Nicotinic Cholinergic Synaptic Mechanisms in the Ventral Tegmental Area Contribute to Nicotine Addiction

Volodymyr I. Pidoplichko, Jun Noguchi, Oluwasanmi O. Areola, Yong Liang, Jayms Peterson, Tianxiang Zhang, and John A. Dani

Division of Neuroscience, Baylor College of Medicine, Houston, Texas 77030, USA

Tobacco use is a major health problem that is estimated to cause 4 million deaths a year worldwide. Nicotine is the main addictive component of tobacco. It acts as an agonist to activate and desensitize nicotinic acetylcholine receptors (nAChRs). A component of nicotine’s addictive power is attributable to actions on the mesolimbic dopaminergic system, which serves a fundamental role in the acquisition of behaviors that are inappropriately reinforced by addictive drugs. Here we show that nicotine, in the same concentration and time ranges as obtained from tobacco, has three main actions that regulate the activity of midbrain dopamine (DA) neurons. Nicotine first activates and then desensitizes nAChRs on the DA neurons. This process directly excites the DA neurons for a short period of time before the nAChRs desensitize. Nicotine also enhances glutamatergic excitation and decreases GABAergic inhibition onto DA neurons. These events increase the probability for synaptic plasticity, such as long-term potentiation. The short-lived direct excitation of the DA neurons coupled with the enhanced glutamatergic afferent activity provides the presynaptic and postsynaptic coincidence necessary to initiate synaptic potentiation. In total, these synaptic events lead to a relatively long-lasting heightened activity of midbrain DA neurons. Consistent with other summarized studies, this work indicates that the synaptic changes normally associated with learning and memory can be influenced and commanded during the nicotine addiction process.
of methyllycaconitine (MLA; Alkondon et al. 1992; Castro and Albuquerque 1995; Gray et al. 1996). Although α7* nAChRs are quickly desensitized by very high agonist concentrations (e.g., 500 μM ACh or nicotine), they have a lower affinity for nicotine. Consequently, the α7* nAChRs are not significantly desensitized by the low concentrations of nicotine obtained from tobacco (see Quick and Lester 2002; Wooltorton et al. 2003).

The predominant nAChR-mediated currents from VTA and SNc neurons have relatively slow kinetics and are inhibited by the β2-selective inhibitor, dihydro-β-erythroidine (DHβE; Pidoplichko et al. 1997; Picciotto et al. 1998; Klink et al. 2001; Wooltorton et al. 2003). Those characteristics indicate that the vast majority of the nicotinic currents on midbrain neurons are mediated by β2-containing (β2*) nAChRs. The β2 subunit is in combination with other nicotinic subunits that are expressed in these areas, particularly α4, α6, and β3 (Wada et al. 1989, 1990; Le Novère et al. 1996; Goldner et al. 1997; Charpantier et al. 1998; Klink et al. 2001). That conclusion was verified using β2-null mice in which nicotinic currents were dramatically decreased in the midbrain neurons (Picciotto et al. 1998; Wooltorton et al. 2003). In midbrain slices from the β2-null mice, the only consistent nicotinic current remaining was a small minority current mediated by α7* nAChRs. Therefore, the α7* nAChRs are present on VTA/SNc neurons, but at a much lower density than β2* nAChRs (Wooltorton et al. 2003).

The purpose of this report is to review and further examine nicotinic synaptic mechanisms in midbrain dopaminergic areas. New data are presented along with review material to show that the majority subtypes of nAChRs on DA neurons are directly activated by nicotine, but after a short time, they desensitize. Glutamatergic afferents into this region supply convergent excitation from a number of brain areas. The predominant subtype of nAChR located on the glutamatergic presynaptic terminals is not significantly desensitized by the concentration of nicotine obtained from smoking. Thus, nicotine causes a persistent enhancement of the afferent glutamatergic excitation onto DA neurons. The GABAergic inhibition in this midbrain region arises mainly from the NAc, the ventral pallidum, and midbrain interneurons (Kalivas et al. 1993; Steffensen et al. 1998). The predominant nAChR subtypes on the GABAergic neurons desensitize after some exposure to nicotine, thereby decreasing the inherent inhibition onto DA neurons. The consequence of these synaptic events is a prolonged firing of DA neurons in response to nicotine. In summary, nicotine as obtained from smoking interacts with multiple nAChR subtypes on DA neurons and on afferent neurons, fibers, and presynaptic terminals to produce synaptic events much like those that underlie the synaptic changes associated with learning and memory.

RESULTS

Nicotine Induces a Long-lasting DA Signal in the Nucleus Accumbens

A common feature of addictive drugs such as cocaine, amphetamine, and nicotine is that at the doses they are self-administered, those drugs increase the concentration of DA in the NAc (Di Chiara and Imperato 1988; Clarke 1991; Corrigall et al. 1992; Di Chiara 1999, 2000; Pontieri et al. 1996; Balfour et al. 2000; Dani and De Biasi 2001). Using microdialysis in the rat NAc shell, we found that nicotine causes more than a doubling in the background DA concentration (Fig. 1). Furthermore, the DA concentration remains elevated for well over an hour (Imperato et al. 1986). The main dopaminergic projections to the NAc arise from VTA neurons of the mesolimbic dopamine system. The long-lasting elevation of DA in the NAc presents a problem because the direct activation of nAChRs on VTA DA neurons is usually much shorter. There is significant (but not uniform) desensitization of nAChRs on DA neurons in a few minutes (Pidoplichko et al. 1997; Dani et al. 2000; Wooltorton et al. 2003).

Cigarette smoking delivers ~50 nM to 500 nM nicotine throughout the brain on a time scale of many seconds to minutes (Russell 1987; Benowitz et al. 1989; Henningfield et al. 1993; Gourlay and Benowitz 1997; Karan et al. 2003). Significantly lower concentrations of nicotine will linger in the human brain for hours. When 100 nM nicotine is applied to the bath of a midbrain slice, VTA DA neurons display inward-going (depolarizing or activating) current (Fig. 2; Pidoplichko et al. 1997). However, this relatively low concentration of nicotine also causes a great deal of nAChR desensitization because the subsequent addition of 500 nM nicotine causes no further current (Fig. 2). The desensitization is further documented by brief pressure-puff applications of ACh (1 μM for 30 msec; downward arrows in Fig. 2). Before nicotine is applied, the ACh puff activates a 50-pA current (I1 of Fig. 2), but after 3 min in 100 nM nicotine, the ACh puff activates a barely detectable current (I2 of Fig. 2; Pidoplichko et al. 1997). Despite the long-lasting DA signal detected by microdialysis (Fig. 1), the results of Figure 2 demonstrate that after a few minutes, many nAChRs on the surface of midbrain DA neurons are desensitized. The prolonged DA signal can be explained by considering the afferent drive onto the VTA neurons (Mansvelder and McGehee 2000, 2002; Dani et al. 2001; Mansvelder et al. 2002; Wooltorton et al. 2003).

Nicotine Increases Glutamatergic Excitation and Decreases GABAergic Inhibition Onto DA Neurons

Upon smoking, it is estimated that the nicotine in the brain reaches ~500 nM (possibly as high as 1 μM), and nicotine lingers in brain tissue longer than the time it takes to consume the tobacco (Russell 1987; Benowitz et al. 1989; Henningfield et al. 1993; Gourlay and Benowitz 1997). To mimic this situation in vitro, we bath-applied 500 nM nicotine for 25 min to midbrain slices while recording spontaneous glutamatergic afferent excitatory postsynaptic currents (sEPSCs) arriving at VTA DA neurons (Fig. 3). In eight out of 12 neurons (four showed no change),
Nicotine caused an increase in the frequency (but not the amplitude) of sEPSCs (Fig. 3; Mansvelder and McGehee 2000, 2002; Dani et al. 2001; Mansvelder et al. 2002). The frequency of the sEPSCs remained elevated throughout the 25-min period, indicating that desensitization did not terminate the nicotinic effect.

Nicotine also increased the amplitude of electrically evoked EPSCs on DA neurons (Fig. 4). The enhancement was favored by cutting sagittal slices and stimulating with a low stimulus strength (~10% of the maximal response). In five out of 11 neurons (six showed no change), the amplitude of the sEPSCs increased (Mansvelder and McGehee 2000, 2002; Dani et al. 2001; Mansvelder et al. 2002). It is interesting to note that both the spontaneous and evoked EPSCs remain elevated after the nicotine was washed away, consistent with the induction of long-term potentiation of glutamatergic afferents (Mansvelder and McGehee 2000, 2002; Dani et al. 2001; Ji et al. 2001; Mansvelder et al. 2002). This result is consistent with presynaptic nAChRs that boost EPSC frequency without changing the amplitude, as has been demonstrated in the hippocampus and elsewhere (McGehee and Role 1995; McGehee et al. 1995; Gray et al. 1996; Role and Berg 1996; Albuquerque et al. 1997; Wonnacott 1997; Guo et al. 1998; Li et al. 1998; Radcliffe and Dani 1998; Jones et al. 1999; Radcliffe et al. 1999; Mansvelder and McGehee 2000, 2002; Dani et al. 2001; Mansvelder et al. 2002). The nicotine-induced long-lasting potentiation of glutamatergic afferent excitation onto DA neurons is similar to the synaptic plasticity that is normally thought to underlie learning and memory (Martin et al. 2000).

The response to nicotine by the spontaneous GABAergic afferent inhibitory postsynaptic currents (sIPSCs) was markedly different from the long-lasting boost of glutamatergic spontaneous or evoked EPSCs. Bath-applied 500 nM nicotine for 25 min briefly boosted sIPSCs, but that was followed by a strong long-lasting inhibition \((n = 13 \text{ of } 18; \text{five showed no change; Fig. 5})\). The amplitudes of the sIPSCs also responded. During the increase in sIPSC frequency, there were larger sIPSCs and the average increase in amplitude was 16% ± 6% (Mansvelder et al. 2002). This amplitude increase is consistent with the interpretation that the nAChRs are located on preterminal and somal locations where nicotine can boost the fraction of action potential-dependent IPSCs (Lena et al. 1993; McMahon et al. 1994; Alkondon et al. 1997; Ji and Dani 2000; Mansvelder et al. 2002). Activation of nAChRs located on the soma or preterminally has been found to depolarize the membrane locally, leading to activation of voltage-dependent channels that directly mediate action potentials. Thus, adding nicotine to the bath briefly boosts the action potential firing of GABAergic neurons, decreasing the relative contribution from the smaller-amplitude miniature IPSCs, which arise from the stochastic release of a single quantum of neurotransmitter. After a short time, the nAChRs desensitize, removing the nicotine-derived excitation as well as removing any endogenous nicotinic cholinergic drive onto the GABAergic neurons.

**Endogenous Cholinergic Activity Influences GABAergic and Glutamatergic Afferents**

Nicotine activates and desensitizes nAChRs, and in that way directly influences afferent activity into the midbrain and the firing of the DA neurons (Pidoplichko et al. 1997; Picciotto et al. 1998; Mansvelder and McGehee 2000, 2002; Dani et al. 2001; Mansvelder et al. 2002). Desensitization also can have a long-lasting effect on the normal nicotinic mechanisms driven by endogenous cholinergic activity. For example, the long-lasting decrease of sIPSC frequency (Fig. 5) is consistent with nicotine-desensitizing cholinergic afferents that partially drive the GABAergic activity, as has been seen in the hippocampus (Alkon-
don et al. 1998; Frazier et al. 1998; Hefft et al. 1999). Cholinergic innervation into the midbrain DA areas arises from neurons in the pedunculopontine tegmentum (PPT) and the laterodorsal pontine tegmentum (LDT), which provide widespread innervation mainly to the thalamus and midbrain areas and descending innervation that reaches to the brain stem. When we cut horizontal slices and electrically stimulated rostral inputs to the VTA, we found that inhibiting nAChRs had little or no effect on the amplitude of GABAergic-evoked IPSCs (Mansvelder et al. 2002; data not shown). Neither 1 µM DHβE (which is selective for non-β2*, mainly β2* nAChRs) nor 5 nM MLA (which is selective for β7* nAChRs) influenced the eIPSCs. Therefore, to better preserve the cholinergic inputs into the VTA from the PPT/LDT, we cut parasagittal slices and stimulated caudally to the VTA. Figure 6 shows that the cholinergic neurons of the PPT/LDT (labeled red for ChAT activity) are near to the DA neurons of the VTA/SNc (labeled green for TH activity). For our electrophysiological studies, we tried to select DA neurons near the interface with the cholinergic neurons, and stimulated near the border between the PPT/LDT and the VTA. Under those conditions, we found that the β2-selective inhibitor, 1 µM DHβE, inhibited the amplitude of the GABAergic eIPSCs (n = 4 out of 10 neurons; six showed no effect; Fig. 7). MLA (5 nM), which inhibits α7* nAChRs, did not influence the eIPSCs when it was added separately (data not shown) or when it was added prior to the DHβE (Fig. 7). The results indicate that non-α7, mainly β2* nAChRs, are activated by endogenous cholinergic activity that helps to drive GABAergic IPSCs onto DA neurons. Thus, ongoing endogenous nicotinic cholinergic activity contributes to the background GABAergic inhibition onto DA neurons.

When we inhibited nAChRs while recording glutamatergic eEPSCs onto VTA DA neurons, we found that α7* nAChRs were important. Inhibition with DHβE had no effect on the eEPSC amplitude (data not shown), but inhibition of α7* nAChRs with 5 or 10 nM MLA decreased the eEPSC amplitude (n = 3 out of 10; seven showed no effect; Fig. 8). The results are consistent with sparse endogenous cholinergic innervation stimulating presynaptic α7* nAChRs on some glutamatergic terminals, and in that way boosting excitatory eEPSCs onto DA neurons.

Nicotine Differentially Desensitizes the nAChR Subtypes
Midbrain DA neurons express different nAChR subunits, but the pharmacological and physiological characteristics of nAChRs currents indicate that β2* nAChRs are by far the predominant subtypes (Pidoplichko et al. 1997; Picciotto et al. 1998; Klink et al. 2001; Wooltorton et al. 2003). Nicotine at the concentration achieved by smokers desensitizes nAChR currents from VTA DA neurons (Pidoplichko et al. 1997; Dani et al. 2000; Wooltorton et al. 2003). An important characteristic, however, is the difference in desensitization of nAChR subtypes (Wooltorton et al. 2003).

Nicotine Increases Then Decreases the Frequency of Spontaneous IPSCs Recorded from VTA DA Neurons
Figure 5 Nicotine increases then decreases the frequency of spontaneous IPSCs recorded from VTA DA neurons. Example traces (upper) show that bath-applied nicotine (0.5 µM) first increases the frequency (and amplitude) of sIPSCs, but the average (13 of 18; five showed no change) shows the more potent, long-lasting effect is an inhibition of sIPSC frequency (lower). The recordings were at room temperature at a holding potential of ~ 65 mV, and a weak simulation strength was used. The scale bars represent 20 pA and 0.5 msec.

Figure 4 Nicotine increases the amplitude of evoked EPSCs recorded from VTA DA neurons. Example traces (upper) show that bath-applied nicotine (1 µM) increases the amplitude of eEPSCs, and the average (five of 11; six showed no change) shows the long-lasting effect of the amplitude increase (lower). A low concentration of EGTA (0.4 µM) was used in the patch pipette to enhance the long-lasting increase in amplitude. The recordings were at room temperature at a holding potential of ~ 65 mV, and a weak simulation strength was used. The scale bars represent 100 pA and 10 msec.
Nearly all VTA DA neurons display an ACh-induced current with slow kinetics that is consistent in most cases with α4β2*-type nAChRs, sometimes in combinations with α6 (Le Novère et al. 1996; Picciotto et al. 1998; Arroyo-Jimenez et al. 1999; Klink et al. 2001; Azam et al. 2002; Champtiaux et al. 2002; Wooltorton et al. 2003). Bath application of 20 nM nicotine for 20 min causes about 45% desensitization of this slow component of ACh-induced currents (Fig. 9A; Wooltorton et al. 2003). The slow component was nearly completely desensitized by 500 nM nicotine. This result is consistent with those from others who have estimated that α4β2* nAChRs have an IC50 for nicotine-induced desensitization of ~1–60 nM (Lippiello et al. 1987; Wonnacott 1987; Peng et al. 1994; Rowell 1995; Fenster et al. 1999; Quick and Lester 2002). However, as indicated by the arrow in Figure 9A, a fast component of the ACh-induced current is not desensitized by 500 nM nicotine, but that current is inhibited by MLA (Wooltorton et al. 2003; data not shown), indicating that α7* nAChRs are not desensitized by the low concentrations of nicotine.

In most cases, the fast and slow components of the current are not easily separable because the small amplitude of the fast component is contaminated by the rising phase of the slow component, which is the predominant current. Using mutant mice lacking the β2 nAChR subunit almost always eliminates all the contributions to the slow component of the current, revealing the fast, α7 component. The fast, α7 component of the current is not significantly desensitized by bath-applied nicotine in the range experienced by smokers (20–500 nM; Fig. 9B). Although α7* nAChRs desensitize rapidly when exposed to high concentrations of nicotine or ACh (Alkondon and Albuquerque 1991; Bertrand et al. 1992; Dani et al. 2000; Picciotto et al. 1998; Arroyo-Jimenez et al. 1999; Klink et al. 2001; Le Novère et al. 1996; Azam et al. 2002; Champtiaux et al. 2002; Wooltorton et al. 2003), they have a relatively low affinity for desensitization by nicotine. The results of Figure 9 are consistent with measurement of α7* nAChRs from rodent hippocampus or expressed in oocytes, where estimates of IC50 for nicotine-induced desensitization of α7* nAChRs range from about 0.5 to 7 μM (Fenster et al. 1997; Frazier et al. 1998; McQuiston and Madison 1999; Alkondon et al. 2000; Quick and Lester 2002). In summary, the β2* subtypes of nAChRs are strongly desensitized by the concentration of nicotine obtained from tobacco, but the α7* nAChRs are not.

**DISCUSSION**

**Nicotine as Obtained From Tobacco**

Nicotine obtained from tobacco is initially at a significant concentration in the arterial blood, lung, and brain (roughly 100–500 nM), and then it distributes to storage adipose and muscle tissue (Karan et al. 2003). The distribution half-life of ~8 min determines the initial action of nicotine within the central nervous system. The elimination half-life is ~2 h, which allows nicotine to accumulate with ongoing smoking and persist for many hours after the cessation of smoking. Although there is significant individual variability, the steady-state plasma concentration of nicotine plateaus in the early afternoon roughly in the range of 10–50 ng/ml (Karan et al. 2003). Thus, smokers often deliver a small pulse of nicotine with each episode of smoking, and nicotine accumulates and lingers in the body (and brain) as the day progresses. This situation will initially cause some activation of most nAChR subtypes, but then the prolonged low levels of nicotine will favor desensitization of most non-α7 nAChR subtypes.

**Desensitization of nAChR Subtypes in the Ventral Tegmental Area**

Midbrain DA regions contain many nAChR subunits: α3–α7 and β2–β6. Although α7* nAChRs are commonly expressed at a low density on the DA neurons, the vast majority of subtypes contain β2, often in combinations with α4 and α6 (Le Novère et al. 1996; Picciotto et al. 1998; Arroyo-Jimenez et al. 1999; Klink et al. 2001; Azam et al. 2002; Champtiaux et al. 2002; Wooltorton et al. 2003). These β2* nAChRs also are the predominant subtype on the midbrain GABAergic interneurons, but again, there are other minority subtypes. On the other hand, α7* nAChRs are the predominant subtype on the presynaptic terminals of glutamatergic afferents onto DA neurons. This difference in the distribution of nAChRs is important because nicotine does not identically activate or desensitize the subtypes. The β2* subtypes have a higher affinity for nicotine than the α7* subtypes. Therefore, the β2* subtypes are activated, but then they strongly proceed into desensitization. Wooltorton et al. (2003) showed that 80 nM nicotine caused an 80% desensitiza-
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2* nAChRs in the midbrain. That result is consistent with previous estimates that 2* nAChRs have an IC50 for nicotine-induced desensitization of ∼1–60 nM (Lippiello et al. 1987; Wonnacott 1987; Peng et al. 1994; Rowell 1995; Fenster et al. 1999; Quick and Lester 2002). Thus, nicotine strongly desensitizes the majority subtypes of nAChRs (i.e., 2*) on midbrain DA and GABA neurons. On the other hand, 7* nAChRs are not strongly desensitized by the low concentrations of nicotine obtained from tobacco. Wooltorton et al. (2003) showed that up to 500 nM nicotine caused very little desensitization of VTA 7* nAChRs. That result is consistent with previous estimates that 7* nAChRs have an IC50 for nicotine-induced desensitization of up to 7 µM (Fenster et al. 1997; Frazier et al. 1998; McQuiston and Madison 1999; Alkondon et al. 2000; Quick and Lester 2002). In minutes, significant desensitization affects these (predominantly) 2* nAChRs (see Figs. 2, 5, and 9). The GABAergic activity declines more rapidly because desensitization removes the direct excitation caused by nicotine and decreases the endogenous cholinergic drive onto the GABAergic interneurons (Figs. 5 and 7). Whereas a small subset of DA neurons receives cholinergic in-

The 2* antagonist, DHβE, decreased the amplitude of GABAa-evoked IPSCs onto DA neurons. Example traces (upper) show that bath-applied DHβE (1 µM) decreases the amplitude of eIPSCs, and the average (four out of 10 neurons; six showed no effect) shows the amplitude decrease (lower). The 7* nAChR antagonist, MLA, had no effect in separate experiments (data not shown) or when applied before DHβE. The recordings were at room temperature at a holding potential of −60 mV, and weak simulation strength was used. The scale bars represent 50 pA and 20 msec.

The 7* antagonist, MLA, decreased the amplitude of Glu-evoked EPSCs. Example traces (upper) show that bath-applied MLA (10 nM) decreases the amplitude of eEPSCs, and the average (three out of 10; seven showed no effect) shows the amplitude decrease (lower). The recordings were at room temperature at a holding potential of −65 mV, and a weak simulation strength was used. The scale bars represent 100 pA and 10 msec.

Because of the rapid kinetics, those 7* nAChRs rapidly recover from desensitization when nicotine unbinds. Consequently, only a very small portion of the overall 7* population is desensitized at any moment, leaving the remaining receptors available to activate.

Model of the Synaptic Action of Nicotine in the Ventral Tegmental Area
Although other minority subtypes are present, 2* nAChRs make up the vast majority of subtypes on VTA DA neurons and GABAergic interneurons. Those receptors underlie the initial direct activation of DA neurons by nicotine (see Fig. 10; Mansvelder and McGehee 2000, 2002; Dani et al. 2001; Mansvelder et al. 2002; Wooltorton et al. 2003). When nicotine first arrives, these 2* nAChRs are activated, causing direct excitation of the DA neurons and the GABAergic interneurons (indicated by Figs. 2 and 5; Calabresi et al. 1989; Pidoplichko et al. 1997; Picciotto et al. 1998; Dani et al. 2000, 2001; Mansvelder and McGehee 2002). In minutes, significant desensitization affects these (predominantly) 2* nAChRs (see Figs. 2, 5, and 9). The GABAergic activity declines more rapidly because desensitization removes the direct excitation caused by nicotine and decreases the endogenous cholinergic drive onto the GABAergic interneurons (Figs. 5 and 7). Whereas a small subset of DA neurons receives cholinergic in-
with a large enough postsynaptic response to induce a Ca^{2+} signal through NMDA-type glutamate receptors (NMDARs). When nicotine initially arrives, it excites the DA neurons to increase their action potential firing rate. That postsynaptic DA neuron activity is coupled with a nicotine-induced increase in presynaptic glutamatergic afferent excitation (see Fig. 10). That combination produces the presynaptic and postsynaptic coincidence that boosts the production of LTP (see Mansvelder and McGehee 2000, 2002; Dani et al. 2001; Ji et al. 2001; Mansvelder et al. 2002). Subsequently, non-α7 subtypes desensitize, thereby, decreasing the inhibition onto DA neurons by GABAergic neurons. In addition, the α7 nAChRs on presynaptic glutamate terminals do not desensitize. Thus, they continue to enhance glutamatergic excitation as long as the nicotine signal is present. This seemingly choreographed complex of nicotinic synaptic mechanisms contributes to the prolonged DA signal in the NAc and elsewhere that is thought to be a critical component in the addiction process.

The synaptic changes that are induced by nicotine are much like the normal synaptic plasticity that underlies learning and memory: Presynaptic calcium signals enhance excitatory transmission coupled to a strong postsynaptic response, leading to short-term and long-term potentiation. Nicotine tips the normal balance, inappropriately favoring potentiation of synapses and, ultimately, favoring inappropriate behaviors. In this way, the addictive drug, nicotine, commandeers fundamental synaptic mechanisms that normally subserve learning and memory.

**MATERIALS AND METHODS**

**Brain Slice Preparation and Electrophysiology**

Midbrain horizontal or sagittal slices containing the VTA and SNc were prepared from 14- to 25-day-old Sprague Dawley rats that were anesthetized before decapitation (see Wooltorton et al. 2003). Slices (300–350 µm thick) were cut in ice-cold cutting solution, and all the solutions were saturated with 95% O_{2}, 5% CO_{2} to achieve a pH near 7.4 during the experiments. The cutting solution was either of the following or a 50%/50% mixture of the two solutions. The sucrose cutting solution was 23.0 mM sucrose, 1.0 mM KCl, 1.25 mM NaH_{2}PO_{4}, 30 mM NaHCO_{3}, 1 mM CaCl_{2}, 7 mM MgCl_{2}, and 25 mM D-glucose. The N-methyl-D-glucamine (NMDG) cutting solution was 144 mM NMDG, 1.5 mM KCl, 1.25 mM NaH_{2}PO_{4}, 30 mM NaHCO_{3}, 2 mM CaCl_{2}, 2 mM MgCl_{2}, and 25 mM D-glucose, and the pH was adjusted to 7.4 with 50% D-gluconic acid and sodium bicarbonate. The slices were then transferred to a holding chamber containing the bath solution: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_{2}PO_{4}, (or 1.24 mM KH_{2}PO_{4}), 21 mM NaHCO_{3}, 2.5 mM CaCl_{2} (or 2.1 mM CaCl_{2}), 1 mM MgCl_{2} (or 0.3 mM MgCl_{2}), and 25 mM D-glucose 25; Slices were held either for 1 h at room temperature or for 20 min at 34°C and, then, for a minimum of 20 min at room temperature. The experimental chamber (0.8 mL capacity) had continuously flowing bath solution (~5 mL/min) at room temperature near 23°C. The osmolality of the external solution was adjusted to 320 mOsm with D-glucose. All the experiments studying evoked responses had 0.5 mM atropine added to the...
evoked via a computer-controlled stimulus isolator model A-360 (WPI) applying minimal stimulation intensity. The intensity of the stimulus was adjusted to elicit amplitudes at 10%–20% of the maximum better to see the effect of nicotine (see Mansvelder and McGee 2000). Stimulation currents ranged from 150 to 450 µA and usually lasted 0.7 msec. Currents were amplified and filtered (1 kHz) using Axopatch 200B or 1C amplifiers (Axon Instruments). The filtered signal was sent to a four-pole low-pass Bessel filter and were digitally sampled (up to 5 kHz). Currents were recorded using pClamp software (Axon), and further analyzed using Origin (MicroCal Software). Additional off-line filtering and signal averaging were sometimes used in the figures. Neurons that were patch-clamped were identified as VTA DA based on large I
currents (Pidoplichko et al. 1997; Bonci and Malenka 1999).

**Immunohistochemistry**

Tyrosine hydroxylase (TH, indicating catecholamine synthesis) and choline acetyltransferase (ChAT, indicating ACh synthesis) immunohistochemistry were adapted from published methods (Zhou et al. 2001). For ChAT and TH double immunolabeling, brains were fixed in 0.1 M phosphate buffer containing 4% (w/v) paraformaldehyde and 14% (v/v) picric acid. Sections were cut on a cryostat, and were incubated with goat anti-CHAT and rabbit anti-TAC antibodies. The secondary antibody mixture containing Cy2-conjugated donkey anti-rabbit and rhodamine-conjugated donkey anti-goat IgG antibodies was used. All images were captured with a digital camera and processed in Adobe Photoshop.

**Microdialysis**

Long-Evans male rats (Harlan) were housed together on a 12-h light/dark cycle. Their body weight was from 280–300 g. The rats were anesthetized using a ketamine–xylazine combo injected intraperitoneally (1.8 µL per gram body weight) and subsequently maintained on an isoflurane-gas mixture. The microdialysis CMA/12 (CMA/Microdialysis) guide cannula was aimed at the NAc shell (1.7 mm AP; 0.8 mm L; 6.5 mm DV, with the probe at 7.5 mm) and was secured with bone wax and held in acrylic cement and three screws into the skull. The rat was allowed to recover fully for a minimum of 48 h.

CMA/12 probes (diameter, 0.5 mm; length, 1 mm; monoblock, polycarbonate; cutoff, 20,000 D) were prepared, and filtered degassed artificial cerebrospinal fluid (aCSF; from ESA) was perfused through the probe at a flow rate of 0.1 µL/min using a CMA/100 pump. Following a 2-h recovery period, three 20-min fractions were collected to assess the basal output of dopamine in the dialysate. Subsequently, saline and nicotine (0.6 mg/kg i.p.) were injected, and samples were collected every 20 min for 3 h. After these experiments, rats were killed with an overdose of anesthetics and trans-cardially perfused with PBS and then 10% formalin. The brain was removed and fixed in 10% formalin. The accuracy of probe placement was later confirmed by histological sectioning.

Dopamine contents of microdialysates were determined using a high-performance liquid chromatography (HPLC) system (model 580 pump, Coulochem II electrochemical detector, model 5014B analytical cell; ESA, Inc.). Separation of dopamine was achieved on a 150 × 3 mm column with 3 µm particle size (ESA, Inc.; MD-150). An isotropic mobile phase (pH 4.0) containing 75 mM NaH2PO4, 2 mM 1-octane sulfoninic acid-sodium salt,
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Received September 5, 2003; accepted in revised form November 21, 2003.
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Learn. Mem. 2004 11: 60-69
Access the most recent version at doi:10.1101/lm.70004

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