRs containing $\alpha 7$ and $\beta 2$, as well as non- $\alpha 7$ subunits. Although DH β E at the concentrations that we used can have actions on nAChRs containing $\alpha 4\beta 2$ and $\alpha 4\beta 4$ subunits in other cell populations (Harvey *et al*, 1996), cholinergic neurons seem to be devoid of mRNA for the $\alpha 4$ subunit (Azam *et al*, 2003), suggesting that the antagonistic effect of DH β E was mediated by $\beta 2$ -subunit-containing nAChRs. Failure to detect changes in baseline calcium in the somata of cholinergic neurons could indicate that nAChRs located on the somas are heteropentameric, consisting of both α and β subunits. However, nicotine did elicit dendritic calcium rises. These data suggest that if there are nAChRs at the soma, there may be differences in their subunit composition from those at the dendrites of cholinergic LDT neurons, although it can not be ruled out that the larger surface to volume ratio at the dendrite may have precluded detection of somatic calcium rises. Regardless, such intracellular subunit-specific targeting has been reported (Champtiaux *et al*, 2002; Lena and Changeux, 1999; Lena *et al*, 1999). Furthermore, nicotine-induced increases in dendritic calcium were sensitive to MLA, indicating the presence of the $\alpha 7$ subunit within dendritic nAChRs, as has been shown for hippocampal neurons (Fayuk and Yakel, 2007), and suggesting, although we did not rule out a contribution of VGCCs to nicotine-induced

dendritic calcium changes, that these rises were mediated, at least partly, by $\alpha 7$ -subunit-containing receptors. Calcium influx through $\alpha 7$ -containing nAChRs is linked to several calcium-dependent processes, including developmental-related switching of polarity of electrophysiological actions after GABA_A receptor activation (Liu *et al*, 2006), transcription, and gene regulation (Chang and Berg, 2001), and amplitudes of GABA_A-mediated currents (Zhang and Berg, 2007). Furthermore, in other cell types, nicotine increases amplitudes of mEPSCs through calcium-dependent, plastic changes mediated by $\alpha 7$ -containing nAChRs in the pre-synaptic terminal. These changes lead to a coordinated release of quanta, resulting in heightened excitatory neurotransmitter concentration at the postsynaptic cell (Sharma *et al*, 2008). To sum, elevations of intracellular calcium have been linked to a variety of distinct signaling functions as well as to synapse formation, which makes the spatial and temporal rises in baseline calcium in dendrites of cholinergic neurons after nAChR activation a topic of potential interest.

The ability of MEC, but failure of MLA and DH β E, to reduce the nicotine-induced frequency increase of APV and DNQX-sensitive mEPSPs indicates that nAChRs on glutamate-containing terminals synapsing on cholinergic LDT neurons contain neither $\alpha 7$ nor $\beta 2$ subunits. In contrast, blockade of bicuculline and strychnine-sensitive IPSCs with MEC, MLA, and DH β E suggests the presence of $\alpha 7$, $\beta 2$, as well as non- $\alpha 7$ -subunit-containing receptors on terminals of GABAergic inhibitory neurons synapsing on cholinergic LDT cells. Accordingly, our data suggest differences in subunit compositions of nAChRs located on excitatory and inhibitory presynaptic terminals on cholinergic LDT cells, which may confer differing rates of receptor activation and desensitization (Albuquerque *et al*, 1997; Alkondon and Albuquerque, 2006; Alkondon *et al*, 1997b; Fenster *et al*, 1997). Differing rates of desensitization of nAChRs on terminals directed to DA-containing VTA cells have been elegantly shown to underlie a long-lived enhancement (over 1 h after nicotine exposure) of excitation of glutamate-containing VTA cells with concurrent depression of GABA-containing local neurons within the VTA (Mansvelder *et al*, 2002; Mansvelder and McGehee, 2000). Differing rates of nicotinic activation and desensitization have also been shown to mediate differential actions of nicotine on DA-containing neurons of the VTA and those of the substantia nigra pars compacta (Pidoplichko *et al*, 2004; Woollorton *et al*, 2003). Accordingly, the observed differences in subunits comprising nAChRs in GABAergic and glutamatergic terminals may be physiologically significant for LDT functioning by imparting different regulatory actions of ACh and nicotine on these presynaptic sites.

Functional Considerations

Although drugs of abuse target the entire brain, enhanced DA release from the VTA to the NAcc is a key element in rewarding properties of drugs. Burst firing in DA-containing VTA neurons, resulting in DA release in the NAcc, conveys the physiologically relevant stimulus for assigning reward to behavior, making it critical to understand synaptic input to the VTA, which could regulate behaviorally relevant firing patterns. Within the VTA, cholinergic

agonists, including nicotine, increase the activity of DA- and GABA-containing cells, induce burst firing, and influence the release of DA in the NAcc (Corrigall *et al*, 1994; Grillner and Svensson, 2000; Gronier *et al*, 2000; Nisell *et al*, 1994; Westerink *et al*, 1998, 1996). Very recent data indicate that ACh fluctuations within the VTA underlie cocaine-seeking behavior (You *et al*, 2008), providing further evidence that cholinergic inputs within the VTA are part of important segments of the reward circuitry.

In the most comprehensive anatomical study to date of direct, cholinergic innervation of the VTA, Omelchenko and Sesack (2006) expand upon findings reported by Garzon *et al* (1999) and demonstrate cholinergic input to NAcc-projecting DA-containing VTA neurons and conclude that vesicular acetylcholine transporter (VACHT)-containing terminals more frequently synapsed on this set of neurons and much less commonly on DA-containing, mesoprefrontal-projecting neurons, thereby contradicting a previous hypothesis (Garzon *et al*, 1999). Furthermore, cholinergic axons were found to contribute asymmetrical (presumed excitatory) synapses most commonly on DA-containing, NAcc-projecting VTA cells, whereas, symmetrical (presumed inhibitory) synapses were more common on GABA-containing cells (Omelchenko and Sesack, 2006). The source of endogenous cholinergic input derives in large part from the LDT based on the morphology and synaptology of cholinergic axons within the VTA (Omelchenko and Sesack, 2005). Anatomical findings suggest that endogenous cholinergic input within the VTA from the LDT would most likely be excitatory to NAcc-projecting DA-containing cells both through direct excitation and through inhibition of local circuit GABA cells. In addition to cholinergic projections, the LDT also sends glutamate and GABAergic input to the VTA (Hallanger and Wainer, 1988; Oakman *et al*, 1995). The synapse types of cholinergic terminals within the VTA suggested colocalization of other neuroactive substances (Omelchenko and Sesack, 2006), in particular that of glutamate (Bevan and Bolam, 1995; Clarke *et al*, 1997) and GABA (Jia *et al*, 2003). However, a very recent study showed that the vast majority of cholinergic LDT neurons do not contain GAD or VGluT2 transcripts (Wang and Morales, 2009); furthermore, in a preliminary report, the majority of axon terminals in the VTA, some of which certainly derive from the LDT, that contained VACHT did not contain immunolabel for VGluT2 or for GAD (Wang *et al*, 2008).

The LDT influences DA release within the VTA, which shows functional connectivity between these two nuclei. Electrical stimulation of the LDT results in two phases of increases in DA levels within the VTA: the first phase is due to activation of ionotropic glutamatergic and nicotinic receptors within the VTA, whereas the second and longer-lived phase depends on metabotropic M5 receptors (Forster and Blaha, 2000; Forster *et al*, 2002b). Microdialysis studies in the VTA and NAcc in amphetamine-injected rats revealed an increase in glutamate release within these sites after AMPA injections in the LDT, indicating a heightened excitability, and subsequent facilitation, of synaptic transmission within the LDT-VTA-NAcc pathway, which suggested for the first time that drug-induced plasticity can occur within the LDT (Nelson *et al*, 2007). Heightened transmission from the LDT to the VTA may underlie the

sensitization of post-stereotypy locomotive behaviors; as lesions of the LDT attenuated, this addiction-associated phenotype developed in response to repeated amphetamine injections (Nelson *et al*, 2007). Loss of LDT projections from the LDT to the VTA was speculated to underlie the failure of nicotine to induce normal locomotor actions in LDT-lesioned animals (Alderson *et al*, 2005). Additional evidence for the role that excitatory projections from the LDT to the VTA have in addiction is provided by the finding that burst firing of DA-VTA neurons, which results in large increases in synaptic DA and, therefore, is believed to be the functionally relevant mode of transmission elicited by exposure to behaviorally relevant stimuli, is entirely dependent on a physiologically intact, functioning LDT (Lodge and Grace, 2005). Accordingly, it has been proposed that the LDT serves as a gate that allows VTA-DA neurons to switch to behaviorally relevant, phasic patterns of firing and a role for cholinergic transmission in this gating is indicated (Grace *et al*, 2007). Although GABA-containing afferents from the LDT synapse on DA-containing VTA cells (Charara *et al*, 1996; Omelchenko and Sesack, 2005, 2006), the role they have in the modulation of activity of these cells is unclear. Taken together, the anatomical and physiological data suggest that the LDT through cholinergic and glutamatergic projections to the VTA provides a major excitation of NAcc-projecting DA-containing neurons, and that these projections have a vital role in communicating the saliency properties of natural and drug stimuli serving to initiate, as well as maintain, motivated behavior.

An accumulating body of evidence suggests that neurons of the LDT are important substrates for signaling reward and for modulation of goal-driven behavior. As the LDT seems to exert excitatory regulation of NAcc-projecting, DA-containing neurons, direct excitation of cholinergic and non-cholinergic LDT neurons by nicotine would likely result in heightened excitation of these VTA cells. In addition, input to the LDT from non-VTA areas also included in reward circuitry, such as the prefrontal cortex (Carr and Sesack, 2000a,b), may be scaled differently after nicotine exposure of the LDT. Nicotine-induced increases in GABAergic IPSCs directed to cholinergic neurons might selectively dampen cholinergic output; however, phasic GABA_A-mediated inhibition does not alter excitability induced by tonic depolarization (Nusser and Mody, 2002), suggesting the possibility that the directly mediated depolarizing effect of nicotine on LDT neurons would not be greatly opposed by inhibitory synaptic events. Furthermore, our data show that non-cholinergic neurons, some of which are almost certainly local GABA-containing cells (Ford *et al*, 1995; Jones, 2004), are subject to nicotine-induced inhibitory GABA input, thus potentially limiting their inhibitory actions on cholinergic neurons and other target cells. Nicotine enhanced intracellular calcium levels induced by action potentials and increased the baseline calcium levels in dendrites. Somatic and dendritic calcium rises may be proximal to calcium-dependent effectors suggesting the possibility that nicotine could engender synaptic plasticity, as shown to be induced by amphetamine and suggested to be mediated by induction of LTP (Nelson *et al*, 2007). Taken together, our data provide physiological evidence that nicotine alters excitability in LDT circuitry and suggest that LDT may be involved in imparting

stimulus saliency through nicotine-induced alteration of throughput of this nucleus to circuitry, which is important for signaling reward. The LDT may, therefore, be critically involved in the development of nicotine dependence and maintenance of addiction.

Behavioral State

The pontine cholinergic system is also considered to be a mediator of cortical arousal through rostrally directed projections (Semba and Fibiger, 1989, 1992; Semba *et al*, 1990) and a positive effector of REM sleep through its cholinergic caudal projections to a pontine site known as the REM sleep-induction zone, because of the ability of cholinomimetics to induce a REM-like state when locally injected (Baghdoyan *et al*, 1993). Studies examining nicotine's actions on sleep and wakefulness are contradictory, perhaps because of differences in species, routes of administration, and dose (Gillin *et al*, 1994; Salin-Pascual *et al*, 1999; Vazquez *et al*, 1996; Wetter and Young, 1994). Although the precise action of nicotinic transmission in the LDT on behavioral state remains to be elucidated, given the importance of cholinergic and non-cholinergic LDT neurons in the expression of sleep and wakefulness, our data showing strong electrophysiological actions of this drug on cells in this nucleus suggests that exposure of the LDT to nicotine, either acutely in the form of occasional use of tobacco products or chronically, as experienced by an addicted smoker, could lead to profound changes in behavioral state. Moreover, withdrawal of nicotine from the brain of a recurrent nicotine user, such as during a sleep bout of 7–8 h, would also be expected to influence LDT functioning, which may, in part, contribute to deviations from the normal expression of behavioral state patterns in smokers (Gillin *et al*, 1994; Salin-Pascual *et al*, 1999; Vazquez *et al*, 1996; Wetter and Young, 1994).

Conclusions

The LDT contributes to amphetamine, nicotine, and morphine-induced behaviors associated with drug abuse (Forster *et al*, 2002a; Laviolette *et al*, 2000; Nelson *et al*, 2007), and an appreciation is developing that it may be critically involved in expression of addiction to a wide range of drugs of abuse (Maskos, 2008; Mena-Segovia *et al*, 2008). An important step for incorporation of the LDT into the neural circuitry involved in addiction to nicotine was determination of whether this drug has actions on cells within this nucleus. By showing that nicotine has pre- and postsynaptic actions on virtually every cholinergic and non-cholinergic LDT cell tested, our data contributes to understanding the mechanisms that may underlie the likely role that the LDT, through its projections to reward circuitry, has in the development, and maintenance of, addiction to nicotine. It will now be important to determine whether nicotine-dependent synaptic plasticity occurs in the LDT, as has been suggested by the data of Nelson *et al* (2007) and shown in the VTA (Mansvelder and McGehee, 2000). Actions of nicotine within the LDT incurred in a first time smoker, or those in a chronic smoker, may be long lasting and may result in alteration of cholinergic and non-cholinergic output to the VTA and to other terminal fields.

Accordingly, gaining information about the basic action of nicotine on LDT neurons represents a necessary step toward refinement of the current model of the neurobiology of addiction to include involvement of the LDT.

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