

Synaptic Mechanisms Underlie Nicotine-Induced Excitability of Brain Reward Areas

Huibert D. Mansvelder,^{1,4} J. Russel Keath,²
and Daniel S. McGehee^{1,3}

¹Department of Anesthesia and Critical Care

²Department of Neurobiology, Pharmacology,
and Physiology

University of Chicago

5841 S. Maryland Avenue

MC 4028

Chicago, Illinois 60637

Summary

A single nicotine exposure increases dopamine levels in the mesolimbic reward system for hours, but nicotine concentrations experienced by smokers desensitize nAChRs on dopamine neurons in seconds to minutes. Here, we show that persistent modulation of both GABAergic and glutamatergic synaptic transmission by nicotine can contribute to the sustained increase in dopamine neuron excitability. Nicotine enhances GABAergic transmission transiently, which is followed by a persistent depression of these inhibitory inputs due to nAChR desensitization. Simultaneously, nicotine enhances glutamatergic transmission through nAChRs that desensitize less than those on GABA neurons. The net effect is a shift toward excitation of the dopamine reward system. These results suggest that spatial and temporal differences in nicotinic receptor activity on both excitatory and inhibitory neurons in reward areas coordinate to reinforce nicotine self-administration.

Introduction

Tobacco use is a serious risk factor for cancer, as well as cardiovascular and respiratory disease. Currently, over 4 million deaths each year are attributed to tobacco use (WHO, 1999). Despite these consequences and significant public education efforts, the number of smokers continues to increase worldwide (Peto et al., 1992, 1999). Nicotine is the addictive ingredient in tobacco that drives people to dependence. Many drugs of abuse, including nicotine, cocaine, and amphetamines, stimulate increases in dopamine (DA) levels in the mesoaccumbens reward system (Court et al., 1998; Di Chiara and Imperato, 1988; Imperato et al., 1986). Although nicotinic acetylcholine receptors (nAChRs) are expressed throughout the brain, those found in the ventral tegmental area (VTA) contribute to the rewarding effects of nicotine (Corrigall et al., 1994; Nisell et al., 1994). A single exposure to nicotine increases DA release in the nucleus accumbens (NAcc) from VTA DA neurons for more than an hour in vivo (Di Chiara and Imperato, 1988; Imperato et al., 1986; Schilström et al., 1998a). However, the

nAChRs on the DA neurons desensitize in seconds to minutes in the presence of physiologically relevant nicotine concentrations (Pidoplichko et al., 1997; Dani et al., 2000), so alternative mechanisms must be responsible for the prolonged excitation.

In vivo biochemical data indicate that excitatory glutamatergic synaptic transmission in the VTA contributes to the long-term excitation of the DA system by nicotine (Schilström et al., 1998a). We have recently shown that a single exposure to nicotine can induce long-term potentiation (LTP) of glutamatergic inputs to the VTA DA neurons (Mansvelder and McGehee, 2000). In vivo biochemical data have also indicated that DA neurons are under tonic inhibitory control by GABA, and that removal of this inhibition by VTA infusion of GABA_A antagonists leads to increased DA release in the NAcc and behavioral reinforcement (David et al., 1997; Ikemoto et al., 1997a, 1997b; Westerink et al., 1996). The possible interaction of nicotine with GABAergic input to DA neurons has not been studied.

VTA DA neurons receive GABAergic inputs from local interneurons and projection fibers from the NAcc and the ventral pallidum (Kalivas et al., 1993; Steffensen et al., 1998; Walaas and Fonnum, 1980). Nicotinic AChRs are expressed by GABA neurons throughout the brain in rodents and in humans, where they excite the GABA neuron and increase GABAergic transmission (Alkondon et al., 2000a; Fisher et al., 1998; Jones and Yakel, 1997; Lena and Changeux, 1997; Lena et al., 1993). Recently, it was reported that non-DA neurons in the VTA express nAChRs that excite these neurons upon activation by nicotine (Charpentier et al., 1998; Yin and French, 2000). However, the physiological role of these receptors was not established. In this study, we examine whether nicotine concentrations experienced by smokers (Henningfield et al., 1993) can modulate GABAergic inputs to VTA DA neurons. Moreover, we test the effects of nicotine exposure similar to that experienced during and after cigarette smoking on both glutamatergic and GABAergic inputs to the VTA DA neurons. We show that combined effects of nicotine on inhibitory and excitatory inputs to VTA DA neurons leads to a persistent increase in excitability that may ultimately contribute to the rewarding properties of nicotine.

Results

VTA DA neurons receive GABAergic inputs from local interneurons and afferent projections from the NAcc and the ventral pallidum (Kalivas et al., 1993; Steffensen et al., 1998; Walaas and Fonnum, 1980), which is schematized in Figure 1A. Horizontal slices, cut at the level of the VTA, contain a substantial part of the GABAergic projections from the NAcc and VP, as well as GABAergic interneurons within the nucleus (Figure 1B). Under infrared illumination, cell bodies of VTA neurons are readily visualized (Figure 1C). In whole-cell recordings, DA neurons in the VTA can be distinguished from other cell types in the nucleus by the presence of a prominent

³Correspondence: dmcgehee@midway.uchicago.edu

⁴Present address: Columbia University, Department of Biological Sciences, New York, NY 10027.

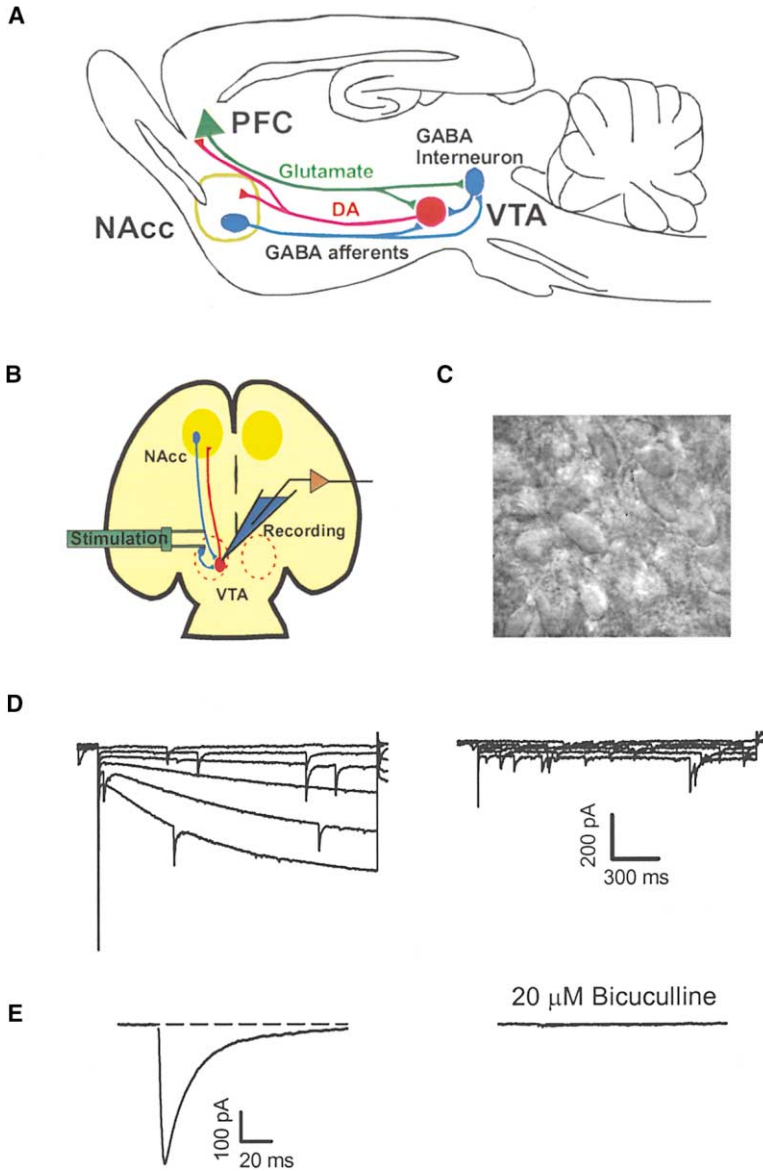


Figure 1. Inhibitory Transmission in Brain Reward Areas

(A) shows a simplified schematic of the VTA and afferent projections. Inhibitory GABAergic innervation of VTA DA neurons originates from NAcc, ventral pallidum (not shown), and local interneurons.

(B) Horizontal slice preparation includes the VTA, Nacc, and rostrocaudal GABAergic projection fibers. Stimulation and recording electrodes are shown. The stimulating electrode was positioned such that both GABAergic projections to the VTA and local interneurons were stimulated.

(C) shows an image of VTA neurons under infrared illumination in a 250 μm thick tissue slice.

(D) The current responses to a series of hyperpolarizing voltage steps are illustrated. The holding potential was -60 mV and the voltage was stepped to -120 mV in 10 mV intervals. The presence of a slowly activating hyperpolarization current (I_h) was used to identify DA neurons. The current response in an I_h -negative neuron is illustrated in the right hand panel.

(E) Extracellular stimulation via electrodes placed $\sim 100\text{ }\mu\text{m}$ rostral to the VTA induces GABAergic synaptic transmission, which elicits an inward current under these recording conditions ($V_m = -70\text{ mV}$). IPSCs were completely blocked by $20\text{ }\mu\text{M}$ bicuculline ($n = 3$). All IPSC recordings were carried out in the presence of DNQX ($10\text{ }\mu\text{M}$).

hyperpolarization activated current, I_h (Figure 1D) (Johnson and North, 1992; Mercuri et al., 1995). All synaptic modulation experiments were performed on cells that express I_h . Extracellular stimulation of the tissue 50 to $100\text{ }\mu\text{m}$ rostral of the recording site in the VTA can result in excitation of both local and projection GABAergic fibers (Figure 1B). Our experiments did not discriminate between these populations. Stimulation of the tissue in the presence of $10\text{ }\mu\text{M}$ DNQX resulted in IPSCs that varied in size from recording to recording (50 pA – 2 nA). Evoked IPSCs were abolished by bath application of $20\text{ }\mu\text{M}$ bicuculline (Figure 1E), indicating that the IPSCs are mediated by GABA_A receptor activation.

Nicotine Facilitates AP Firing in GABA Neurons that Project to VTA DA Neurons

Without stimulation and in the presence of DNQX, spontaneous IPSCs occurred with an average frequency of $1.68 \pm 0.44\text{ Hz}$ ($n = 11$). A 2 min bath application of 1

μM nicotine caused a robust increase in spontaneous IPSC frequency (Figures 2A, 2D, and 2F). Figure 2D illustrates the average frequency normalized to control activity levels for all cells tested. In 7 of 11 cells tested, the spontaneous IPSC frequency decreased after the increase (Figures 2B and 2D), suggesting a desensitizing effect of nicotine. The nicotine-induced increase in IPSC frequency was accompanied by an increase in IPSC amplitudes in all neurons tested. An example of the nicotine-induced shift to larger amplitudes is illustrated in Figure 2C. This effect could be explained by an increase in action potential firing in the GABA neurons that synapse on the DA neurons in the presence of nicotine. To test this, recordings were made in the presence of $1\text{ }\mu\text{M}$ TTX to block action potentials. In all 9 cells tested in the presence of TTX, nicotine had no effect on either the IPSC frequency (Figures 2E and 2F) or amplitude (not shown). We recently reported that nicotine enhances glutamate release onto VTA DA neu-

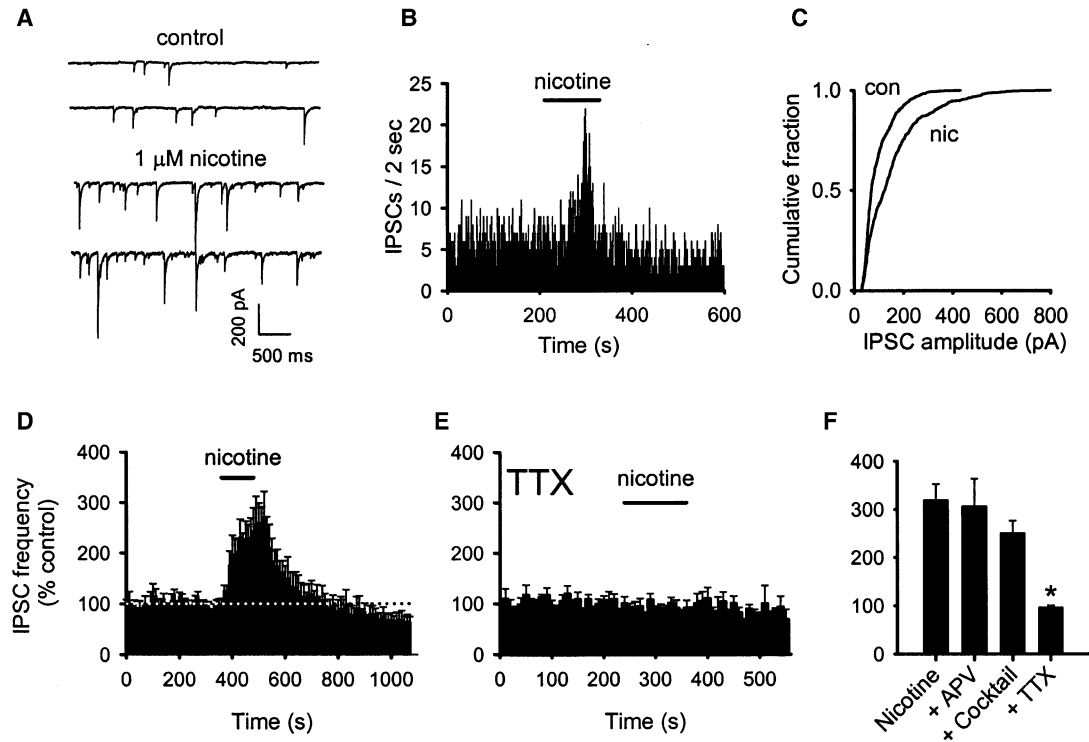


Figure 2. Nicotine Enhances GABAergic Synaptic Transmission

(A) Spontaneous IPSCs recorded during control and in the presence of 1 μ M nicotine.
 (B) The IPSC frequency from one cell is plotted versus the time of the experiment (2 s bins). Perfusion of the recording chamber with 1 μ M nicotine induced an increase in IPSC frequency that was followed by a decrease below baseline.
 (C) An example of the cumulative IPSC amplitude distributions from one neuron compiled from control and nicotine treatment periods, illustrating the shift to larger amplitude events ($p < 0.001$, Kolmogorov-Smirnov test). Similar amplitude effects were seen in all 11 neurons tested.
 (D) Average normalized IPSC frequency data from all 11 neurons tested with nicotine.
 (E) Average normalized IPSC frequency data from 9 neurons recorded in the presence of TTX (1 μ M). Nicotine did not induce a significant change in IPSC frequency in any of the cells tested under these conditions, indicating a dependence upon action potential propagation.
 (F) Population data from control slices, illustrating an average increase of $310\% \pm 37\%$ in 11 cells tested. APV (50 μ M) treatment had no effect on the nicotine-induced increase in IPSC frequency ($n = 6$). "Cocktail" denotes perfusion of the slice with inhibitors of AMPA, NMDA, mGluRs, muscarinic AChRs, D₁, D₂, and 5-HT receptors: DNQX (10 μ M), APV (50 μ M), MCPG (1 mM), atropine (1 μ M), SCH23390 (10 μ M), sulpiride (10 μ M), and methysergide (10 μ M), respectively. This treatment had no effect on the nicotine-induced increase in IPSC frequency ($n = 4$). TTX completely abolished the nicotine response ($n = 9$).

rons. Although we blocked non-NMDA type glutamate receptors in these experiments, we tested whether NMDA receptor activity contributed to the effect of nicotine. Application of APV (50 μ M) had no effect on the nicotine-induced increase in GABAergic transmission (Figure 2F; $n = 6$). To further examine whether indirect effects on neuronal circuitry in the VTA are responsible for the nicotine-induced increase in GABAergic input to the DA neurons, we tested the effect of nicotine on spontaneous IPSCs in the presence of a cocktail of receptor antagonists, including DNQX (10 μ M), APV (50 μ M), MCPG (1 mM), atropine (1 μ M), SCH23390 (10 μ M), sulpiride (10 μ M), and methysergide (10 μ M). Under these conditions, 1 μ M nicotine increased GABAergic transmission by $250\% \pm 27\%$ (Figure 2F, $n = 4$), showing that the effects of nicotine on GABA neuron activity are independent of glutamatergic, muscarinic, dopaminergic, and serotonergic inputs. In 3 of 4 cells tested in the presence of these blockers, we also observed a decrease in IPSC frequency after the initial increase (not shown). These findings indicate that nicotine directly

increases GABAergic transmission by inducing action potential firing in GABA neurons, mediated by nAChRs on the GABA neurons. The TTX sensitivity of this effect suggests that these nAChRs are located away from the terminal, and do not influence GABA release directly.

In several brain areas, including the VTA, activation of nAChRs induces inward currents that cause depolarization and lowering of the threshold for action potential firing (Jones et al., 1999; McGehee and Role, 1995). To test the effect of nicotine on the excitability of GABA neurons, we studied evoked IPSCs with minimal stimulation. The extracellular stimulation intensity was reduced to the point where an IPSC was elicited in only about 50% of the stimulations (Figures 3A and 3B). Apparently, at this stimulus intensity, the GABAergic fibers are depolarized very close to the threshold for action potential firing. When 1 μ M nicotine was bath applied for 200 s, the failure rate decreased dramatically, meaning that the majority of stimulations successfully elicited IPSCs. Nicotine decreased the failure rate in all 14 cells tested (Figures 3B and 3C). This could either result from a more

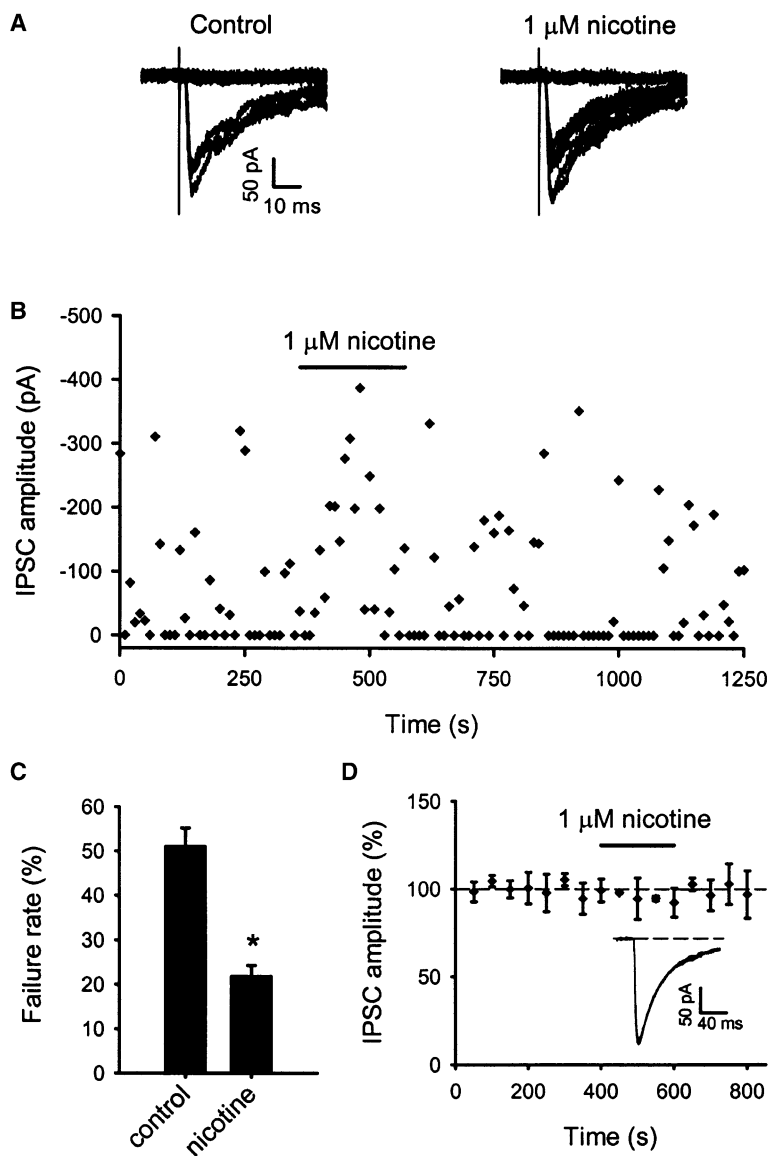


Figure 3. Nicotine Decreases the Firing Threshold of GABA Neurons that Project to VTA DA Neurons

(A) Example of evoked IPSCs with stimulus amplitude set to evoke IPSCs in about 50% of the stimulations. Ten consecutive traces are superimposed. *Left*, control; *right*, in the presence of 1 μ M nicotine. Note the increased number of successful stimulations in the presence of nicotine. Traces filtered at 5 kHz.

(B) IPSC magnitude is plotted versus time with 0.1 Hz extracellular stimulations. Perfusion of the slice with 1 μ M nicotine resulted in a decrease in failures.

(C) One micromolar nicotine decreased the failure rate in 14 cells tested under these conditions ($p < 0.001$).

(D) When stimulus intensity was set to evoke IPSCs in 100% of the stimulations, there was no change in the IPSC amplitudes in response to nicotine ($n = 5$).

successful recruitment of the same GABAergic fibers as stimulated under control, or from the recruitment of more GABAergic fibers. In either case, the excitability of GABA neurons is increased by nicotine. Interestingly, in 10 of these 14 cells, the failure rate increased during the wash out of nicotine (see below). When stimulus intensity was set to evoke IPSCs in 100% of the stimulations, nicotine did not affect the evoked IPSC size ($n = 5$; Figure 3D). These data suggest that nicotine lowers the threshold for action potential firing in GABA neurons.

nAChRs on GABA Neurons and Presynaptic Glutamatergic Terminals

Non-DA neurons in the VTA have been reported to express mRNA coding for the $\alpha 4$, $\beta 2$, and $\beta 3$ nAChR subunits (Charpentier et al., 1998). To test whether these receptor subunits mediate the observed effects of nicotine on spontaneous GABAergic transmission to the DA neurons, we examined the sensitivity of the enhancement to selective nAChR antagonists. Methyllycaconi-

tine citrate (MLA, 10 nM), a selective antagonist of $\alpha 7$ subunit-containing nAChRs (Alkondon et al., 1992; McGehee and Role, 1995; Seguela et al., 1993), had no effect on the increase of spontaneous IPSC frequency induced by nicotine (Figures 4A and 4D). In contrast, mecamylamine (MEC, 1 μ M), a selective antagonist of non- $\alpha 7$ subunit-containing nAChRs (McGehee and Role, 1995), completely abolished the effect of nicotine (Figures 4B and 4D). Another non- $\alpha 7$ antagonist, dihydro- β -erythroidine hydrobromide (DH β E, 500 nM), which is selective for $\beta 2$ -containing nAChRs (Alkondon and Albuquerque, 1993; Luetje et al., 1990), also blocked the effect of nicotine on spontaneous IPSC frequency (Figures 4C and 4D). This pharmacological profile demonstrates that $\beta 2$ -containing nAChRs mediate the enhancement of GABAergic inputs to VTA DA neurons by low nicotine concentrations. Given the strong expression of $\alpha 4$ subunit mRNA in VTA (Charpentier et al., 1998), it is likely that these receptors also contain $\alpha 4$.

Interestingly, the nAChR subtypes expressed by GABA

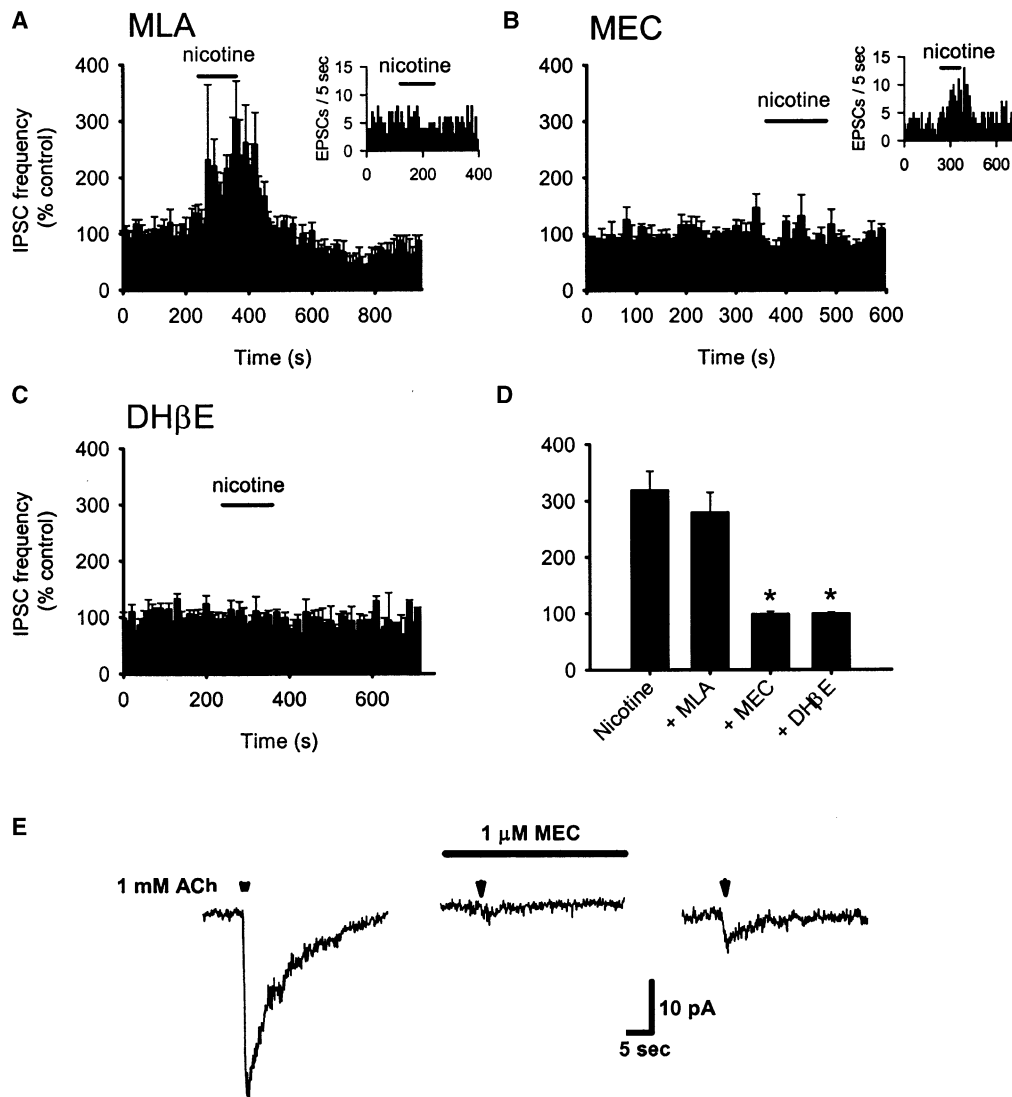


Figure 4. β 2-Containing nAChRs Mediate Nicotinic Modulation of GABAergic Transmission

All IPSC frequency data collected under each condition were normalized, averaged, and plotted versus the time of the experiments. (A) Perfusion of the slice with the α 7-selective antagonist MLA (10 nM) did not affect the 1 μ M nicotine-induced increase in IPSC frequency. Inset shows an example of MLA blockade of nicotine-induced increase in glutamatergic EPSC frequency in a VTA DA neuron. (B) The nicotine-induced increase in IPSC frequency was inhibited by 1 μ M MEC, which is selective for non- α 7 nAChRs. Inset shows an example of nicotine-induced increase in glutamatergic EPSC frequency in the presence of 1 μ M MEC. (C) The nicotine response was also inhibited by 500 nM DH β E, which is selective for β 2-containing nAChRs. (D) Average nicotine-induced increases in IPSC frequency in the presence and absence of different antagonists. (Control, n = 12; MLA, n = 5; MEC, n = 6; Dh β E, n = 7; * p < 0.001.) (E) One millimolar ACh-induced currents in I_h -negative VTA neurons are inhibited by MEC. The example trace shows inhibition of the ACh current following bath perfusion with 1 μ M MEC. A 50 min wash of the slice with control aCSF resulted in partial recovery. 12 of 12 neurons showed similar sensitivity to 1 μ M MEC, with an average inhibition to 11% \pm 4% of control amplitude.

neurons differ markedly from the nAChRs that enhance glutamatergic inputs to VTA DA neurons (Mansvelder and McGehee, 2000). These nAChRs have a different pharmacology and are of the α 7 subtype (or possibly α 6-containing nAChRs found in VTA that are also MLA sensitive; Klink et al., 2001) (see insets of Figures 4A and 4B). Thus, in the VTA, different nAChR subtypes mediate distinct physiological effects by different synaptic inputs.

To assay the pharmacology of the nAChRs expressed by GABA neurons more directly, the effect of focal appli-

cation of acetylcholine (ACh; 1 mM) was tested during voltage-clamp recordings from I_h -negative neurons in the VTA. Within this nucleus, I_h -negative neurons are predominantly GABAergic (Johnson and North, 1992; Klink et al., 2001). As illustrated in Figure 4E, ACh induced inward currents that were at least partially blocked by bath perfusion with 1 μ M MEC. In 12 of 12 neurons tested, MEC inhibited the currents to 11% \pm 4% of control response magnitude on average. MEC washes out slowly from the slice preparation, but the ACh response recovered partially after 50 min wash

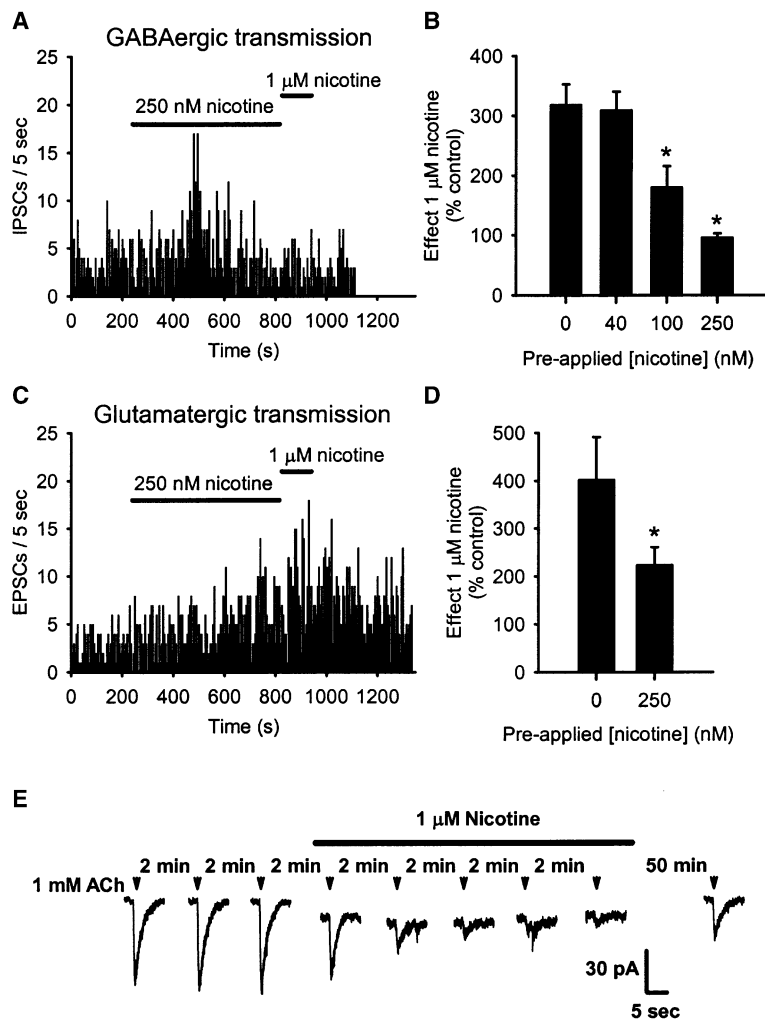


Figure 5. GABAergic and Glutamatergic Transmission Show Different Degrees of Desensitization by Nicotine Concentrations Experienced by Smokers

(A) Perfusion of the slice with 250 nM nicotine induces a small transient increase in spontaneous IPSC frequency. Application of 1 μ M nicotine immediately following the lower concentration did not alter IPSC frequency.

(B) The concentration dependence of the desensitizing effects of 10 min nicotine pretreatments on the IPSC frequency response. In all cases, the test dose was 1 μ M nicotine. (Control, $n = 12$; 40 nM, $n = 5$; 100 nM, $n = 7$, 250 nM, $n = 7$; * $p < 0.02$.)

(C) Spontaneous EPSC frequency data are plotted versus time. Perfusion with 250 nM nicotine induced a small increase in EPSC frequency and subsequent challenge with 1 μ M nicotine induced a further increase.

(D) Following the 250 nM nicotine treatment, 1 μ M nicotine more than doubles EPSC frequency on average. There is, however, a significant decrease from control nicotine sensitivity due to partial desensitization of the nAChRs that mediate this effect ($p < 0.05$; $n = 5$).

(E) Puffer pipette application of 1 mM ACh onto the soma of an I_h -negative VTA neuron induces inward current responses (300 ms applications; $V_h = -70$ mV). Repeated applications at 2 min intervals are illustrated before and during bath perfusion with 1 μ M nicotine. Partial recovery of the response was observed after 50 min wash of the slice with control solution. Similar effects were seen in four neurons tested.

(Figure 4E). These data support the idea that the nAChRs expressed by I_h -negative neurons in the VTA have similar pharmacology to those responsible for the enhancement of GABAergic input to VTA DA neurons.

Desensitization of nAChRs on GABA Neurons

The nicotine concentration in the blood of smokers is elevated to levels between 250 and 500 nM for about 10 min during and after smoking a cigarette (Henningfield et al., 1993). At these nicotine concentrations and exposure times, nAChRs desensitize substantially (Fenster et al., 1997). However, different nAChR subtypes have different desensitization properties. In particular, the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs differ considerably in this regard (Corringer et al., 1998; Fenster et al., 1997). Since glutamatergic terminals express $\alpha 7$ type nAChRs whereas GABA neurons express nAChRs with $\alpha 4$ and $\beta 2$ subunits, we compared the desensitization properties of the nAChRs that modify GABAergic and glutamatergic synaptic transmission in the VTA. These experiments involved exposing the slice to low concentrations of nicotine for 10 min, which is similar to the time course of nicotine exposure experienced by smokers.

Figure 5A shows an example of such a desensitization

experiment. After a control period of several minutes, 250 nM nicotine was applied for 10 min. Immediately following the 250 nM application, the effect of a 2 min, 1 μ M nicotine application was tested. In some cases, the frequency of spontaneous GABAergic IPSCs increased briefly during the 250 nM pre-application, but then returned rapidly to control levels or below. After the 10 min application, the sensitivity of GABAergic transmission to 1 μ M nicotine was completely abolished ($n = 7$; Figures 5A and 5B), indicating that the nAChRs were substantially desensitized and no longer depolarized the GABA neuron enough to fire action potentials. In 3 of 7 cells tested, the frequency of IPSCs during 1 μ M nicotine application was depressed to $80\% \pm 3.2\%$ of control levels; in 4 out of 7, the frequency remained the same or was slightly increased.

Just before smoking a cigarette, the nicotine concentration in the blood of a smoker is around 40 nM (Henningfield et al., 1993). In a similar experiment, a 10 min pretreatment with 40 nM nicotine did not alter the sensitivity of GABAergic transmission to 1 μ M nicotine ($n = 5$; Figure 5B). This suggests that nAChRs on GABA neurons can recover from desensitization with resting levels of nicotine, i.e., between cigarettes. At an intermediate

nicotine concentration (100 nM), nAChRs on the GABA neuron were partially desensitized as the increase in IPSC frequency was only $180\% \pm 35.6\%$ of control ($n = 7$), which is significantly lower than the sensitivity of untreated slices ($310\% \pm 37\%$; Figure 5B).

In contrast, the response of spontaneous glutamatergic transmission to such a nicotine profile is strikingly different. The frequency of spontaneous EPSCs gradually increased during the 10 min 250 nM nicotine pre-application, without any decline (Figure 5C). Subsequent application of 1 μ M nicotine increased the frequency of spontaneous EPSCs to $223\% \pm 37\%$ of control with a slow decline back to baseline ($n = 5$; Figures 5C and 5D). Thus, due to the different desensitization properties of the nAChRs on presynaptic glutamatergic terminals versus those on GABA neurons, glutamatergic transmission can increase under conditions that eliminate the GABAergic enhancement by nicotine.

To further assess the desensitization of nAChR responses in VTA GABA neurons, we tested the effects of nicotine perfusion on whole-cell responses to 1 mM ACh. Figure 5E illustrates the inward current responses to repeated ACh applications at 2 min intervals. Perfusion of the recording chamber with 1 μ M nicotine caused rapid desensitization of the ACh-induced currents, with near complete inhibition of these currents within 5 min of the nicotine application ($n = 4$). Nicotine also induced a slow inward current that returned to baseline within 10–15 min in all four neurons tested in this manner. When the nicotine was washed from the bath for 50 min, the ACh-induced currents showed significant recovery from desensitization (Figure 5E).

Endogenous Cholinergic Transmission Drives DA Neuron Inhibition

As indicated above, in a subset of the dopamine neurons, we observed a decrease of spontaneous IPSCs below control level during and after the application of nicotine (Figure 2B). Furthermore, in a similar subset of dopamine neurons, we found that the failure rate of evoked IPSCs increased during and after nicotine application (Figure 3). Moreover, nAChRs on GABA neurons desensitize rapidly at low nicotine concentrations (Figure 5). Therefore, we reasoned that this nAChR desensitization might block ongoing endogenous cholinergic excitation of these GABA neurons. The VTA receives cholinergic input from the laterodorsal and the pedunculopontine tegmental nuclei (Oakman et al., 1995). Recently, it was found that cholinergic terminals in the VTA selectively target non-DA neurons and a subset of DA neurons (Garzón et al., 1999). If cholinergic transmission normally depolarizes GABA neurons, nicotine-induced nAChR desensitization would remove this excitatory drive.

To address this hypothesis, we first tested the firing threshold of GABA neurons by re-examining the effects of nicotine on IPSC failure rate, as shown in Figure 3. These experiments employed perforated patch recording to ensure stability of the postsynaptic neuron for long recordings. The amplitude of evoked IPSCs was smaller in the amphotericin perforated-patch configuration (Figure 6A) than in normal whole-cell mode (Figure 3B), where the internal chloride concentration is set by the pipette solution. The endogenous chloride concen-

tration in VTA DA neurons is likely to be much lower than the 77 mM used in the internal solution for whole-cell recordings and the poor Cl^- permeability of the amphotericin channels conserves the natural Cl^- concentration gradient. This was supported by measurement of the chloride reversal potential, which was close to -40 mV ($n = 3$; data not shown). As in the normal whole-cell configuration, the failure rate of evoked IPSCs decreased in response to nicotine (1 μ M, 200 s) in all neurons tested ($n = 14$). In 9 of 14 neurons, near the end of the application and shortly after, the failures increased above control levels (Figures 6A and 6B). The time course of the nicotine effect for the nine neurons with increased failure rates is illustrated in Figure 6B. Four of these neurons were recorded from long enough to show recovery back to baseline failure rates (Figure 6B). Thus, in two thirds of the GABA neurons, nicotine first decreases and then increases the threshold for action potential firing relative to control conditions. This observation is in agreement with the decrease in spontaneous IPSC frequency to below baseline levels following nicotine application in a subset of dopamine neurons (8 of 12 neurons; Figures 2B and 2D).

Additional support for a direct effect of nicotine on GABA neuron activity was obtained from current clamp recordings on I_h -negative neurons in the VTA. The bath solution contained DNQX (10 μ M), APV (50 μ M), and bicuculline (20 μ M) to limit the effects of nicotine-induced enhancement of glutamate and GABA transmission. In 4 of 4 cells tested, nicotine induced a transient increase in AP activity to $180\% \pm 42\%$ (Figure 6C), in agreement with previous studies (Yin and French, 2000). In addition, 3 of 4 cells displayed a decrease in AP frequency to $66\% \pm 12\%$ of control levels following the increased activity (Figure 6C). In control recordings from I_h -negative neurons ($n = 4$), the AP frequency did not vary more than 7.5% over similar recording times. This observation is consistent with the idea that nAChR activation and desensitization control the AP threshold of a subset of GABA neurons.

If the loss of cholinergic drive to a subset of GABA neurons can contribute to the excitability of a subset of DA neurons, then it follows that inhibiting nAChRs should increase AP frequency in some VTA DA neurons. To test this possibility, current clamp recordings were carried out on I_h -positive neurons to assess the effect of MEC on action potential frequency. In these experiments, the bath solutions included DNQX (10 μ M), APV (50 μ M) to block the endogenous effects of glutamate transmission. In 4 of 11 current clamp recordings from I_h -positive VTA neurons, MEC (1 μ M) significantly increased AP firing frequency to $321\% \pm 129\%$ of control (Figure 6C). These effects were slow in onset, with the peak response occurring after 5–7 min of continuous MEC application. In all cases, the increase continued until the end of the recording, which averaged 10.25 ± 2.1 min from the onset of the increase. Thus, the non- $\alpha 7$ nAChR antagonist MEC increased the firing rate of a subset of dopamine neurons. This is consistent with a decrease in the inhibitory GABAergic input to these neurons, due to loss of cholinergic drive to the GABA neurons.

The prevalence of this effect (4 of 11, or 36%) is significantly lower than the nicotine-induced depression of

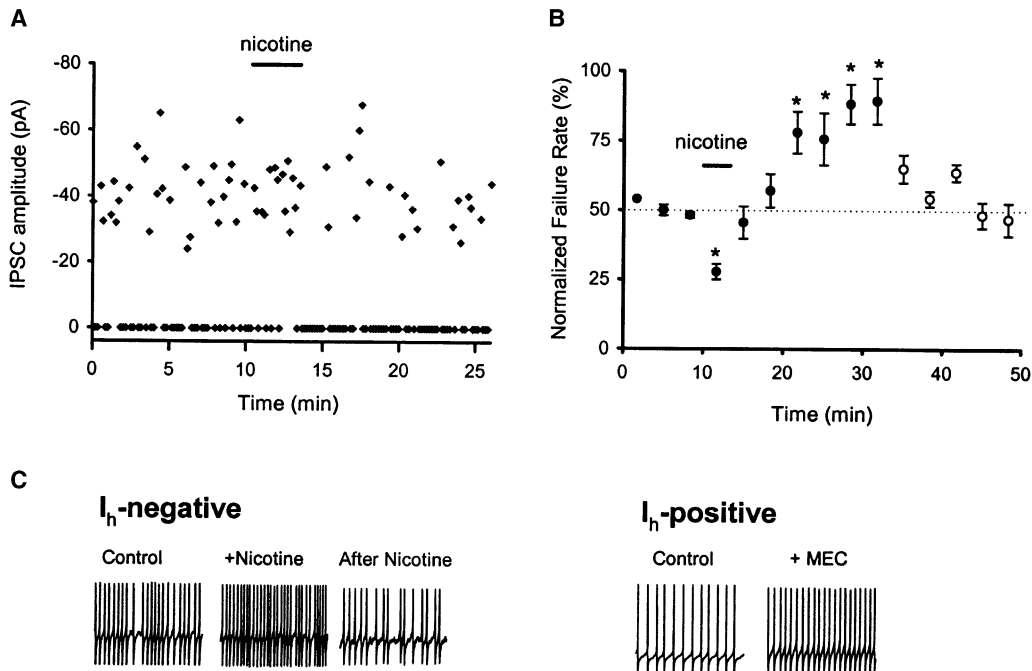


Figure 6. Nicotine Increases and then Decreases GABAergic Input to VTA DA Neurons

(A) Perforated-patch recording of evoked IPSC amplitudes before, during, and after 1 μ M nicotine treatment.

(B) The time course of the nicotine effect is illustrated for 9 of 14 neurons tested that showed a decrease followed by an increase in failures in response to 1 μ M nicotine. Data from the 5 cells that only showed a decrease in failures were not included in this average. Failure rates during 200 s epochs were averaged and normalized to 50% and plotted versus the time of the experiment (* $p < 0.05$ relative to baseline rate; $n = 9$). The open symbols are averaged from four neurons that were recorded from long enough to illustrate recovery to baseline.

(C) Action potential recording from an I_h -negative neuron shows that nicotine induces an increase followed by a decrease in AP frequency. 4/4 neurons showed significant increases in AP frequency in response to nicotine ($p < 0.005$). Significant decreases in AP frequency following nicotine treatment were observed in 3/4 neurons tested ($p < 0.005$). In the right panel, AP recording from an I_h -positive DA neuron is illustrated before and after treatment with MEC (1 μ M). 4 of 11 neurons displayed significant increases in activity relative to control ($p < 0.005$) and this increase continued until the end of each recording (average 10.25 ± 2.1 min from the onset of the increase).

IPSCs shown above ($\sim 70\%$). However, an increased firing rate in 36% of VTA DA neurons *in vivo* would likely cause significant increases in DA release.

To further test whether endogenous cholinergic transmission contributes to the circuitry of the VTA, we studied the effects of acetylcholinesterase inhibition on GABAergic transmission. In the brain, ACh is rapidly broken down to choline and acetate by acetylcholinesterase (Feldberg, 1945). By inhibiting acetylcholinesterase activity, the longevity of ACh as an active neurotransmitter is increased. Methamidophos has been reported to inhibit acetylcholinesterase activity in hippocampal tissue without affecting neurotransmitter release or postsynaptic receptors (Camara et al., 1997). Therefore, we expected methamidophos to have an effect similar to nicotine application by increasing ACh concentration in the VTA. Methamidophos (200 μ M), applied for 10 min, decreased the failure rate of evoked IPSCs ($n = 6$; Figures 7A and 7B), thus confirming our hypothesis. Similar to the effects of nicotine, 3 of 6 methamidophos-treated cells had increases in failure rate above control levels following the decrease in failures. The effect of increased endogenous ACh levels by methamidophos on evoked IPSCs was not blocked by 1 μ M atropine, but was blocked by 1 μ M MEC (Figures 7C and 7D). This suggests that the same nAChR subtype mediates the increase in GABA neuron excitability by

both nicotine and endogenous ACh. These findings also support the idea that a subset of GABA neurons are depolarized by endogenous ACh inputs, and this excitation is removed when either nicotine or chronic high ACh levels desensitize the nAChRs. The subsequent decrease of the inhibitory inputs effectively "disinhibits" the DA neuron, thus increasing its excitability.

Recovery from Nicotine-Induced Desensitization Takes over an Hour

Nicotine induces [3 H]GABA release from mouse brain synaptosomes via $\beta 2$ -containing nAChRs, and desensitization of this effect has a recovery $T_{1/2}$ of 4.95 min (Lu et al., 1999). In oocyte expression, nAChRs of defined subunit composition recover from desensitization with time constants that vary from minutes to tens of minutes. For instance, the $\alpha 4\beta 2$ receptor recovers with a time constant of approximately 90 min at room temperature, whereas the $\alpha 7$ homomeric receptor recovers with a time constant of only 2 min (Fenster et al., 1997). Since nicotine-induced disinhibition of DA neurons is due to nAChR desensitization, recovery from desensitization could restore inhibition of DA neurons and terminate the enhanced DA output to the NAcc. It is important to note that in the slice preparation, recovery from desensitization is likely a combination of the time required for diffu-

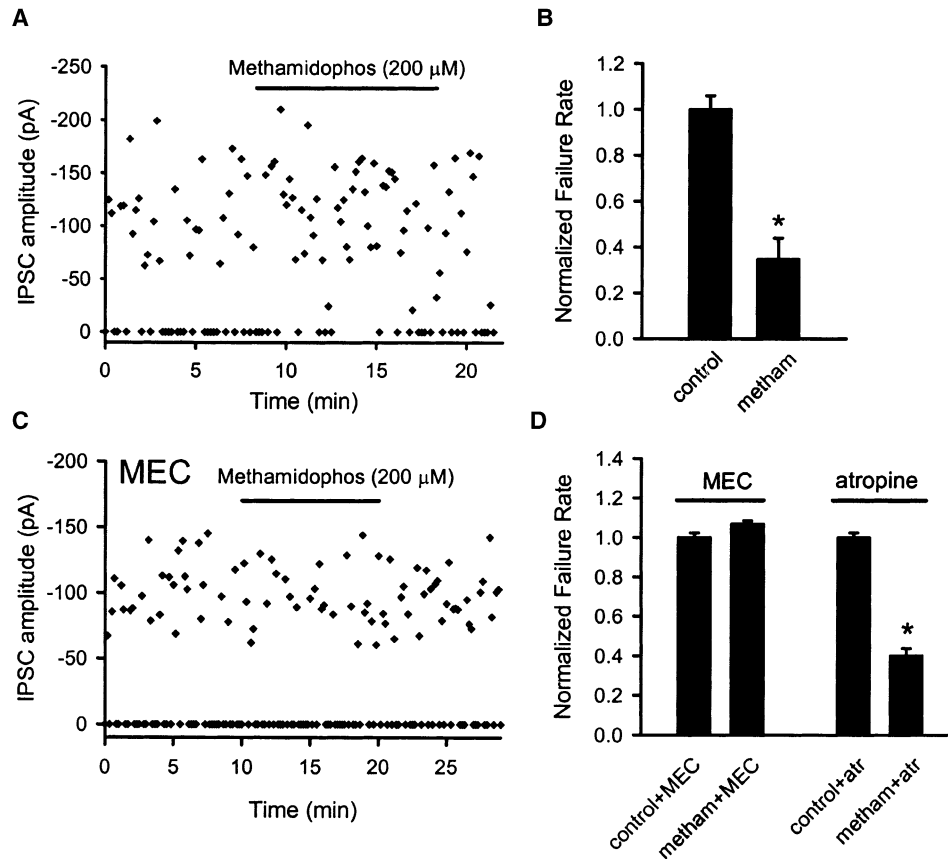


Figure 7. Endogenous ACh Transmission to GABAergic Neurons Is Modified by Acetylcholinesterase Inhibition

(A) Perfusion of the slice with the selective acetylcholinesterase inhibitor, methamidophos (200 μM), resulted in a decrease in failures of inhibitory transmission.

(B) Average IPSC failure rate normalized to the control levels plotted versus time. Methamidophos decreased failures relative to control (* $p < 0.03$; $n = 6$). In 3 of the 6 cells tested, there was also an increase in failures above control levels following the methamidophos-induced decrease in failures (not shown).

(C) MEC (1 μM) blocked the effect of methamidophos ($n = 3$).

(D) Average IPSC failure rate normalized to control levels for MEC and atropine (1 μM) treated slices. Atropine did not affect the decrease in failure rate induced by methamidophos ($n = 3$). For the antagonist tests, baseline failure rate was set close to 50% in the presence of each drug, prior to testing the effects of methamidophos.

sion of the nicotine out of the tissue and receptor desensitization properties.

We tested the recovery of the nicotine sensitivity of GABAergic transmission in the slice, as follows. After making the horizontal slices, the tissue was allowed to recover from the procedure at 34°C for at least 1 hr. After this hour, brain slices were exposed to 250 nM nicotine for 10 min at 34°C. Subsequently, they were incubated in a nicotine-free bath at 34°C for variable lengths of time. After this recovery period, the slices were transferred to the recording chamber and nicotine responsiveness was tested at room temperature (Figure 8A). During the first 13 min of recovery, 1 μM nicotine had no effect on the IPSC frequency (Figure 8B), suggesting that although nAChRs may be recovering, they are not able to depolarize the GABA neuron above threshold. After this refractory period, the response to nicotine recovered with a time constant of 20 min. From the beginning of the wash, complete restoration of the nicotine sensitivity of GABA neurons took more than an

hour. This time course is in agreement with the recovery of the ACh response in I_h -negative neurons in the VTA following nicotine-induced desensitization (Figure 5E). These recovery kinetics are considerably longer than those observed in [^3H]GABA release studies, which likely reflects the time required for nicotine to diffuse from the slice during the wash period. Independent of the underlying causes, our results indicate that DA neurons are disinhibited by nicotine for more than an hour when exposed to similar time course and concentration as those experienced by smokers. The time course of restoration of inhibition may determine the timing of the next cigarette.

Discussion

Nicotine induces a persistent enhancement of DA release from the afferent terminals of VTA DA neurons in the NAcc (Di Chiara and Imperato, 1988; Imperato et al., 1986; Schilstrom et al., 1998a), even though the nAChRs

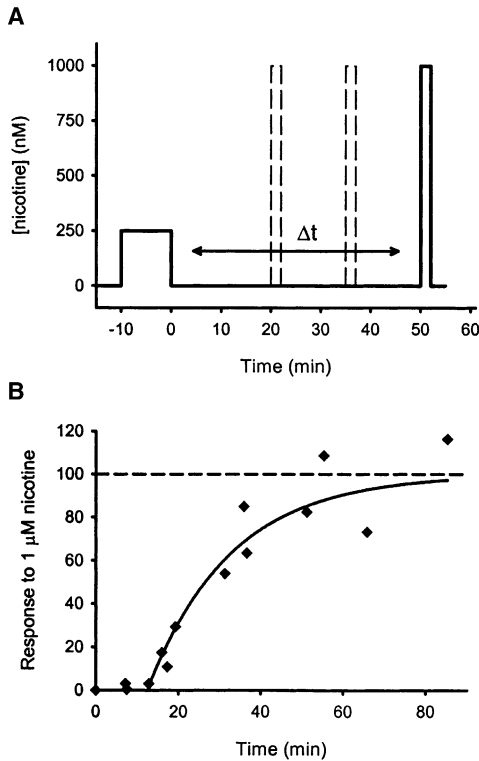


Figure 8. Complete Recovery from Desensitization by Nicotine Takes more than an Hour

(A) Diagram of experimental design where nicotine concentration is plotted versus time. (B) IPSC frequency responses to 1 μM nicotine are plotted versus the time after a 10 min 250 nM nicotine pretreatment. The responses are normalized to the control increase of spontaneous IPSC frequency by nicotine, as described in Figure 2. Note that during the first 13 min after the 250 nM application, nicotine is unable to affect spontaneous IPSC frequency. The solid line is an exponential function fit to the data ($\tau = 20$ min).

on DA neurons desensitize rapidly (Pidoplichko et al., 1997). Our results on nicotine-induced synaptic modulation may help resolve this conundrum. Here we report that GABAergic and glutamatergic synaptic inputs to VTA DA neurons are modulated by different nAChR subtypes with distinct desensitization properties. Nicotine can enhance glutamatergic transmission while the nAChRs on GABA neurons are desensitized, thus shifting the balance of synaptic inputs to excitation. This shift is even greater for a subset of DA neurons as nAChR desensitization silences endogenous cholinergic drive to the GABAergic inputs, which ultimately disinhibits the DA neurons. This coordinated disinhibition and enhanced excitation likely contributes to prolonged increases in DA release and ultimately behavioral reinforcement.

Synaptic Modulation by Nicotine in the VTA

Our results demonstrate that GABAergic inputs to VTA DA neurons express functional nAChRs. These receptors have a similar pharmacology to those expressed by VTA DA neurons themselves and are likely to contain the $\alpha 4$ and $\beta 2$ subunits (Charpentier et al., 1998; Pidoplichko et al., 1997). The nAChRs on VTA GABA neu-

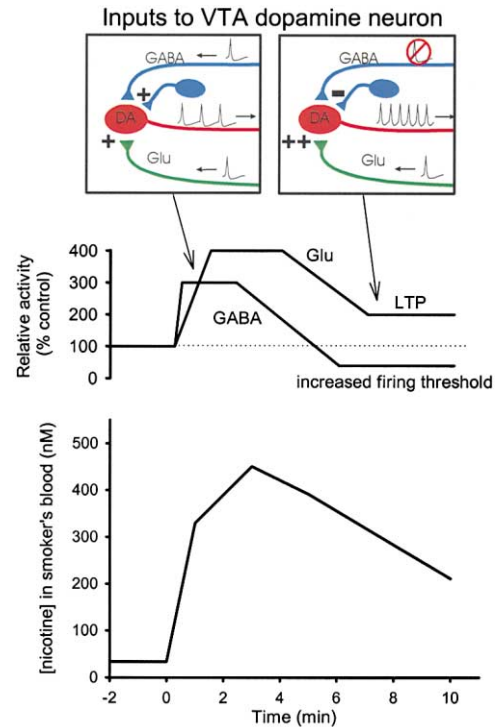


Figure 9. Excitatory and Inhibitory Inputs to VTA DA Neurons Have a Different Time Course of Activity in Response to Nicotine Concentrations in a Smoker's Arterial Blood

The upper diagram depicts the changes in activity of the different VTA cell types following nicotine exposure. The middle panel schematizes the relative activity of GABAergic and glutamatergic inputs to VTA DA neurons during a physiologically relevant nicotine concentration profile, as depicted in the lower panel. Time course of nicotine concentrations measured in smoker's arterial blood during cigarette smoking is adapted from Henningfield et al. (1993).

rons are distinct from those found on the presynaptic terminals of glutamatergic fibers in the VTA that contain the $\alpha 7$ subunit (Mansvelder and McGehee, 2000). As a result of different desensitization properties of different nAChR subtypes, we found that GABAergic and glutamatergic inputs respond very differently to nicotine profiles that are experienced by smokers.

The time course of the nicotinic modulation of GABAergic and glutamatergic inputs to VTA DA neurons is schematized in Figure 9. During the first minute of cigarette smoking, the arterial blood concentration of nicotine in humans rises rapidly above 250 nM (Figure 9, lower panel; adapted from Henningfield et al., 1993). During this increase, the non- $\alpha 7$ nAChRs on the DA neurons (Dani et al., 2000; Picciotto et al., 1998; Pidoplichko et al., 1997) as well as on the GABA neurons are activated and then desensitized rapidly (Figure 9, upper panel). The direct depolarization of the DA neurons by somatic nAChRs coincides with the increase in GABA synaptic transmission. Once these nAChRs desensitize, the inhibitory input to a subset of DA neurons will be suppressed. At the same time, $\alpha 7$ nAChR-mediated enhancement of glutamatergic transmission increases during the nicotine exposure, as there is much less desensitization of these receptors (Figure 9, upper

panel). If the DA neuron is depolarized sufficiently, the enhancement of glutamatergic transmission can induce a long-term potentiation of these inputs, as we reported previously (Mansvelder and McGehee, 2000). Complete recovery of the enhancement of GABA transmission takes more than an hour. In our experiments, there was a 13 min refractory period during which nicotine did not enhance GABA transmission, which was then followed by recovery of this effect with a time constant of 20 min. Thus, the DA neurons receive a net increase in excitatory drive from the synaptic inputs that outlasts both the presence of nicotine and the time course of nAChR receptor activation. These synaptic mechanisms may explain the prolonged excitation of the mesolimbic DA system after a single nicotine exposure, as observed in vivo (Di Chiara and Imperato, 1988; Imperato et al., 1986; Schilstrom et al., 1998b).

Nicotinic Modulation of GABA Neurons: Disinhibition by Desensitization

Our studies on AP frequency in both I_h -negative and I_h -positive VTA neurons support the role of nAChRs in the control of DA neuron excitability. In line with the observations of nicotinic modulation of inhibitory inputs to DA neurons, nicotine induced an increase followed by a decrease in AP frequency in I_h -negative VTA neurons (Figure 6C). This supports the idea that endogenous ACh contributes to the baseline excitability of a subpopulation of these cells, and that desensitization of nAChRs by nicotine removes that excitatory drive in this group. Furthermore, AP frequency in a subset of VTA DA neurons increased following treatment of the slice with MEC (Figure 6C). Again, removal of cholinergic drive to the GABA neurons tips the balance toward excitation of VTA DA neurons.

The proportion of neurons that showed disinhibition is likely a function of the sparse cholinergic projections to the VTA. Despite the high prevalence of nAChR expression, our data suggest that not every GABAergic neuron receives cholinergic inputs. We found that all I_h -negative neurons express nAChRs, but only a subset of DA neurons showed an increase in failure rate of GABAergic input, or a decrease in IPSC frequency, after nicotine application. There is precedence for this idea in other studies. For example, while nearly all DA neurons in the VTA express nAChRs (Pidoplichko et al., 1997), only 5% of the neurons actually receive cholinergic projections (Fiorillo and Williams, 2000). Together, these findings support the idea that there are only sparse cholinergic inputs to the VTA, as shown in anatomical studies by Garzón et al. (1999) and Oakman et al. (1995).

Although the nicotine-induced increase in AP frequency in non-DA VTA neurons is in agreement with the observations of Yin and French (2000), they did not report a depression of AP activity following nicotine exposure. This may be due to the different orientation of the slice preparations used in these two studies. Our horizontal slices include the pedunculopontine tegmental nucleus, which is a major source of cholinergic projections to the VTA. It is unlikely that this structure was included in the coronal slices used by Yin and French (2000). Another important difference between the studies was that MEC had no effect on DA neuron AP firing

rates in their experiments. Again, the lack of cholinergic drive to the GABA neurons in their preparation may explain the different results, along with the low prevalence of the MEC effect in our hands (36%). It is important to note that in both studies, the transmitter phenotype and postsynaptic targets of the non-DA neurons remains unknown. Our model is based primarily upon the nicotine sensitivity of GABAergic inputs to DA neurons. This will ultimately influence DA release in the NAcc, which is central to reward and reinforcement (Koob, 1992).

Nicotinic AChRs have been reported to modulate excitatory glutamatergic transmission in several brain regions (Gray et al., 1996; McGehee et al., 1995). In addition, nAChRs can modulate GABAergic transmission in multiple brain areas, such as thalamus, cortex, hippocampus, and interpeduncular nucleus (Alkondon et al., 1997, 2000a; Fisher et al., 1998; Lena and Changeux, 1997; Lena et al., 1993; Radcliffe et al., 1999). Modulation of GABA neurons by nAChRs has been most extensively studied in the hippocampus, where GABAergic interneurons express multiple nAChR subtypes (Alkondon et al., 1997, 1999; Frazier et al., 1998a; Ji and Dani, 2000; Jones and Yakel, 1997; McQuiston and Madison, 1999). Subcellular localization of nAChRs determines the physiological impact of their activation. There is evidence for nAChR expression both on presynaptic terminals, where they directly modulate GABA release, independent of action potential firing (Fisher et al., 1998; Radcliffe et al., 1999; Lu et al., 1999), and away from the synaptic terminal, where modulation of GABA release is TTX sensitive (Alkondon et al., 1997, 1999; Frazier et al., 1998a). In the VTA, nAChR-induced modulation of GABAergic transmission was completely TTX sensitive, implying that the receptors are expressed at a distance from the terminals.

Activation of nAChRs on cortical and hippocampal interneurons results either in inhibition or disinhibition of the pyramidal neurons (Alkondon et al., 2000a; Ji and Dani, 2000; Ji et al., 2001). In these areas, disinhibition of pyramidal neurons results from an increase of inhibitory GABAergic transmission to GABAergic interneurons by activation of nAChRs. Consequently, the pyramidal neurons receive less GABAergic input and are disinhibited. We found that in the VTA, low concentrations of nicotine can also disinhibit DA neurons, but by a different mechanism. Here, nicotine desensitizes nAChRs on GABA neurons, which makes them insensitive to ongoing endogenous cholinergic transmission, thereby reducing GABA neuron excitability. As a result, there is less GABAergic transmission and the DA neurons are disinhibited. A similar mechanism may disinhibit hippocampal GABA interneurons (Alkondon et al., 2000b). Prolonged exposure to low concentrations of nicotine decreases the ACh sensitivity of the GABAergic input to interneurons. Endogenous cholinergic input to GABAergic interneurons can be evoked in hippocampal slices (Frazier et al., 1998b; Hefft et al., 1999). It will be interesting to learn whether this endogenous cholinergic input contributes to the depolarization of the interneurons, and whether this is also removed by nicotine exposure as we found in the VTA.

Disinhibition of VTA DA Neurons and Reward

The depression of GABAergic input to VTA DA neurons by nAChR desensitization occurs at a nicotine concentration profile that is experienced by tobacco smokers. An important question is whether a reduction in VTA GABAergic transmission actually contributes to nicotine addiction. There are several lines of evidence that link behavioral reinforcement to GABAergic transmission. Both rats and mice will readily self-administer GABA_A receptor antagonists when they are infused focally into the VTA (David et al., 1997; Ikemoto et al., 1997a). Given the importance of DA release in self-administration, it is not surprising that GABA_A receptor blockade in the VTA also increases DA levels in the NAcc (Ikemoto et al., 1997b; Westerink et al., 1996). Thus, it is likely that the reduction in GABAergic transmission by nAChR desensitization we observed in the VTA contributes to the reinforcing effects of cigarette smoking. It is important to note that $\beta 2$ subunits, which mediate the effects of nicotine on GABA neurons, have been shown to be necessary for the maintenance of nicotine self-administration in both rats and mice (Grottick et al., 2000; Picciotto et al., 1998).

Inhibition of acetylcholinesterase was used to demonstrate that endogenous ACh transmission modulates GABA neuron excitability in the VTA. It has been shown in vivo that cholinesterase inhibition in the VTA will augment DA release in the NAcc (Blaha et al., 1996). One interpretation of this data is that endogenous ACh transmission in the VTA activates the DA system. However, physiological experiments indicate very sparse cholinergic inputs to VTA DA neurons (Fiorillo and Williams, 2000), while ultrastructural analyses of cholinergic projections to the VTA found that only a very small proportion of cholinergic terminals make contact on DA neurons (Garzón et al., 1999). The vast majority of the cholinergic neurons in the laterodorsal tegmental and the pedunculopontine nuclei project to GABA neurons in the VTA (Garzón et al., 1999). Therefore, the in vivo application of the cholinesterase inhibitor in the VTA by Blaha et al. (1996), which was maintained for several hours, may induce increased DA levels in the NAcc by disinhibition of VTA DA neurons due to nAChR desensitization on GABA neurons.

Synaptic Mechanisms and Behavior

Our results were obtained from brain slices of naïve rats, i.e., the tissue was exposed to nicotine for the first time during these experiments. Thus, nAChRs on GABA neurons are desensitized, and in about two thirds of the cases, GABAergic transmission is depressed, by a nicotine exposure similar to the levels experienced by a person smoking one cigarette. This depression lasts much longer than the nicotine exposure. In a previous study, we found that the first exposure to a similar dose of nicotine can induce LTP of glutamatergic transmission, when combined with depolarization of the DA neuron (Mansvelder and McGehee, 2000). The reduction of GABAergic transmission reported in this study might provide sufficient depolarization of the DA neuron for nicotine to induce LTP. Together, these studies emphasize that a limited exposure to nicotine is sufficient to induce lasting changes in the circuitry of the mesolimbic reward system.

Recently, it was reported that in human adolescents, the initial symptoms of nicotine dependence are already present after smoking of only a few cigarettes (DiFranza et al., 2000). These first symptoms appear during occasional use, before the onset of daily smoking. This demonstrates that for an adolescent to become nicotine dependent, occasional use for a short period of time may be sufficient. These behavioral findings support the observations of lasting changes in synaptic activity by a single exposure to nicotine, or to other drugs of abuse, as reported by our laboratory and that of others (Hamid et al., 1997; Mansvelder and McGehee, 2000; Vanderschuren et al., 1999). The synaptic mechanisms that nicotine activates within the DA reward system are likely to underlie the early steps of nicotine dependence.

Experimental Procedures

Horizontal brain slices were prepared from Sprague-Dawley rats (10–14 days of age). Following rapid decapitation, the brain was removed, the olfactory bulbs were cut away, and the midbrain was cut at the level of the 4th ventricle. Then the brain was placed in ice-cold artificial CSF solution (in mM: NaCl 125, KCl 2.5, MgCl₂ 1, CaCl₂ 2.5, Glucose 20, NaH₂PO₄ 1, NaHCO₃ 25, ascorbic acid 1; bubbled continuously with 95% O₂/5% CO₂, pH 7.3). Two or three slices (thickness 250–300 μ m) were cut in the cold solution and were placed in a holding chamber (32°C–34°C) to recover for at least 1 hr. For recording, the slice was transferred to a chamber superfused (2 ml min⁻¹) with aCSF lacking ascorbic acid at room temperature.

Neurons were visualized under infrared illumination using an upright microscope (Axioskop, Zeiss). When recording GABAergic transmission, electrodes (2.5–4 M Ω) contained (in mM): K-Gluconate 78, KCl 77, EGTA 1, HEPES 10, Glucose 10, ATP 5, GTP 100 μ M (pH 7.4 with KOH). When recording glutamatergic transmission, electrodes were filled with (in mM): K-Gluconate 154, KCl 1, EGTA 1, HEPES 10, Glucose 10, ATP 5 (pH 7.4 with KOH). When recording action potential frequency, internal K⁺ was substituted with Cs⁺. Standard whole-cell recordings were made using an Axopatch 200B amplifier, a Digidata 1320A interface, and pCLAMP 8 software (Axon Instruments Inc.). For evoked transmission, the current was filtered at 5 kHz and digitized at 20 kHz. Spontaneous transmission was filtered at 1 kHz and digitized at 5 kHz. For perforated patch recordings (Horn and Marty, 1988; Rae et al., 1991), amphotericin B (660 μ g/ml final concentration, Sigma) dissolved in DMSO was added to the pipette solution (Bonci and Malenka, 1999). Experiments were started when the series resistance dropped below 40 M Ω , but was typically 15–30 M Ω . Series resistance in normal whole-cell recording was 4–6 M Ω . Neurons were held at -60 mV to assess the presence of I_h, but were held at -70 mV throughout the rest of the voltage-clamp experiments. A bipolar tungsten electrode (FHC) was placed 50–100 μ m rostral of the recording site to evoke synaptic transmission at 0.1 Hz. With this electrode placement, it is likely that we stimulate both GABAergic projection neurons and local interneurons within the VTA.

To isolate GABAergic transmission, the external solution contained 10 μ M DNQX. Glutamatergic transmission was isolated by adding 20 μ M bicuculline to the external solution. Spontaneous IPSC analysis was carried out using MiniAnalysis software (Synaptosoft, Inc.). Amplitude and area thresholds were used to acquire events and each event was visually inspected to protect against software errors. Graphical representations of the results were constructed with SigmaPlot 5.0 (SPSS, Inc.). Average nicotine effects on IPSC frequency were presented as mean \pm SEM of the frequency during the nicotine application (2 min), while average control frequency is the mean \pm SEM of the complete baseline (typically 4 to 6 min). A Student's *t* test was used to compare the population means for IPSC frequency obtained under different conditions (SigmaPlot 7.0). A Kolmogorov-Smirnov test was used to assess statistical differences between the spontaneous IPSC amplitude distributions for control and nicotine treatment.

The IPSC frequency data are collected as counts and are therefore

best described using Poisson statistics (Cameron and Trivedi, 1998). The data from each cell were tested for the goodness-of-fit of the baseline data to a Poisson distribution with constant mean using Stata 6.0 software. Data that did not conform to a Poisson distribution due to frequency fluctuations or low counts were excluded from the analysis. Following selection, a Poisson regression analysis was used to identify significant changes in IPSC frequency between control, nicotine, and post-nicotine periods for each recording. Responsive cells were defined as those that had significant differences between control and drug treatment ($p < 0.05$).

Evoked IPSCs were differentiated from failures by the same amplitude criteria used in the analysis of spontaneous transmission: deflections from baseline $> 5 \times$ RMS noise, with appropriate rise and decay characteristics, were considered to be successful transmission. Current fluctuation less than threshold were failures. The amplitude, rise time, and decay time of the evoked IPSC were determined in real time by the pCLAMP 8 software (Axon Instruments). Care was taken that the holding current and series resistance were stable through the entire experiment. Every IPSC was visually inspected to ensure that the software determined the parameters correctly. Delay between the stimulus artifact and the IPSC was never more than 10 ms in our experiments, and histograms of delay times were well-fit with a Gaussian distribution with standard deviation less than 1 ms in all cases. These observations support the idea that these were monosynaptic connections. In addition, excitatory glutamatergic activity is inhibited by DNQX in these experiments, further limiting the possibility for polysynaptic connections following extracellular stimulation. A paired *t* test was used for comparison between control and both nicotine as well as post-nicotine periods for the failure experiments (SigmaPlot 7.0).

Action potential frequencies were determined using MiniAnalysis software, and the Poisson regression test described above was used to assess differences between control and drug-treated time periods. "Responsive cells" were defined as those that showed significant differences between control and experimental time periods ($p < 0.05$).

Nicotine tartrate, acetylcholine chloride, mecamylamine (MEC), bicuculline methiodide, methyllycaconitine citrate (MLA), dihydro- β -erythroidine hydrobromide (DH β E), SCH 23390, sulpiride, methysergide, atropine were obtained from Sigma/RBI (St. Louis, MO). 6,6-dinitroquinoxaline-2,3(1H,4H)dione (DNQX), 2-amino-5-phosphonovalerate (APV), (S)- α -methyl-4-carboxyphenylglycine (MCPG) were obtained from Tocris (Ellisville, MO) and TTX from Alomone Labs (Jerusalem, Israel). All compounds were applied through bath perfusion, with the exception of ACh, which was applied by focal puffer pipette application system (Picospritzer, General Valve Corporation). All antagonists and channel blockers were present in the bath at least 15 min before the effect of nicotine or ACh was assessed. A new slice was used for each experiment, so that neurons were exposed only once to 1 μ M nicotine.

Acknowledgments

We thank Zara Fettig and Jonathan Genzen for comments on earlier drafts of the manuscript and Dr. Robin Lester for helpful discussions. We also thank Lindy Nakamura and Dr. Vitas Bindokas for imaging assistance. This work was supported by funding from the Netherlands Organization for Scientific Research to H.D.M. (NWO, S 93-334), the National Institutes of Health DA07255 to J.R.K., and NS 35090 to D.S.M., and by the Brain Research Foundation to D.S.M.

Received: July 25, 2001

Revised: February 4, 2002

References

Alkondon, M., and Albuquerque, E.X. (1993). Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. *J. Pharmacol. Exp. Ther.* 265, 1455–1473.

Alkondon, M., Pereira, E.F., Wonnacott, S., and Albuquerque, E.X. (1992). Blockade of nicotinic currents in hippocampal neurons de-

fines methyllycaconitine as a potent and specific receptor antagonist. *Mol. Pharmacol.* 41, 802–808.

Alkondon, M., Pereira, E.F., Barbosa, C.T., and Albuquerque, E.X. (1997). Neuronal nicotinic acetylcholine receptor activation modulates gamma-aminobutyric acid release from CA1 neurons of rat hippocampal slices. *J. Pharmacol. Exp. Ther.* 283, 1396–1411.

Alkondon, M., Pereira, E.F., Eisenberg, H.M., and Albuquerque, E.X. (1999). Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. *J. Neurosci.* 19, 2693–2705.

Alkondon, M., Pereira, E.F., Eisenberg, H.M., and Albuquerque, E.X. (2000a). Nicotinic receptor activation in human cerebral cortical interneurons: a mechanism for inhibition and disinhibition of neuronal networks. *J. Neurosci.* 20, 66–75.

Alkondon, M., Pereira, E.F., Almeida, L.E., Randall, W.R., and Albuquerque, E.X. (2000b). Nicotine at concentrations found in cigarette smokers activates and desensitizes nicotinic acetylcholine receptors in CA1 interneurons of rat hippocampus. *Neuropharmacology* 39, 2726–2739.

Blahe, C.D., Allen, L.F., Das, S., Inglis, W.L., Latimer, M.P., Vincent, S.R., and Winn, P. (1996). Modulation of dopamine efflux in the nucleus accumbens after cholinergic stimulation of the ventral tegmental area in intact, pedunculopontine tegmental nucleus-lesioned, and laterodorsal tegmental nucleus-lesioned rats. *J. Neurosci.* 16, 714–722.

Bonci, A., and Malenka, R.C. (1999). Properties and plasticity of excitatory synapses on dopaminergic and GABAergic cells in the ventral tegmental area. *J. Neurosci.* 19, 3723–3730.

Camara, A.L., Braga, M.F., Rocha, E.S., Santos, M.D., Cortes, W.S., Cintra, W.M., Aracava, Y., Maelicke, A., and Albuquerque, E.X. (1997). Methamidophos: an anticholinesterase without significant effects on postsynaptic receptors or transmitter release. *Neurotoxicology* 18, 589–602.

Cameron, A.C., and Trivedi, P.K. (1998). Regression analysis of count data (Cambridge, UK: Cambridge University Press).

Charpentier, E., Barneoud, P., Moser, P., Besnard, F., and Sgard, F. (1998). Nicotinic acetylcholine subunit mRNA expression in dopaminergic neurons of the rat substantia nigra and ventral tegmental area. *Neuroreport* 9, 3097–3101.

Corrigall, W.A., Coen, K.M., and Adamson, K.L. (1994). Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. *Brain Res.* 653, 278–284.

Corringer, P.J., Bertrand, S., Bohler, S., Edelstein, S.J., Changeux, J.P., and Bertrand, D. (1998). Critical elements determining diversity in agonist binding and desensitization of neuronal nicotinic acetylcholine receptors. *J. Neurosci.* 18, 648–657.

Court, J.A., Lloyd, S., Thomas, N., Piggott, M.A., Marshall, E.F., Morris, C.M., Lamb, H., Perry, R.H., Johnson, M., and Perry, E.K. (1998). Dopamine and nicotinic receptor binding and the levels of dopamine and homovanillic acid in human brain related to tobacco use. *Neuroscience* 87, 63–78.

Dani, J.A., Radcliffe, K.A., and Pidoplichko, V.I. (2000). Variations in desensitization of nicotinic acetylcholine receptors from hippocampus and midbrain dopamine areas. *Eur. J. Pharmacol.* 393, 31–38.

David, V., Durkin, T.P., and Cazala, P. (1997). Self-administration of the GABA_A antagonist bicuculline into the ventral tegmental area in mice: dependence on D2 dopaminergic mechanisms. *Psychopharmacology (Berl.)* 130, 85–90.

Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. USA* 85, 5274–5278.

DiFranza, J.R., Rigotti, N.A., McNeill, A.D., Ockene, J.K., Savageau, J.A., Cyr, D.S., and Coleman, M. (2000). Initial symptoms of nicotine dependence in adolescents. *Tob. Control* 9, 313–319.

Feldberg, W. (1945). Present views on the mode of action of acetylcholine in the central nervous system. *Physiol. Rev.* 25, 596–642.

Fenster, C.P., Rains, M.F., Noerager, B., Quick, M.W., and Lester,

- R.A. (1997). Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine. *J. Neurosci.* *17*, 5747–5759.
- Fiorillo, C.D., and Williams, J.T. (2000). Cholinergic inhibition of ventral midbrain dopamine neurons. *J. Neurosci.* *20*, 7855–7860.
- Fisher, J.L., Pidoplichko, V.I., and Dani, J.A. (1998). Nicotine modifies the activity of ventral tegmental area dopaminergic neurons and hippocampal GABAergic neurons. *J. Physiol. (Paris)* *92*, 209–213.
- Frazier, C.J., Rollins, Y.D., Breese, C.R., Leonard, S., Freedman, R., and Dunwiddie, T.V. (1998a). Acetylcholine activates an alpha-bungarotoxin-sensitive nicotinic current in rat hippocampal interneurons, but not pyramidal cells. *J. Neurosci.* *18*, 1187–1195.
- Frazier, C.J., Buhler, A.V., Weiner, J.L., and Dunwiddie, T.V. (1998b). Synaptic potentials mediated via alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal interneurons. *J. Neurosci.* *18*, 8228–8235.
- Garzón, M., Vaughan, R.A., Uhl, G.R., Kuhar, M.J., and Pickel, V.M. (1999). Cholinergic axon terminals in the ventral tegmental area target a subpopulation of neurons expressing low levels of the dopamine transporter. *J. Comp. Neurol.* *410*, 197–210.
- Gray, R., Rajan, A.S., Radcliffe, K.A., Yakehiro, M., and Dani, J.A. (1996). Hippocampal synaptic transmission enhanced by low concentrations of nicotine. *Nature* *383*, 713–716.
- Grottick, A.J., Trube, G., Corrigan, W.A., Huwyler, J., Malherbe, P., Wyler, R., and Higgins, G.A. (2000). Evidence that nicotinic alpha(7) receptors are not involved in the hyperlocomotor and rewarding effects of nicotine. *J. Pharmacol. Exp. Ther.* *294*, 1112–1119.
- Hamid, S., Dawe, G.S., Gray, J.A., and Stephenson, J.D. (1997). Nicotine induces long-lasting potentiation in the dentate gyrus of nicotine-primed rats. *Neurosci. Res.* *29*, 81–85.
- Hefft, S., Hulo, S., Bertrand, D., and Muller, D. (1999). Synaptic transmission at nicotinic acetylcholine receptors in rat hippocampal organotypic cultures and slices. *J. Physiol. (Lond.)* *515*, 769–776.
- Henningfield, J.E., Stapleton, J.M., Benowitz, N.L., Grayson, R.F., and London, E.D. (1993). Higher levels of nicotine in arterial than in venous blood after cigarette smoking. *Drug Alcohol Depend.* *33*, 23–29.
- Horn, R., and Marty, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.* *92*, 145–159.
- Ikemoto, S., Murphy, J.M., and McBride, W.J. (1997a). Self-infusion of GABA(A) antagonists directly into the ventral tegmental area and adjacent regions. *Behav. Neurosci.* *111*, 369–380.
- Ikemoto, S., Kohl, R.R., and McBride, W.J. (1997b). GABA(A) receptor blockade in the anterior ventral tegmental area increases extracellular levels of dopamine in the nucleus accumbens of rats. *J. Neurochem.* *69*, 137–143.
- Imperato, A., Mulas, A., and Di Chiara, G. (1986). Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats. *Eur. J. Pharmacol.* *132*, 337–338.
- Ji, D., and Dani, J.A. (2000). Inhibition and disinhibition of pyramidal neurons by activation of nicotinic receptors on hippocampal interneurons. *J. Neurophysiol.* *83*, 2682–2690.
- Ji, D., Lape, R., and Dani, J.A. (2001). Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. *Neuron* *31*, 131–141.
- Johnson, S.W., and North, R.A. (1992). Two types of neurons in the rat ventral tegmental area and their synaptic inputs. *J. Physiol. (Lond.)* *450*, 455–468.
- Jones, S., and Yakel, J.L. (1997). Functional nicotinic ACh receptors on interneurons in the rat hippocampus. *J. Physiol. (Lond.)* *504*, 603–610.
- Jones, S., Sudweeks, S., and Yakel, J.L. (1999). Nicotinic receptors in the brain: correlating physiology with function. *Trends Neurosci.* *22*, 555–561.
- Kalivas, P.W., Churchill, L., and Klitenick, M.A. (1993). GABA and enkephalin projection from the nucleus accumbens and ventral pallidum to the ventral tegmental area. *Neuroscience* *57*, 1047–1060.
- Klink, R., de Kerchove d'Exaerde, A., Zoli, M., and Changeux, J.P. (2001). Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. *J. Neurosci.* *21*, 1452–1463.
- Koob, G.F. (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol. Sci.* *13*, 177–184.
- Lena, C., and Changeux, J.P. (1997). Role of Ca²⁺ ions in nicotinic facilitation of GABA release in mouse thalamus. *J. Neurosci.* *17*, 576–585.
- Lena, C., Changeux, J.P., and Mulle, C. (1993). Evidence for “preterminal” nicotinic receptors on GABAergic axons in the rat interpeduncular nucleus. *J. Neurosci.* *13*, 2680–2688.
- Lu, Y., Marks, M.J., and Collins, A.C. (1999). Desensitization of nicotinic agonist-induced [³H]gamma-aminobutyric acid release from mouse brain synaptosomes is produced by subactivating concentrations of agonists. *J. Pharmacol. Exp. Ther.* *291*, 1127–1134.
- Luetje, C.W., Patrick, J., and Seguela, P. (1990). Nicotine receptors in the mammalian brain. *FASEB J.* *4*, 2753–2760.
- Mansvelder, H.D., and McGehee, D.S. (2000). Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron* *27*, 349–357.
- McGehee, D.S., and Role, L.W. (1995). Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu. Rev. Physiol.* *57*, 521–546.
- McGehee, D.S., Heath, M.J.S., Gelber, S., Devay, P., and Role, L.W. (1995). Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. *Science* *269*, 1692–1696.
- McQuiston, A.R., and Madison, D.V. (1999). Nicotinic receptor activation excites distinct subtypes of interneurons in the rat hippocampus. *J. Neurosci.* *19*, 2887–2896.
- Mercuri, N.B., Bonci, A., Calabresi, P., Stefani, A., and Bernardi, G. (1995). Properties of the hyperpolarization-activated cation current I_h in rat midbrain dopaminergic neurons. *Eur. J. Neurosci.* *7*, 462–469.
- Nisell, M., Nomikos, G.G., and Svensson, T.H. (1994). Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. *Synapse* *16*, 36–44.
- Oakman, S.A., Faris, P.L., Kerr, P.E., Cozzari, C., and Hartman, B.K. (1995). Distribution of pontomesencephalic cholinergic neurons projecting to substantia nigra differs significantly from those projecting to ventral tegmental area. *J. Neurosci.* *15*, 5859–5869.
- Peto, R., Chen, Z.M., and Boreham, J. (1999). Tobacco—the growing epidemic. *Nat. Med.* *5*, 15–17.
- Peto, R., Lopez, A.D., Boreham, J., Thun, M., and Heath, C., Jr. (1992). Mortality from tobacco in developed countries: indirect estimation from national vital statistics. *Lancet* *339*, 1268–1278.
- Picciozzo, M.R., Zoli, M., Rimondini, R., Lena, C., Marubio, L.M., Pich, E.M., Fuxe, K., and Changeux, J.P. (1998). Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. *Nature* *391*, 173–177.
- Pidoplichko, V.I., DeBiasi, M., Williams, J.T., and Dani, J.A. (1997). Nicotine activates and desensitizes midbrain dopamine neurons. *Nature* *390*, 401–404.
- Radcliffe, K.A., Fisher, J.L., Gray, R., and Dani, J.A. (1999). Nicotinic modulation of glutamate and GABA synaptic transmission of hippocampal neurons. *Ann. NY Acad. Sci.* *868*, 591–610.
- Rae, J., Cooper, K., Gates, P., and Watsky, M. (1991). Low access resistance perforated patch recordings using amphotericin B. *J. Neurosci. Methods* *37*, 15–26.
- Schilström, B., Nomikos, G.G., Nisell, M., Hertel, P., and Svensson, T.H. (1998a). N-methyl-D-aspartate receptor antagonism in the ventral tegmental area diminishes the systemic nicotine-induced dopamine release in the nucleus accumbens. *Neuroscience* *82*, 781–789.
- Schilström, B., Svensson, H.M., Svensson, T.H., and Nomikos, G.G. (1998b). Nicotine and food induced dopamine release in the nucleus accumbens of the rat: putative role of alpha7 nicotinic receptors in the ventral tegmental area. *Neuroscience* *85*, 1005–1009.
- Seguela, P., Wadiche, J., Dineley-Miller, K., Dani, J.A., and Patrick, J.W. (1993). Molecular cloning, functional properties, and distribu-

tion of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.* *13*, 596–604.

Steffensen, S.C., Svingos, A.L., Pickel, V.M., and Henriksen, S.J. (1998). Electrophysiological characterization of GABAergic neurons in the ventral tegmental area. *J. Neurosci.* *18*, 8003–8015.

Vanderschuren, L.J., Schmidt, E.D., De Vries, T.J., Van Moorsel, C.A., Tilders, F.J., and Schoffelmeer, A.N. (1999). A single exposure to amphetamine is sufficient to induce long-term behavioral, neuroendocrine, and neurochemical sensitization in rats. *J. Neurosci.* *19*, 9579–9586.

Walaas, I., and Fonnum, F. (1980). Biochemical evidence for gamma-aminobutyrate containing fibres from the nucleus accumbens to the substantia nigra and ventral tegmental area in the rat. *Neuroscience* *5*, 63–72.

Westerink, B.H., Kwint, H.F., and deVries, J.B. (1996). The pharmacology of mesolimbic dopamine neurons: a dual-probe microdialysis study in the ventral tegmental area and nucleus accumbens of the rat brain. *J. Neurosci.* *16*, 2605–2611.

WHO (1999). Chapter 5, Combating the Tobacco Epidemic. In *The World Health Report 1999. Making a Difference*. World Health Organization: Available through: <http://www.who.int/>.

Yin, R., and French, E.D. (2000). A comparison of the effects of nicotine on dopamine and non-dopamine neurons in the rat ventral tegmental area: an in vitro electrophysiological study. *Brain Res. Bull.* *51*, 507–514.