Intermittent nicotine exposure upregulates nAChRs in VTA dopamine neurons and sensitizes locomotor responding to the drug

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Abstract

Dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) mediate the behavioral and motivational effects of many drugs of abuse, including nicotine. Repeated intermittent administration of these drugs, a pattern often associated with initial drug exposure, sensitizes the reactivity of dopamine (DA) neurons in this pathway, enhances the locomotor behaviors the drugs emit, and promotes their pursuit and self-administration. Here we show that activation of nicotinic acetylcholine receptors (nAChRs) in the VTA, but not the NAcc, is essential for the induction of locomotor sensitization by nicotine. Repeated intermittent nicotine exposure (4×0.4 mg/kg, base, i.p., administered over 7 days), a regimen leading to long-lasting locomotor sensitization, also produced upregulation of nAChRs in the VTA, but not the NAcc, in the hours following the last exposure injection. Functional nAChR upregulation was observed selectively in DA but not GABA neurons in the VTA. These effects were followed by long-term potentiation of excitatory inputs to these cells and increased nicotine-evoked DA overflow in the NAcc. Withdrawal symptoms were not observed following this exposure regimen. Thus, intermittent activation and upregulation by nicotine of nAChRs in DA neurons in the VTA may contribute to the development of behavioral sensitization and increased liability for nicotine addiction.

Keywords

dopaminergic neurons; GABAergic neurons; LTP; nAChR upregulation; nucleus accumbens; sensitization; ventral tegmental area

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Introduction

Nicotine and other psychomotor stimulants produce their motivating effects by stimulating the mesoaccumbens dopamine (DA) system. Repeated intermittent injection of these drugs, a pattern often associated with initial exposure, enhances drug-induced DA neurotransmission and locomotor activity and these effects can persist for weeks to months (Vezina *et al.*, 2007). The persistence of these effects and their extension to enhanced drug self-administration in animals has led to the proposal that drug sensitization has mechanistic overlap with the development of addiction in humans (Robinson & Berridge, 1993; Vezina, 2004). Ultimately, drug-induced changes in mesoaccumbens DA neurotransmission and associated behaviors arise from cellular and synaptic changes that we are only starting to decipher (Dani *et al.*, 2001; Changeux, 2010). Adaptations occur at glutamatergic synapses in the VTA following exposure to psychomotor stimulants including nicotine (Mansvelder & McGehee, 2000; Ungless *et al.*, 2001; Mansvelder *et al.*, 2002; Saal *et al.*, 2003; Mao *et al.*, 2011). In addition, nicotinic acetylcholine receptors (nAChRs) undergo an upregulation in ligand binding and function following nicotine exposure (Buisson & Bertrand, 2001; Mugnaini *et al.*, 2002; Vallejo *et al.*, 2005; Govind *et al.*, 2009, 2012).

nAChRs are expressed both on the cell bodies and terminals of mesoaccumbens DA neurons as well as by non-DA cells and afferent terminals in the VTA (Changeux, 2010). Activation of nAChRs in either the VTA or the NAcc can regulate DA release (Nisell *et al.*, 1994; Zhang & Sulzer, 2004) and the motivation to self-administer nicotine (Maskos *et al.*, 2005; Brunzell *et al.*, 2010). Thus, alterations in nAChR function and neuroadaptations initiated in these sites by repeated nAChR activation could conceivably change the ability of nicotine to stimulate mesoaccumbens DA activity, increase locomotion and maintain self-administration behaviors.

In many studies examining the effects of nicotine exposure, rodents are exposed to intense continuous nicotine exposure regimens lasting days to weeks (Vezina *et al.*, 2007). When discontinued, these regimens produce withdrawal symptoms with magnitude and duration corresponding to the intensity and length of exposure (Epping-Jordan *et al.*, 1998; Skjei & Markou, 2003). These regimens can upregulate radioligand binding to nAChRs in a number of brain regions including the VTA and NAcc (Mugnaini *et al.*, 2002). They also specifically upregulate α4-containing nAChRs in midbrain GABA neurons and increase inhibition of mesoaccumbens DA activity in the period immediately following exposure, when withdrawal symptoms are prevalent (Nashmi *et al.*, 2007). These preclinical findings have been argued to support opponent process views of drug taking (Koob & Le Moal, 1997) and, in particular, to model the maintenance of tobacco use once it is established.

Here we explore the cellular, synaptic and behavioral consequences of exposure to a moderate, intermittent nicotine exposure regimen, a pattern often associated with initial exposure to the drug that is not associated with withdrawal symptoms but known to produce long-lasting sensitization. Our findings identify nicotine-induced neuroadaptations that may contribute to the development of increased liability for nicotine addiction.

Materials and Methods

Subjects and surgery

Male Sprague Dawley rats (180–250g on arrival from Harlan Sprague-Dawley, Madison, WI) were housed in a reverse light-dark cycle room with free access to food and water. They were allowed to acclimate for 1 week before any procedures. In some experiments, rats were anesthetized with a mix of ketamine (100 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.) and surgically implanted with chronic guide cannulae either aimed unilaterally at the NAcc core

[anteroposterior (A/P), +3.4; lateral (L), ±1.5; dorsoventral (D/V), −6.5 to −8.5; angled at 10°] for microdialysis or bilaterally either at the VTA (A/P, −3.6; L, ±0.6; D/V, −8.9; 16°) or the NAcc core (AP, +3.4; L, ±1.5; DV, −7.5; 10°) for locomotion, using procedures described previously (Vezina *et al.*, 2002). Coordinates are in mm from bregma and skull with the incisor bar positioned 5.0 mm above the interaural line. Only rats with the active portion of the microdialysis probe or both injection cannula tips positioned in the targeted nucleus were included in the data analyses. All experimental procedures were performed in accordance with an approved Institutional Animal Care and Use Committee protocol.

Design and procedure

Experiments involved two phases: drug exposure and test. During the exposure phase, rats received 4 injections of nicotine (0.4 mg/kg, i.p., 1 every other day). This moderate intermittent regimen is known to produce long-lasting sensitization (Schofelmeer *et al.*, 2002). Control rats received saline. After the first and fourth injections, rats were placed in locomotor activity monitoring chambers for 2 hrs. The remaining injections were administered in the rats' home cage. The test phase involved administering nicotine at various times following the last exposure injection. Nicotine [(−) nicotine tartrate; Sigma Inc., Saint Louis, MO] was dissolved in saline and its pH adjusted to 7.0 with NaOH. Nicotine doses are expressed as free base.

Locomotion

To assess the effect of nAChR blockade in the VTA and NAcc, the broad-spectrum nAChR antagonist mecamylamine (0, 10 or 30 μg/0.5μl/side; Tocris, Ellisville, MO) was administered intracranially immediately before each nicotine exposure injection. Testing for sensitization was conducted 3 weeks later. After 1 hr of habituation to the locomotor chambers, rats were injected with nicotine (0.4 mg/kg, i.p.) and locomotor activity measured for 2 hrs. No receptor antagonists were administered on this test. The locomotor activity monitoring chambers and intracranial microinjections were as described previously (Vezina *et al.*, 2002).

As there is evidence that mecamylamine may block NMDA receptors at high concentrations (Snell & Johnson, 1989), cases in which it was found to block the induction of sensitization by nicotine (e.g., after VTA infusion) were confirmed in a separate experiment testing the effect of bilateral infusion of a combination of dihydro-β-erythroidine (DHβE; 10 μg/side; Tocris, Ellisville, MO) and methyllycaconitine (MLA; 3 μg/side; Tocris, Ellisville, MO), receptor antagonists with non-α7 and α7 nAChR profiles, respectively. Doses were selected from published reports of dose-effect functions for these ligands (Panagis *et* al., 2000; Bruijnzeel & Markou, 2004).

nAChR upregulation: ^{125}I-epibatidine binding

Rats were sacrificed by rapid decapitation 2 hrs, 3 days, or 3 weeks after the last exposure injection and brains rapidly removed and flash-frozen on dry ice. Punches of VTA (1 mm) and NAcc (2 mm) were subsequently obtained bilaterally from 1mm-thick coronal slices. These were homogenized on ice in hypotonic lysis buffer containing protease inhibitors. Supernatant was processed to isolate the membrane portion of the sample by ultracentrifugation. The pellet was re-suspended in PBS and washed three times. Binding to membrane fractions was measured at room temperature by incubating triplicate samples in PBS with 0.5 nM ^{125}I-epibatidine (PerkinElmer, Waltham, MA) for 30 min on a table top rotator. It was terminated by vacuum filtration through Whatman (Clifton, NJ) GF/B filters presoaked in 0.5% polyethyleneimine using a Brandel (Gaithersburg, MD) 24 channel cell harvester. Bound ^{125}I-epibatidine (2200 Ci/mmol) was determined by gamma counting (PerkinElmer Wallac 1470, Gaithersburg, MD) with nonspecific binding estimated by ^{125}I-

epibatidine binding in a saturating concentration of nicotine (1mM). Total protein concentration in each brain membrane sample was measured by micro BCA (Pierce). ^{125}I-epibatidine binding was subsequently expressed as fmol/mg protein.

nAChR upregulation: Slice electrophysiology

Electrophysiology procedures were as described previously (Fagan *et al.*, 2007). Immediately after the last nicotine exposure injection, rats were anesthetized with isofluorane and the brain removed to ice-cold sucrose-artificial cerebrospinal fluid (sucrose-aCSF) with 10 mM ascorbic acid (in mM: 252 sucrose, 2.5 KCl, 1 $MgCl_2$, 2.5 CaCl, 20 glucose, 25 $NaHCO_3$; bubbled with 95%O_2/5%CO_2; pH=7.4). Horizontal slices (250 μm) including the VTA were made with a Vibrating Microtome (Leica). Slices were transferred to a 32°C holding chamber with normal aCSF (sucrose replaced by 125 mM NaCl) and perfused at ~20 ml/min for at least 15 min prior to recording.

Cells were visualized using infrared illumination on an upright microscope (Axioskop FS, Zeiss). Recording electrodes (5–8MΩ) were filled with potassium-gluconate internal solution (in mM: 154 K-gluconate, 1 KCl, 1 EGTA, 10 HEPES, 10 Glucose, 5 ATP, 0.1 GTP; pH adjusted to 7.4 with KOH). Series resistance for recordings was 5–30MΩ. Whole-cell voltage-clamp recordings were conducted at room temperature with constant bath perfusion at ~3 ml/min with normal aCSF containing DNQX (10 μM), bicuculine (20 μM), atropine (1 μM), TTX (1 μM), and Cd^{2+} (100 μM) to isolate nAChR currents. All cells were analyzed for the presence of the Ih current while holding at −50mV, and stepping to −115mV at −15mV increments. Cells with Ih 150 pA at −115mV (large Ih currents), slow pacemaker-like firing rate (<5 Hz), long action potential duration (>2 ms), and anatomical location within the VTA were considered dopaminergic. The population of neurons with Ih<100 pA at −115mV (small Ih currents) were considered GABA-enriched. Neurons were selected within an area approximately 0.6 mm to 0.1 mm from the medial border of the medial terminal nucleus (MT), extending approximately 0.3 mm in A/P directions from the center of the MT (in mm from bregma: L, 0.75 to 1.25; A/P, −5.1 to −5.6; D/V, −7.9 to −8.4). This region has a small degree of overlap with the medial extreme of the substantia nigra compacta and a small number of DA neurons in this area may have been included in the data analyses. These methods of cell identification are supported by recent reports from our group and others (Zhang *et al.*, 2010; Mao *et al.*, 2011). To elicit nAChR currents, 1 mM acetylcholine (ACh) was focally applied (300 ms) using a Picospritzer II (General Valve). To limit response variability, pipettes had uniform tip diameter, pressures for drug delivery were equal, and the pipette tip was always 50–60 μm from the soma. Currents were analyzed off-line using Clampfit (Axon Instruments, Inc.). Only nAChR responses with a rise time of <50ms were included in the analyses to limit confounding effects of receptor desensitization. ACh-induced currents were converted to current density (pA/pF), pooled in time bins following the last nicotine exposure injection and averaged.

Synaptic plasticity

The ratio of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) to N-methyl-D-aspartate (NMDA) receptor (AMPAR/NMDAR) components of excitatory postsynaptic currents (EPSCs) were determined in brain slices taken from rats 17–26 hrs after the last nicotine exposure injection. This assay provides a relative assessment of AMPA receptor trafficking using previously described methods (Mao *et al.*, 2011).

Nicotine withdrawal symptoms

The day after the last nicotine exposure injection, rats were administered the nAChR antagonist mecamylamine (1.0 mg/kg, s.c.; O'Dell *et al.*, 2006), placed in a circular plastic observation chamber and the frequency of the following somatic signs of precipitated

withdrawal recorded at three times (20–30, 60–70, and 110–120 minutes after the mecamylamine injection) and summed for each rat: writhes, cheek tremors, teeth chattering, yawns, and body shakes (Epping-Jordan *et al.*, 1998). To provide comparative data, separate rats were exposed to an intense continuous exposure regimen lasting two weeks. These rats were surgically prepared with osmotic mini-pumps (2ML2; Alzet, Cupertino, CA) set to deliver 3.0 mg/kg/day s.c. of nicotine (Epping-Jordan *et al.*, 1998). Control rats were fitted with a dummy pump. Pumps were removed after two weeks and precipitated withdrawal signs recorded the following day.

NAcc DA

The day after the last exposure injection, extracellular levels of DA in the NAcc core were assessed with microdialysis before and after a challenge injection of nicotine (0.4 mg/kg, i.p.). Comparative data were again provided by rats exposed to 3.0mg/kg/day of nicotine for two weeks and tested the day following pump removal. The day before testing, concentric microdialysis probes were lowered into the NAcc core and perfused with modified Ringer's dialysate at 0.3 µl/min overnight and 1.5 µl/min during testing the following day. Microdialysis samples were collected at 20 minute intervals, 3 before and 9 after the nicotine challenge injection. No significant group differences were detected in the basal DA levels observed in the 1 hour before the nicotine challenge injection [intermittent: exposure, $F_{1,12}=0.01$, *NS*, time, $F_{2,24}=0.9$, *NS*, exposureXtime, $F_{2,24}=2.54$, *NS*; continuous: exposure, $F_{1,20}=0.01$, *NS*, time, $F_{2,40}=2.76$, *NS*, exposureXtime, $F_{2,40}=1.79$, *NS*; analyzed by 2-way between-within ANOVA with exposure as the between factor and time as the within factor]. The maximal DA level achieved after the nicotine challenge was recorded. All microdialysis and chromatography procedures were as described previously (Vezina *et al.*, 2002).

Data analyses

Locomotor data were analyzed with 2-way between analyses of variance (ANOVA) with exposure and mecamylamine (or DHβE+MLA) as the two factors. The nAChR epibatidine binding data were also analyzed with 2-way between ANOVA but with exposure and test time as the two factors. Current density data were analyzed with between-within ANOVA with exposure as the between factor and test time as the within factor. All post-hoc comparisons were conducted with the Scheffé test. Remaining data (somatic signs, NAcc DA) were analyzed using independent samples t-tests.

Results

Activation of nAChRs in the VTA but not the NAcc is necessary for locomotor sensitization by nicotine

As expected, previous exposure to repeated intermittent nicotine produced robust locomotor sensitization. A subsequent nicotine challenge (0.4 mg/kg, IP) given 3 weeks after the final exposure injection produced a greater locomotor response in nicotine vs. saline exposed animals (Fig. 1; up to 2-fold increase; *P*<0.05–0.01). Bilateral infusion of the broad-spectrum nAChR antagonist mecamylamine into the VTA prior to each exposure injection dose-dependently blocked the induction of locomotor sensitization, as evidenced by the lack of enhanced responding on this test (Fig. 1A; exposure, $F_{1,30}=13.19$, *P*<0.01; mecamylamine, $F_{2,30}=6.06$, *P*<0.01; exposure x mecamylamine interaction, $F_{2,30}=7.15$, *P*<0.01). Similarly blocking nAChRs in the NAcc during nicotine exposure had no effect on the induction of sensitization (Fig. 1B; exposure, $F_{1,22}=24.48$, *P*<0.001; mecamylamine, $F_{1,22}=0.002$, *NS*; exposure x mecamylamine interaction, $F_{1,22}=0.28$, *NS*). As with VTA mecamylamine, infusing DHβE+MLA into the VTA prior to each nicotine exposure injection also blocked the induction of locomotor sensitization. These receptor antagonists exhibit non-α7 (DHβE) and α7 (MLA) nAChR profiles. No evidence for sensitization was

observed in these rats. Means ± SEMs obtained were 554.0±33.17 (VTA vehicle IP saline, n=6), 1014.67±132.08 (VTA vehicle IP nicotine, n=6), 483.57±74.74 (VTA DHβE+MLA IP saline, n=7), and 642.25±110.51 (VTA DHβE+MLA IP nicotine, n=8). The ANOVA conducted on these data revealed significant effects of exposure ($F_{1,23}=9.25$, $P<0.01$) and DHβE+MLA ($F_{1,23}=4.73$, $P<0.05$) but a non-significant exposure x DHβE+MLA interaction ($F_{1,23}=2.20$, NS). Again, VTA vehicle rats exposed to nicotine showed greater nicotine-evoked locomotor counts on the sensitization test compared to their saline exposed controls ($P<0.05$). This was not observed in VTA DHβE+MLA rats. These results indicate that nAChR activation in the VTA but not the NAcc is critical for the induction of locomotor sensitization by nicotine.

Intermittent nicotine exposure upregulates nAChR radioligand binding in the VTA but not the NAcc

To test whether nicotine exposure alters nAChRs in either of these regions, tissue punches were obtained from the VTA and NAcc of nicotine- and saline-exposed rats for binding assays with radiolabeled epibatidine, a high affinity nAChR agonist. Repeated intermittent nicotine exposure increased epibatidine binding in the VTA at 2 hours, but not 3 days or 3 weeks following nicotine exposure (Fig. 1C; >2-fold increase relative to saline-exposure; $P<0.05$). The ANOVA conducted on these data (expressed as % of saline-exposed controls) revealed significant effects of exposure ($F_{1,52}=4.31$, $P<0.05$) and time ($F_{2,52}=3.96$, $P<0.05$) as well as a significant exposure x time interaction ($F_{2,52}=3.96$, $P<0.05$). No significant effects of nicotine exposure on epibatidine binding were observed in the NAcc at any of the times tested (Fig. 1D; exposure, $F_{1,67}=2.56$, NS; time, $F_{2,67}=0.13$, NS, exposure x time interaction, $F_{2,67}=0.13$, NS). Thus, transient upregulation was observed in VTA nAChRs that are critical for the induction of sensitization by nicotine.

Intermittent nicotine exposure functionally upregulates nAChRs specifically in VTA DA neurons

To further assess the extent and time course of nicotine induced functional upregulation of nAChRs in the VTA, electrophysiological measures of nicotinic currents were obtained in horizontal brain slices prepared from rats previously exposed to repeated intermittent nicotine or saline. Pharmacologically isolated whole-cell nAChR inward currents were elicited by pressure application of 1mM ACh onto identified VTA neuronal somata in the presence of atropine (1μM) to block muscarinic effects. DA neurons were identified on the basis of cell size, location, action potential properties, and large Ih currents (Mao et al 2011), while cells with small Ih currents were considered GABA-enriched neurons. The ACh responses were larger in the DA (large Ih) neurons of nicotine- relative to saline-exposed rats (Fig. 2A–B; exposure, $F_{(1,86)}=6.03$, $P<0.05$, time, $F_{(2,86)}=3.81$, $P<0.05$, exposure-time interaction, $F_{(2,86)}=6.68$, $P<0.01$). The effect decayed rapidly and was seen only in recordings performed 2 hrs after the last nicotine exposure injection ($P<0.05$). Studies conducted in cultured rat cortical neurons have observed similar transient nAChR upregulation that manifests rapidly, decays within 2 hrs, and corresponds to rapid changes in the conformation of the receptor (Govind et al., 2012). Thus, paralleling the epibatidine binding results in the present experiments, exposure to a sensitizing nicotine regimen produced a transient functional upregulation of nAChRs in VTA DA neurons. Notably, functional upregulation was not observed in the small Ih current expressing GABA-enriched neurons (Fig. 2C–D; exposure, $F_{(1,42)}=0.86$, NS; time, $F_{(2,42)}=0.02$, NS; exposure-time interaction, $F_{(2,42)}=0.33$, NS). These findings are consistent with enhanced momentary excitability in VTA DA neurons that could favor longer-lasting potentiation of excitatory inputs to these cells by facilitating NMDA receptor dependent plasticity (Mao et al., 2011). Thus, the status of synaptic plasticity was assessed by determining the AMPAR/NMDAR ratios of the excitatory inputs to VTA DA (large Ih) neurons. When tested 17–26 hrs after

the last exposure injection, the AMPAR/NMDAR ratios were larger in tissue from nicotine relative to saline exposed rats (Fig. 2E–F; $t_{(25)}=2.05$, $P<0.05$). These results suggest higher synaptic levels of AMPA relative to NMDA receptors following repeated intermittent nicotine injections and are consistent with the induction of long term potentiation at these synapses (Ungless *et al.*, 2001). No differences in rectification of AMPA receptor EPSCs were observed between nicotine and saline exposed rats, suggesting that the relative contribution of GluR1- vs GluR2-containing AMPA receptors was similar following synaptic modulation (data not shown).

Absence of withdrawal symptoms and enhanced DA neuron reactivity following moderate intermittent nicotine exposure

Unlike more intense continuous exposure regimens often used to model the effects of smoking in humans (high nicotine concentrations delivered by osmotic minipump over 7 or more days; Epping-Jordan *et al.*, 1998; Rahman *et al.*, 2004; Nashmi *et al.*, 2007), the moderate intermittent exposure regimen used in the present experiments (4×0.4 mg/kg, i.p., 1 injection every other day) did not produce withdrawal symptoms and increased nicotine-induced DA overflow in the period soon after exposure. One day following exposure, the nAChR antagonist mecamylamine failed to elicit somatic signs of withdrawal in nicotine relative to saline exposed rats (Fig. 3A; $t_{(10)}=0.82$, *NS*). In contrast and as expected, rats exposed to an intense continuous exposure regimen (3.0 mg/kg/day over two weeks) exhibited a significant increase in evoked withdrawal signs at this time (Fig. 3A; $t_{(9)}=1.84$, $P<0.05$). In addition, nicotine induced NAcc DA overflow was significantly enhanced relative to controls one day following moderate intermittent exposure (Fig. 3B; $t_{(12)}=2.27$, $P<0.05$), a time when increased synaptic levels of AMPA receptors could have acted synergistically with nAChR stimulation to potentiate activation by nicotine of VTA DA neurons (Carlezon & Nestler, 2002; Saal *et al.*, 2003). This effect was not observed following the intense continuous exposure regimen (Fig. 3B; $t_{(19)}=0.39$, *NS*). To ease comparison of the different effects, data are illustrated and were analyzed as % of saline-exposed control values. These findings are consistent with the VTA DA neuron specific transient upregulation of nAChRs and the subsequent increase in the AMPAR/NMDAR ratio observed following intermittent nicotine exposure in the present experiments as well as the VTA GABA neuron specific nAChR upregulation and concurrent manifestation of withdrawal symptoms reported by others after intense continuous nicotine exposure (Epping-Jordan *et* al., 1998; Rahman *et al.*, 2004; Nashmi *et al.*, 2007).

Discussion

The present results show that the induction of sensitization by intermittent nicotine requires nAChR activation in the VTA but not the NAcc. Further, VTA but not NAcc nAChRs showed nicotine-induced upregulation of radioligand binding in the hours following the last nicotine exposure injection and functional upregulation was observed selectively in DA but not GABA neurons in the VTA. These findings provide a mechanistic basis whereby repeated intermittent exposure to nicotine, a pattern often associated with initial exposure to tobacco, can ultimately lead to long-lasting predisposition to pursue the drug.

Intense continuous nicotine exposure regimens often used to model smoking in humans are associated with somatic signs of withdrawal (Epping-Jordan *et al.*, 1998; present results). Together with upregulation of nAChRs in VTA GABA neurons (Nashmi *et al.*, 2007) and inhibition of neighboring DA neurons (Rahman *et al.*, 2004; present results), these could conceivably lead to a dysphoric state that contributes to maintenance of nicotine intake once it is established (Koob & Le Moal, 1997). Conversely, in the present experiments, exposure to moderate intermittent nicotine was not associated with somatic withdrawal signs but with transient upregulation of nAChRs in VTA DA neurons followed by long-term potentiation

of excitatory inputs to these cells and increased nicotine-evoked DA overflow. These conditions in turn promote long-lasting sensitization of DA neuron reactivity, drug-induced locomotion and self-administration (Vezina, 2004; Vezina *et al.*, 2007). Indeed, exposure to nicotine under these conditions has been shown to enhance the subsequent self-administration of nicotine (Neugebauer *et al.*, 2012), amphetamine (Cortright *et al.*, 2012), and cocaine (Horger *et al.*, 1992).

How different nicotine exposure conditions cause such different patterns of nAChR upregulation in DA versus GABA neurons in the VTA remains an open question. Repeated intermittent nicotine exposure may favor upregulation of nAChRs in DA neurons as they, in contrast to GABA and glutamate neurons, express functional α6-containing nAChRs (Gotti *et al.*, 2010; cf, Yang *et al.*, 2011) which may require shorter nicotine exposure than α4β2-containing nAChRs for upregulation (Walsh *et al.*, 2008). Different patterns of nAChR upregulation in brain are also observed following moderate intermittent and intense continuous nicotine exposure regimens. In the present experiments, nAChRs exposed to intermittent nicotine showed a transient increase in ligand binding in the VTA but not the NAcc or in the prefrontal cortex, another A10 DA neuron projection field (data not shown). On the other hand, following exposure to intense continuous nicotine regimens, nAChRs show increased ligand binding in a greater number of brain regions including the NAcc and the prefrontal cortex (Collins *et al.* 1988, 1990; Rowell & Li, 1997; Mugnaini *et al.*, 2002) and it persists for a longer period of time (Collins *et al.* 1988, 1990; for more references and discussion, see Vezina *et al.*, 2007). Similarly, exposing cultured rat cortical neurons to nicotine for 25-hrs leads to both transient upregulation corresponding to nAChR conformational changes and long-lasting upregulation correlating with increased nAChR numbers whereas exposure to nicotine for a shorter period (e.g., 4 hr) produces only transient nAChR upregulation (Govind *et al.*, 2012). These findings reflect the differential recruitment by different patterns of nicotine exposure of multiple processes in the upregulation nAChRs (Govind *et al.*, 2012). Determining the subunit composition of nAChRs found in different neurons and brain regions should provide additional insights into how and where nAChR upregulation occurs and help decipher the immediate and long-term consequences for behavior of upregulation in multiple brain regions. Interestingly, continuous stimulant exposure regimens are not known to produce sensitization (Robinson & Becker, 1986), suggesting that the nAChR upregulation observed in the NAcc and in VTA GABA neurons following continuous nicotine exposure may be associated with other endpoints. The present findings are consistent with others showing that intermittent exposure to stimulants initiates neuroadaptations in VTA DA neurons that lead ultimately to sensitized responding (Ferrari *et al.*, 2002; Vezina, 2004).

It has been known for some time that nAChR upregulation does not always correlate either with tolerance to the locomotor depressant (Clarke & Kumar, 1983; Collins *et al.*, 1988, 1990) or sensitization of the locomotor activating effects of nicotine (Ksir *et al.*, 1987). The relatively transient time course of nAChR upregulation observed here (hours) and the subsequent increase in AMPAR-mediated excitatory transmission reported previously in the VTA (days; Ungless *et al.*, 2001) clearly preclude these phenomena as primary mechanisms underlying the expression of sensitized locomotion. It is possible however that nAChR activation might initiate a series of sequential neuroadaptations in the VTA that lead ultimately to the expression of long-lasting behavioral sensitization known to persist for weeks to months. The upregulation of nAChRs observed selectively in VTA DA neurons in the present experiments would be expected to increase the excitability of these neurons and likely contributed to the induction of long-term potentiation of their excitatory synaptic inputs. This potentiation of excitatory inputs onto VTA DA neurons has been proposed to contribute to the induction of longer lasting sensitization as both are prevented by NMDA receptor blockade during drug exposure (Wolf *et* al., 1994; Vezina & Queen, 2000; Ungless

et al., 2001; Saal *et al.*, 2003). As such, transient nicotine-induced nAChR upregulation in DA neurons of the midbrain may initiate a cascade of neuroadaptations that come to support the long-lasting changes in the intensity and repertoire of motivated behaviors an organism ultimately displays. Proof of this possibility awaits development and expression in the VTA of a nAChR mutant that prevents upregulation without compromising receptor functionality.

Acknowledgments

Abbreviations

ACh	acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
DA	dopamine
DHβE	dihydro-β-erythroidine
EPSC	excitatory postsynaptic current
MLA	methyllycaconitine
NAcc	nucleus accumbens
nAChR	nicotine acetylcholine receptor
NMDA	N-methyl-D-aspartate
VTA	ventral tegmental area

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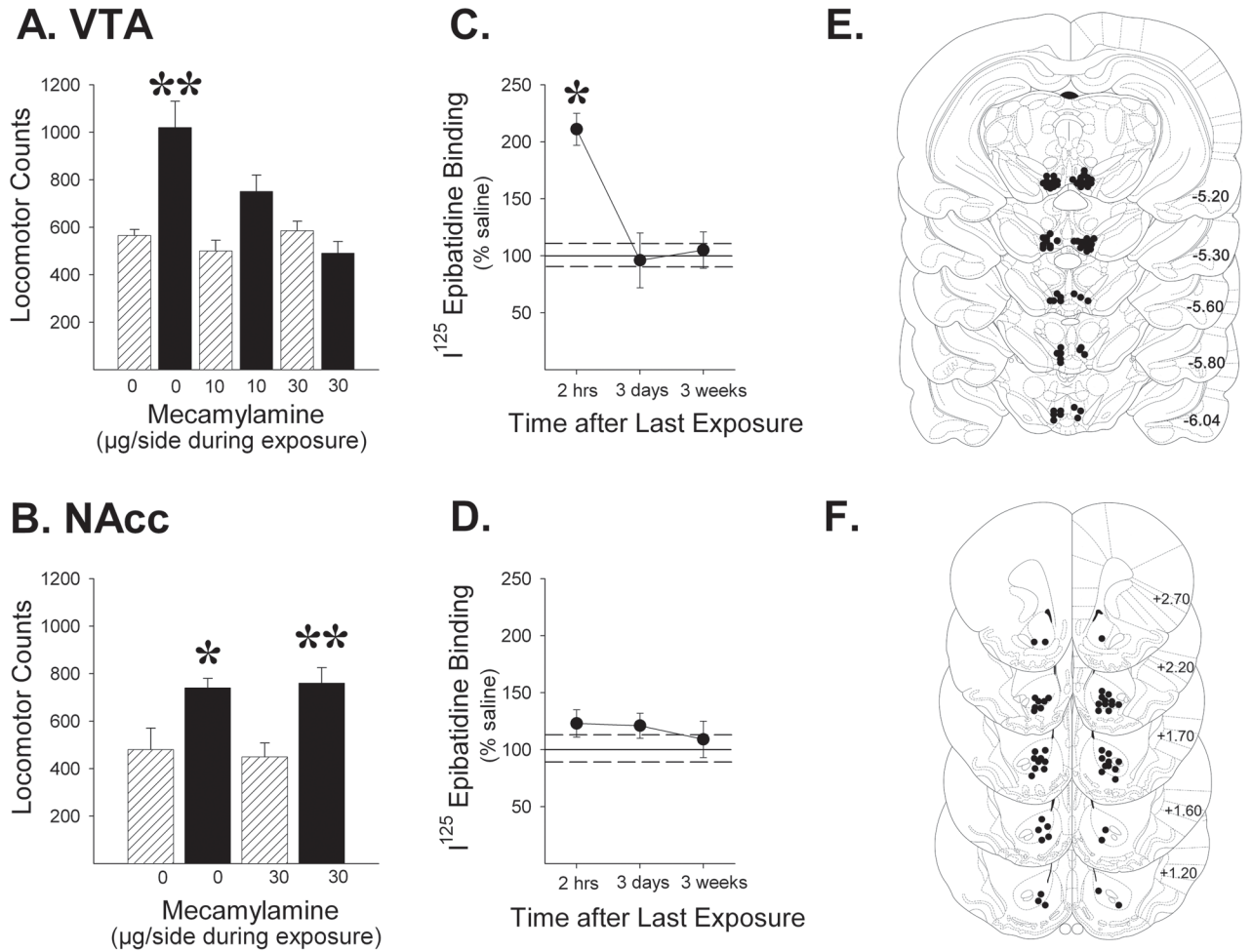
Figure 1.
Blockade of nAChRs in the VTA (**A**), but not the NAcc (**B**), prevents locomotor sensitization by nicotine. Rats were exposed to 4 injections of nicotine (0.4mg/kg over 7 days, i.p.; ■) or saline (▨), with or without an i.c. injection of the nAChR antagonist mecamylamine and tested 3 weeks later for their locomotor response to nicotine (0.4 mg/ kg). Mecamylamine was not injected on this test. n/group=6–7. Data are shown as group mean (+SEM) 2-hr session total locomotor counts obtained after the challenge injection. Line drawings (Paxinos & Watson, 1997; numbers indicate mm from bregma) indicating location of injection cannula tips in the VTA (**E**) and NAcc (**F**) are shown for rats included in the data analyses. **C.** Exposure to the same regimen increased ^{125}I-epibatidine binding in the VTA observed at 2-hrs but not 3 days or 3 weeks following exposure. **D.** Significant nAChR upregulation was not observed in the NAcc at any time tested. n/group=7–10. Data (mean±SEMs) are expressed as % of saline controls (solid and dashed lines). Values for the saline control groups ranged from 90.0±15.81–130.09±10.22 fmol/mg protein in VTA and 55.68±6.96–64.62±8.91 fmol/mg protein in NAcc. No significant differences between control groups were observed in either the VTA ($F_{(2,24)}$=2.07, *NS*) or the NAcc ($F_{(2,32)}$=0.40, *NS*). *****, $p<0.05$, ******, $p<0.01$, compared to respective saline exposure controls.

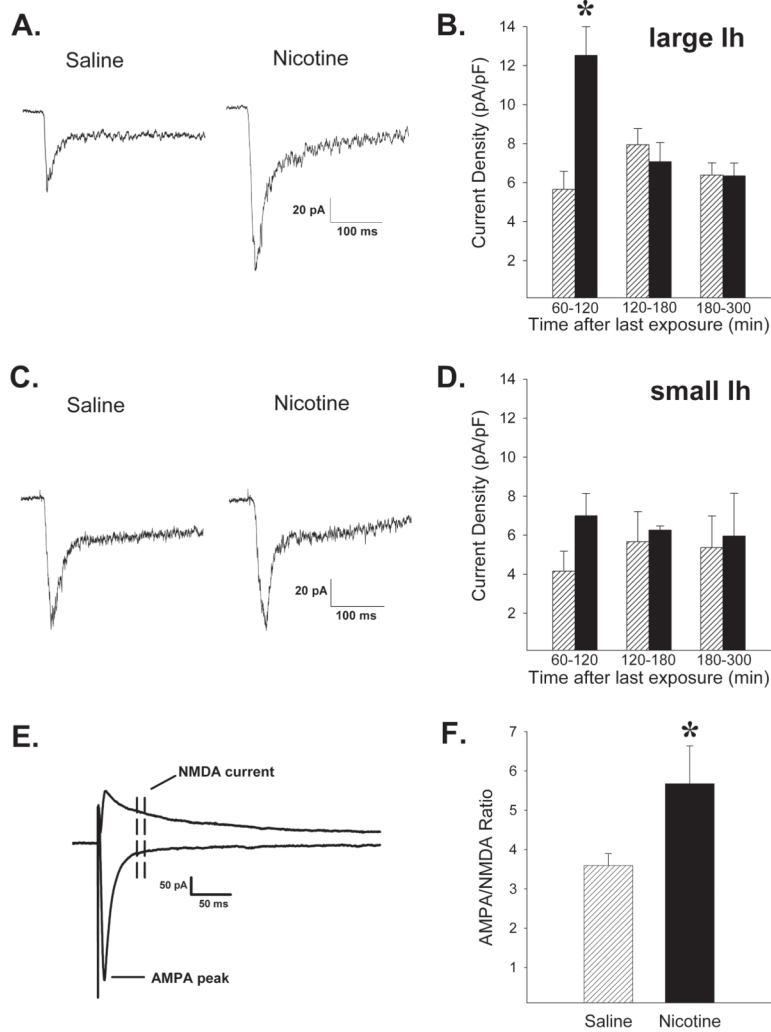**Figure 2.**
Exposure to sensitizing nicotine injections (4 × 0.4mg/kg over 7 days, i.p.; ■) leads to functional upregulation of nAChRs in VTA DA neurons and potentiation of excitatory inputs to these cells compared to saline exposed controls (▨).Traces of nAChR current responses to 300 msec focal application of 1 mM ACh onto (**A**) DA (large Ih) and (**C**) GABA-enriched (small Ih) neurons   120 min after the last saline or nicotine exposure injection. **B.** Average peak currents normalized to cell capacitance were significantly greater in DA (large Ih) neurons of nicotine exposed rats up to 2 hours following the last nicotine exposure injection. n=38,54 cells from 10,10 rats exposed to nicotine, saline, respectively. **D.** No significant effects of nicotine exposure were detected in GABA-enriched neurons (small Ih). n=23,25 cells from 14,15 rats. **E.** Traces of evoked AMPA and NMDA EPSCs. Peak AMPA current was measured at -70 mV and the NMDA current was averaged from a window 30–40 msec after the peak of the EPSC at $+40$ mV. **F.** Mean AMPAR/NMDAR ratios were significantly greater in DA (large Ih) neurons from nicotine relative to saline exposed animals. n=13,14 cells from 8,9 rats. *, $p<0.05$, compared to respective saline exposure controls.

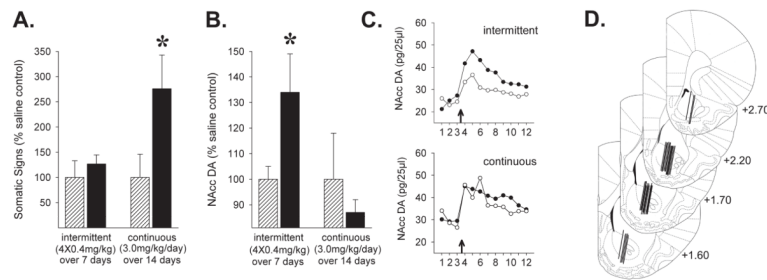**Figure 3.**
Evoked somatic signs of withdrawal (**A**) and maximal nicotine-evoked NAcc DA overflow
(**B**) following an intermittent (4 × 0.4mg/kg, i.p. over 7 days) or continuous (3.0 mg/kg/day,
s.c. over 14 days) nicotine exposure regimen (■). Compared to saline exposed controls
(▨), evoked withdrawal signs were elevated 1-day following continuous but not
intermittent nicotine. Nicotine-induced NAcc DA overflow was enhanced 1-day following
intermittent but not continuous nicotine. n/group=5–11. Data (mean±SEMs) are expressed
as % of saline-exposed controls. Values for the saline control groups were 4.5±2.1
(continuous) and 6.0±1.78 (intermittent) for number of somatic signs and 54.8±10.6 pg/25µl
(continuous) and 38.5±1.7 pg/25µl (intermittent) for maximal nicotine-evoked NAcc DA
overflow. No significant differences between control groups were observed for either
somatic signs ($t_{(10)}$=0.54, *NS*) or NAcc DA ($t_{(17)}$=1.29, *NS*). *, p<0.05, compared to
respective saline exposure controls. **C.** Time course for DA levels (pg/25µl) in the NAcc
before and after the nicotine challenge injection (arrows at abscissae) for both exposure
conditions. Numbers at abscissae indicate 20-min sampling intervals. ●, nicotine exposed;
○, saline exposed. **D.** Line drawings (Paxinos & Watson, 1997; numbers indicate mm from
bregma) illustrating the location of the active portion of the microdialysis probes in the
NAcc of rats included in the data analyses.