α4α6β2* Nicotinic Acetylcholine Receptor Activation on Ventral Tegmental Area Dopamine Neurons Is Sufficient to Stimulate a Depolarizing Conductance and Enhance Surface AMPA Receptor Function

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ABSTRACT

Tobacco addiction is a serious threat to public health in the United States and abroad, and development of new therapeutic approaches is a major priority. Nicotine activates and/or desensitizes nicotinic acetylcholine receptors (nAChRs) throughout the brain. nAChRs in ventral tegmental area (VTA) dopamine (DA) neurons are crucial for the rewarding and reinforcing properties of nicotine in rodents, suggesting that they may be key mediators of nicotine’s action in humans. However, it is unknown which nAChR subtypes are sufficient to activate these neurons. To test the hypothesis that nAChRs containing α6 subunits are sufficient to activate VTA DA neurons, we studied mice expressing hypersensitive, gain-of-function α6nAChRs (α6L9’S mice). In voltage-clamp recordings in brain slices from adult mice, 100 nM nicotine was sufficient to elicit inward currents in VTA DA neurons via α6β2* nAChRs. In addition, we found that low concentrations of nicotine could act selectively through α6β2* nAChRs to enhance the function of 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptors on the surface of these cells. In contrast, α6β2* activation did not enhance N-methyl-d-aspartic acid receptor function. Finally, AMPA receptor (AMPAR) function was not similarly enhanced in brain slices from α6L9’S mice lacking α4 nAChR subunits, suggesting that α4α6β2* nAChRs are important for enhancing AMPAR function in VTA DA neurons. Together, these data suggest that activation of α4α6β2* nAChRs in VTA DA neurons is sufficient to support the initiation of cellular changes that play a role in addiction to nicotine. α4α6β2* nAChRs may be a promising target for future smoking cessation pharmacotherapy.

Introduction

Compared with the 20th century, the number of deaths worldwide from tobacco use is estimated to be 10-fold greater at the completion of the 21st century—with possibly as many as 1 billion lives lost (Peto and Lopez, 2001). Development of better smoking cessation therapies is, therefore, a major priority. Most current therapies seek to interfere with the action of nicotine, the primary psychoactive compound in cigarette smoke. Nicotine activates and/or desensitizes nicotinic acetylcholine receptors (nAChRs) found on neuronal axon terminals, dendrites, and somata (Pidoplichko et al., 1997; Picciotto et al., 2008). The mesolimbic dopamine (DA) pathway, including DA neurons in the ventral tegmental area (VTA) and their terminals in the nucleus accumbens, is a key brain circuit involved in nicotine addiction (Laviolette and van der Kooy, 2004). Nicotine acts through nAChRs in this pathway to stimulate DA neuron firing (Calabresi et al., 1989) and produce long-lasting increases in nucleus accumbens DA release (Di Chiara and Imperato, 1988).

Long-lived enhancement of drug-induced DA release is thought to be mediated by changes in synaptic plasticity at VTA DA neurons (Wolf et al., 2004; Kauer and Malenka, 2007). This involves the abused drug causing enhanced excitability of VTA DA neurons and long-term potentiation (LTP) of excitatory inputs to these cells (Unsgless et al., 2001; Saal et al., 2003). In particular, nicotine acts through VTA nAChRs on DA neuron soma, as well as presynaptic nAChRs, to depolarize...
these cells, facilitate N-methyl-d-aspartic acid (NMDA) receptor activation, and enhance glutamate-induced excitatory post synaptic currents (EPSCs) (Saal et al., 2003; Gao et al., 2010; Jin et al., 2011; Mao et al., 2011). Understanding which proteins—including which nAChR subtypes—mediate these effects could lead to new pharmacotherapy approaches designed to disrupt or reverse the addictive process at the molecular, cellular, or circuit level (Drenan and Lester, 2012).

Heteromeric nAChRs in the brain are pentamers containing two or more 2 subunits (2 and/or 3) and two or more 4 subunits (2-6) (Itoier and Bertrand, 2001). “Auxiliary” subunits 5 or 3 do not contribute to formation of a functional binding site, but nevertheless exert powerful modulatory effects on nAChR function (Cui et al., 2003; Drenan et al., 2008b; Fowler et al., 2011). 4/2 (the asterisk indicates nAChR pentamers that contain the indicated subunits, and may or may not contain other subunits as well) nAChRs are expressed in DAergic and GABAergic neurons in VTA (Nashmi et al., 2007), and activation of these receptors can produce increased firing of VTA DA neurons (Tapper et al., 2004; Liu et al., 2012) as well as increased GABA release onto these cells (Mansvelder et al., 2002). Homomeric 7 nAChRs expressed on glutamatergic axon terminals that synapse onto VTA neurons can enhance glutamatergic excitation of VTA neurons (Mansvelder and McGehee, 2000), thereby potentiating nicotine’s direct action at 4/2 nAChRs on the soma of these cells (Mansvelder et al., 2002).

Interest in nAChRs containing 6 subunits is strong due to their high sensitivity to nicotine (Salminen et al., 2007), and their selective expression in DA and norepinephrine-producing cells (Le Novère et al., 1996; Léna et al., 1999; Champtiaux et al., 2002; Mackey et al., 2012). 6 nAChRs require 2 subunits for proper expression and function (Grady et al., 2002; Champtiaux et al., 2003; Salminen et al., 2004). Tapper and colleagues demonstrated that activation of 4/2 nAChRs in VTA DA neurons induces prolonged depolarization of these cells, an effect that was sensitive to an 6 nAChR antagonist (Liu et al., 2012). Using a similar antagonist, Wu and colleagues reported that GABAergic transmission onto VTA DA neurons may be mediated by 6 nAChRs (Yang et al., 2011). These approaches relied on pharmacological blockade to discern the role of 6 nAChRs, and the results indicate that more experiments are needed to better understand 6 nAChRs in the VTA.

In the present study, we studied transgenic mice expressing 6 nAChR subunits with increased sensitivity to nicotine (Drenan et al., 2008a), which provided a complementary approach to pharmacological inactivation (Drenan and Lester, 2012). Using low concentrations of nicotine that moderately increase firing of VTA neurons (Tapper et al., 2008a), which provided a complementary approach to pharmacological inactivation, nicotine was used to assess the effects of nicotine on nicotine-naive mice as well as on nicotine-tolerant mice. These approaches relied on pharmacological blockade to discern the role of 6 nAChRs, and the results indicate that more experiments are needed to better understand 6 nAChRs in the VTA.

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1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 25 mM glucose, 5 mM Na⁺ ascorbate, 2 mM thiourea, 3 mM Na⁺ pyruvate, 2 mM MgSO4·7H2O, and 2 mM CaCl2·2H2O (300–310 mM, pH 7.3–7.4). Coordinates for recordings in VTA were approximately ~3.5 mm from bregma, 4.0–4.5 mm from the surface, and 0.5–1.0 mm from the midline. In adult C57 mice, these coordinates correspond to nucleus accumbens lateral shell-projecting VTA neurons, which are expected to be approximately 96% tyrosine hydroxylase-positive (Lammel et al., 2008).

Patch-Clamp Electrophysiology. Patch-clamp electrophysiology was carried out as previously described (Engle et al., 2012). A single slice was transferred to a 0.8-mL recording chamber (RC-27L bath with PH-6R heated platform; Warner Instruments, Hamden, CT), and slices were superfused throughout the experiment with standard recording artificial cerebrospinal fluid (1.5–2.0 mL/min) containing the following: 124 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 24 mM NaHCO3, 12.5 mM glucose, 2 mM MgSO4·7H2O, and 2 mM CaCl2·2H2O (300–310 mM, pH 7.3–7.4). Cells were visualized with an upright microscope (FN-1; Nikon Instruments, Melville, NY) using infrared or visible differential interference contrast optics. Patch electrodes were constructed from Kwik-Fil borosilicate glass capillary tubes (1B150F-4; World Precision Instruments, Inc., Sarasota, FL) using a programmable microelectrode puller (P-97; Sutter Instrument Company, Novato, CA). The electrodes had tip resistances of 4.5–8.0 MΩ when filled with internal pipette solution (pH adjusted to 7.25 with Tris base, osmolarity adjusted to 290 mM with sucrose). Two internal pipette solutions were used. The following solution was used when recording nicotine- or ACh-activated currents: (bath application or puff-applied): 135 mM K⁺ gluconate, 5 mM EGTA, 0.5 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 2 mM MgATP, and 0.1 M GTP. The following solution was used when recording AMPA- or NMDA-activated currents: 177 mM NaCl, 20 mM HEPES, 0.4 mM EGTA, 2.8 mM NaCl, 5 mM TEA-Cl, 2.5 mM MgATP, 100 μM spermine, and 0.25 mM MgGTP (pH 7.25 with Tris base). Whole-cell recordings were taken at 32°C with an Axopatch 200B amplifier, a 16-bit Digitida 1440A/D converter, and pCLAMP 10.3 software (all from Molecular Devices, Sunnyvale, CA). Data were sampled at 5 kHz and low-pass filtered at 1 kHz. The junction potential between the patch pipette and the bath solution was nullified immediately prior to gigaseal formation. Series resistance was uncompensated.

DA neurons in VTA were identified according to previously published methods (Woolston et al., 2003; Nashmi et al., 2007; Drenan et al., 2008a). We avoided recording from neurons on the slice surface and neurons deep in the slice that were difficult to visualize. Briefly, DA neurons were identified via several electrophysiological characteristics: 1) broad spike width (>2 milliseconds), 2) slow spontaneous firing (<5 Hz), and 3) expression of hyperpolarization-activated cation current (Ih). To examine the function of somatic ligand-gated ion channels, agonists were locally applied using K⁺ gluconate-based internal solution (see recipe above) made with diethylpyrocarbonate-treated water. After whole-cell recording, the recorded cell was aspirated into the pipette, under visual control, with gentle negative pressure. Input resistance was monitored during aspiration. Successful PCR reactions were typically only attained when a cell resistance of >1 GΩ was maintained after aspiration. Cellular contents were expelled into 75% ethanol, and RNA was precipitated and isolated by centrifugation at 4°C. CDNA was formed from RNA via reverse transcription (RT) (Senscript RT; Qiagen, Germantown, MD) using oligo-dT primers, and a nested PCR strategy was subsequently used to detect target mRNA species. In round 1 of nested PCR, tyrosine hydroxylase (TH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified (1 cycle: 94°C for 2 minutes; 20 cycles: 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1.5 minutes; 1 cycle: 72°C for 10 minutes) with the following primers: TH_F (CATGTGATGCCAGGACGAC), TH_R2 (GAAAGUGGCT-GGAAACTTT), GAPDH_F2 (AACTTGGCATGTGAGAAG), and GAPDH_R2 (CCCTGGTCTGTAGCCGAT). Subsequently, TH and GAPDH signals were further amplified (1 cycle: 94°C for 2 minutes; 36 cycles: 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1.5 minutes; 1 cycle: 72°C for 10 minutes) in round 2 with the following primers: TH_F, TH_R1 (CCT GTG GGT GGT ACC CTA TG), GAPDH_F1 (GTG TCT CTA CCC CCA ATG TG), and GAPDH_R1 (GGT CCT CAG TGT AGC CCA AG). PCR primers were synthesized by IDT (Coralville, IA). Final PCR products were detected by electrophoresis in 1.6% agarose gels with ethidium bromide staining.

Immunohistochemistry and Confocal Microscopy. Transgenic mice expressing α6 nAChR subunits fused-in-frame with GFP (α6GFP mice; n = 3), along with α6 GFP mice homozygous for the α4KO allele, were anesthetized with sodium pentobarbital (100 mg/kg i.p.) and transcardially perfused with 15 mL ice-cold phosphate-buffered saline (PBS) followed by 25 mL ice-cold 4% paraformaldehyde in PBS. Brains were removed and postfixed for 2 hours at 4°C. Coronal sections (50 μm) were cut on a microslicer and collected into PBS. Sections were permeabilized (20 mM HEPES, pH 7.4, 0.5% Triton X-100, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose) for 1 hour at 4°C, blocked (0.1% Triton X-100, 5% donkey serum in Tris-buffered saline (TBS), pH 7.4) for 1 hour at room temperature, and incubated overnight at 4°C in solutions containing primary antibodies (diluted in 0.1% Triton X-100, 5% donkey serum in TBS). Sections were stained with rabbit anti-GFP primary antibodies (A11212; Invitrogen, Carlsbad, CA) with a final dilution of 1:500. Sections were washed three times for 10 minutes each in TBS/Tween 20 (0.1% Triton X-100 in TBS) followed by incubation at room temperature for 1 hour with goat anti-rabbit Alexa 488 secondary antibodies (A11080; Invitrogen) diluted in 0.1% Triton X-100, 5% donkey serum in TBS. Sections were then washed three times in TBS/Tween 20 for 10 minutes each. Sections were stained with Qnuclear Deep Red Stain (1:1000, Q10363; Invitrogen) in PBS for 20 minutes at room temperature followed by three 5-minute washes in PBS. All sections were mounted on slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA), and then imaged with a Nikon A1 laser-scanning confocal microscope system (Nikon Instruments). Nikon Plan Apo 10× oil objectives were used. Alexa 488 was excited with an argon laser at 488 nm. VTA DA neurons were imaged at 60× , and mean pixel intensity per cell was measured for >100 cells in both α6GFP and α6GFPα4KO slices.

Statistical Analysis. Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Data are reported as the mean ± S.E.M. To determine whether data caused slight to modest cell movement, and 2) the seal parameters remained stable for multiple responses. Under these conditions, the 10–90% rise time for AMPA application was 222 ± 17 milliseconds. Faster rise times and excessive cell movement were commonly associated with loss of a stable seal. Responses were much slower (10–90% rise time was 546 ± 75 milliseconds) when the cell did not move during the application.

Single-Cell Reverse-Transcription PCR. These methods were adapted from Zhao-Shea et al. (2011). VTA neurons were studied using K⁺ gluconate-based internal solution (see recipe above) made with diethylpyrocarbonate-treated water. After whole-cell recording, the recorded cell was aspirated into the pipette, under visual control, with gentle negative pressure. Input resistance was monitored during aspiration. Successful PCR reactions were typically only attained when a cell resistance of >1 GΩ was maintained after aspiration. Cellular contents were expelled into 75% ethanol, and RNA was precipitated and isolated by centrifugation at 4°C. CDNA was formed from RNA via reverse transcription (RT) (Senscript RT; Qiagen, Germantown, MD) using oligo-dT primers, and a nested PCR strategy was subsequently used to detect target mRNA species. In round 1 of nested PCR, tyrosine hydroxylase (TH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified (1 cycle: 94°C for 2 minutes; 20 cycles: 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1.5 minutes; 1 cycle: 72°C for 10 minutes) with the following primers: TH_F (CATGTGATGCCAGGACGAC), TH_R2 (GAAAGUGGCT-GGAAACTTT), GAPDH_F2 (AACTTGGCATGTGAGAAG), and GAPDH_R2 (CCCTGGTCTGTAGCCGAT). Subsequently, TH and GAPDH signals were further amplified (1 cycle: 94°C for 2 minutes; 36 cycles: 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1.5 minutes; 1 cycle: 72°C for 10 minutes) in round 2 with the following primers: TH_F, TH_R1 (CCT GTG GGT GGT ACC CTA TG), GAPDH_F1 (GTG TCT CTA CCC CCA ATG TG), and GAPDH_R1 (GGT CCT CAG TGT AGC CCA AG). PCR primers were synthesized by IDT (Coralville, IA). Final PCR products were detected by electrophoresis in 1.6% agarose gels with ethidium bromide staining.
sets were normally distributed, all data sets were subjected to a D'Agostino and Pearson omnibus normality test. Only when all data sets to be compared passed this normality test (α level = 0.05) were parametric statistical tests used. For data sets that were either not normally distributed or not large enough for a normality test, statistical significance (P < 0.05) was assessed with nonparametric tests. A Mann–Whitney test was used for comparisons between two groups, and a Kruskal–Wallis (nonparametric one-way analysis of variance) test followed by a Dunnett’s post hoc test was used for comparisons between three or more groups. Concentration-response curve data were fitted to the Hill equation. Error bars for plotted EC_{50} values indicate 95% confidence intervals.

Results

To study VTA DA neurons in adult mice (aged ≥60 days), we prepared coronal slices and recorded from VTA cells residing in the lateral aspect of the VTA. Although the VTA is emerging as a heterogeneous structure (Lammel et al., 2011), 96.3% of neurons in this area test positive for TH expression in adult C57 mice, and these cells exhibit I_h currents (Lammel et al., 2011). VTA neurons in this study typically fired spontaneous (Fig. 1A), wide action potentials (with a width of approximately 2–5 milliseconds; Fig. 1B). Hyperpolarizing current injections induced “sag” responses in the transmembrane voltage record (I = −120 pA; Fig. 1A), and these cells exhibited inward currents in response to hyperpolarizing voltage steps (I_h currents; Fig. 1C). To provide further confirmation that these neurons are DAergic, we conducted single-cell RT-PCR reactions from a subset of recorded neurons. All recorded neurons (n = 5) in lateral VTA (see Materials and Methods for coordinates) with a PCR signal for GAPDH were also positive for TH mRNA (Fig. 1D), and all of these TH(+) cells exhibited electrophysiological features as shown above (Fig. 1, A–C). On the basis of these results and supporting studies in the literature (Lammel et al., 2008, 2011; Zhang et al., 2010), we proceeded with reasonable confidence that cells with these characteristics, and in this lateral part of the VTA, were DAergic neurons.

First, we tested the hypothesis that activation of α6* nAChRs is sufficient to elicit inward currents in VTA DA neurons by recording from VTA DA neurons from adult α6L9’ and non-Tg littermate mice. Whole-cell voltage-clamp recordings from VTA DA neurons were established using a K+ gluconate-based internal recording solution, and an inhibitor cocktail containing CNQX (10 μM), picrotoxin (75 μM), and tetrodotoxin (0.5 μM) was bath-applied to the cell to eliminate most external influences on membrane potential. We measured inward currents in response to a 10-minute bath exposure to nicotine. We previously reported that brief (250 milliseconds) puff-application of 100 nM nicotine elicited small (approximately 10 pA) inward currents (Drenan et al., 2008a). We reasoned that sustained exposure of VTA DA neurons to 100 nM nicotine could be sufficient to provide prolonged activation of these cells. Nicotine (100 nM) elicited a significant inward current in α6L9’ VTA DA neurons (mean change in holding current value relative to prenicotine baseline = −18.0 ± 3.0 pA; Fig. 2, A and B). Coapplication of αCtxMII (100 nM) with nicotine (100 nM) eliminated these inward currents (mean change in holding current value relative to prenicotine baseline = −2.7 ± 4.1 pA; Mann–Whitney test, P < 0.05; Fig. 2, A, B, and E), suggesting that α6* nAChRs mediate inward currents in response to 100 nM nicotine. To determine whether responses to 100 nM nicotine were selective for α6* nAChRs in α6L9’ slices, 100 nM nicotine was applied to VTA DA neurons from non-Tg littermate slices. Nicotine (100 nM) only slightly increased inward currents in non-Tg littermate VTA DA neurons in this assay (mean change in holding current value relative to

![Figure 1](image.png)

**Fig. 1.** Electrophysiological identification of VTA DA neurons. (A) Whole-cell current-clamp recordings of VTA DA neurons show spontaneous (I = 0 pA), pacemaker firing (1–5 Hz), and “sag” responses in the membrane potential in response to hyperpolarizing (I = −120 pA) current injections. (B) VTA DA neurons have wide action potentials. The neuron in (A) indicated with an arrow is shown on an expanded time scale to better view the action potential width (typically 2–5 milliseconds) seen in the neurons under study. (C) I_h currents in VTA DA neurons. VTA cells were held at −60 mV in voltage-clamp mode and membrane current was recorded at baseline and during a voltage step to −120 mV. (D) Single-cell RT-PCR. VTA neurons recorded in whole-cell mode were aspirated into the recording pipette, followed by RT of RNA and subsequent PCR reactions to detect TH and GAPDH (positive control) expression. Expected band sizes are as follows: TH = 207 bp and GAPDH = 138 bp (the asterisk indicates a spurious PCR reaction, possibly generated from external primer pairs). As a negative control, a pipette was lowered into the slice and mild negative pressure was applied. The pipette was removed from the slice and assayed with RT-PCR as for a recorded cell.
The same concentration of nicotine significantly increased inward currents in a6L9S neurons (Mann–Whitney test, P < 0.05; Fig. 2C and E). CtxMII did not alter this response in non-Tg littermate cells (Fig. 2E). As a positive control, we applied 300 nM nicotine to non-Tg VTA DA neurons. This concentration was sufficient to moderately increase inward currents in these cells (mean change in holding current value relative to prenicotine baseline = −7.0 ± 1.0 pA; Fig. 2D and E), consistent with a previous report (Liu et al., 2012). CtxMII did not block these responses (mean change in holding current value relative to prenicotine baseline = −8.7 ± 2.2 pA; Fig. 2D and E).
D and E), presumably because responses in non-Tg cells are mediated by both α6* and non-α6* (α4β2) nAChRs. Together, these results demonstrate that selective activation of α6* nAChRs is sufficient to increase inward currents in VTA DA neurons. Application of 100 nM nicotine to α6L9′S slices was used in subsequent experiments to study the effects of selectively activating α6* nAChRs.

The initial exposure of brain cells to smoking-relevant concentrations of nicotine results in activation of high-sensitivity nAChRs, including those on VTA DA neurons (Calabresi et al., 1989). This exposure to nicotine leads to upregulation of AMPA receptor (AMPAR) function in these cells (Saal et al., 2003), which could support behavioral changes that lead to nicotine dependence. Because high-sensitivity nAChRs are expressed on VTA DA neurons, terminals from GABA neurons that synapse onto VTA DA neurons, and other glutamatergic fibers, it is not known whether activation of nAChRs specifically on VTA DA neurons can lead to increased AMPAR function. We previously demonstrated that α6* nAChRs are expressed only in DA neurons in VTA (Mackey et al., 2012). We hypothesized that selective activation of α6* nAChRs in VTA, which should stimulate DA neurons but not other VTA nAChRs (such as those on GABA or glutamatergic terminals) (Drenan et al., 2008a), is sufficient to enhance AMPAR function in these cells.

To measure AMPAR function on the cell surface, we applied AMPA to VTA DA neurons using a drug-filled pipette (Li et al., 2008; Kobayashi et al., 2009; Sanchez et al., 2010) that was positioned using a piezoelectric translator (Engle et al., 2012). A cell was voltage clamped and a stable recording was established. The drug-filled pipette remained stationary above/outside the slice until our recording software delivered an analog signal to the piezoelectric translator, triggering movement of the pipette to a predetermined position approximately 20–40 μm from the recorded cell. A digital transistor–transistor logic (TTL) pulse (5 V, 250-millisecond duration) activated the picospritzer, resulting in drug delivery to the recorded cell. After the TTL pulse, the piezoelectric translator withdrew the drug-filled pipette. This procedure is summarized in schematic form in Fig. 3A. Figure 3B shows a representative record of the movement of the piezoelectric translator, the TTL pulse, and a response to 100 μM AMPA in a VTA DA neuron.

To test the hypothesis that selective activation of α6* nAChRs is sufficient to enhance AMPAR function, we prepared coronal slices from α6L9′S mice and their non-Tg littermates. Slices were cut and allowed to recover for 60 minutes, followed by exposure of the slices to 100 nM nicotine (or a control solution containing no nicotine) for 60 minutes similar to previous studies (Jin et al., 2011; Mao et al., 2011) (Fig. 4A). After a washout period (>60 minutes), whole-cell recordings were established in VTA DA neurons using a Cs-methanesulfonate–based internal solution. AMPA currents were evoked at holding potentials of ~60, 0, and +40 mV. Whereas nicotine (100 nM) exposure did not alter AMPAR function in non-Tg VTA DA neurons, this treatment was sufficient to robustly increase AMPA-evoked currents in α6L9′S VTA DA neurons (Fig. 4B). Mean AMPA-evoked current amplitude was not altered by nicotine (100 nM) at ~60, 0, or +40 mV in non-Tg littermates (Fig. 4C). In contrast, there was a significant increase in AMPA-evoked current amplitude at ~60 and +40 mV in α6L9′S neurons (~60 mV: control = −173.5 ± 29.4 pA, 100 nM nicotine = −358.4 ± 48.5 pA; Mann–Whitney test, $P = 0.004$) (+40 mV: control = 82.2 ± 14.3 pA, and 100 nM nicotine = 167.1 ± 23.5 pA; Mann–Whitney test, $P = 0.0045$) (Fig. 4D). As a positive control, we incubated non-Tg slices in a higher concentration of nicotine (500 nM). This treatment led to a significant increase in AMPA-evoked currents at a holding potential of ~60 mV (control = −184.2 ± 18.3 pA and 500 nM nicotine = −283.4 ± 35.8 pA; unpaired t test, $P = 0.0487$) (Fig. 4A, B and C), consistent with previously published experiments with VTA DA neurons in slices (Jin et al., 2011).

We next sought to determine whether enhanced AMPA-evoked currents in α6L9′S slices treated with nicotine (100 nM) were due to a change in the efficacy versus the potency of AMPA. First, we constructed an AMPA concentration-response curve to confirm that changes in AMPA-evoked currents between non-Tg and α6L9′S slices were not due to differences in initial AMPAR sensitivity. Multiple concentrations of AMPA

![Fig. 3. AMPA-evoked current methodology. (A) A drug-filled pipette is positioned above/next to the cell being recorded. A piezoelectric translator brings the pipette close (20–40 μm) to the cell, a TTL pulse triggers a pressure ejection that dispenses drug (AMPA) onto the cell, and the piezoelectric translator withdraws the pipette away from the cell. (B) Representative recording showing the timing of the TTL pulse, piezo drive movement, and resulting inward current elicited by application of 100 μM AMPA to a VTA DA neuron.]
Fig. 4. Activation of α6* nAChRs is sufficient to enhance AMPAR function on the surface of VTA DA neurons. (A) Slices treatment procedure. Brain slices from adult α6L9'S and non-Tg littermate mice were cut, recovered for 60 minutes, and incubated for 60 minutes in control recording solution or recording solution plus nicotine (100 nM). Nicotine was washed out for 60 minutes, and whole-cell recordings were established in VTA DA neurons. (B) AMPA currents were evoked by puff-application of AMPA (100 μM) at holding potentials of −60, 0, and +40 mV. Representative recordings from incubation of slices in control and nicotine solutions are shown for α6L9'S and non-Tg littermate mice. (C and D) Summary showing mean AMPA-evoked currents ([AMPA] = 100 μM) in non-Tg littermate (C) and α6L9'S (D) VTA DA neurons in response to control incubation or nicotine incubation at the indicated concentration. The numbers of observations were as follows: non-Tg control (−60 mV, n = 10; 0 mV, n = 7; +40 mV, n = 7); non-Tg 100 nM nicotine (−60 mV, n = 4; 0 mV, n = 4; +40 mV, n = 4), non-Tg 500 nM nicotine (−60 mV, n = 16; 0 mV, n = 12; +40 mV, n = 12), α6L9'S control (−60 mV, n = 14; 0 mV, n = 13; +40 mV, n = 13), and α6L9'S 100 nM nicotine (−60 mV, n = 11; 0 mV, n = 11; +40 mV, n = 11). (E) AMPA concentration-response curve in VTA DA neurons. AMPA-evoked currents were measured in non-Tg and α6L9'S neurons. AMPA concentrations and number of observations at each data point are as follows: non-Tg (1 μM, n = 2; 10 μM, n = 6; 50 μM, n = 5; 100 μM, n = 10; 250 μM, n = 5; 500 μM, n = 14; 1000 μM, n = 11), and α6L9'S (1 μM, n = 2; 10 μM, n = 4; 50 μM, n = 4; 100 μM, n = 14; 250 μM, n = 5; 500 μM, n = 4; 1000 μM, n = 5; 3000 μM, n = 2). Data (mean ± S.E.M.) were fitted to the Hill equation, and the EC50 (± 95% confidence interval) for each curve is plotted. (F) AMPA concentration-response curve in α6L9'S VTA DA neurons. AMPA-evoked currents were measured in α6L9'S control slices or slices incubated in 100 nM nicotine for 60 minutes followed by >60 minutes washout prior to recording. Control treated α6L9'S data from (E) are replotted here for reference. AMPA concentrations and number of observations at each data point for α6L9'S slices treated with nicotine are as follows: α6L9'S (1 μM, n = 2; 10 μM, n = 3; 50 μM, n = 2; 100 μM, n = 11; 300 μM, n = 3; 1000 μM, n = 3). Data (mean ± S.E.M.) were fitted to the Hill equation and the EC50 (± 95% confidence interval) for each curve is plotted. *P < 0.05; **P < 0.01.
were applied to α6L9’S and non-Tg neurons, and the data were fitted to the Hill equation (non-Tg: $R^2 = 0.9467$; α6L9’S: $R^2 = 0.9819$). There was no substantial difference in AMPA EC$_{50}$ in α6L9’S VTA DA neurons compared with non-Tg neurons (EC$_{50}$ = 174 μM for non-Tg, and EC$_{50}$ = 182 μM for α6L9’S; Fig. 4E). Figure 4E plots these EC$_{50}$ values along with their respective 95% confidence intervals. Similarly, we constructed a concentration-response curve for AMPA-evoked currents in α6L9’S slices exposed to nicotine. AMPA at a range of concentrations was applied to cells in slices exposed to nicotine, and the data were fitted to the Hill equation (α6L9’S nicotine: $R^2 = 0.9942$). The EC$_{50}$ for AMPA-evoked currents in nicotine-exposed α6L9’S slices was shifted to the left compared with α6L9’S slices not exposed to nicotine (EC$_{50}$ = 37 μM; Fig. 4F), suggesting an increase in the sensitivity of AMPARs to AMPA.

Next, we studied the time dependence for enhancement of AMPAR function in VTA DA neurons. As with previous experiments, slices were cut and allowed to recover for 60 minutes. We then compared AMPA-evoked current amplitudes from neurons treated in four different ways: 1) incubated for 60 minutes in a control solution without nicotine followed by a washout period of 60–240 minutes prior to recording, 2) incubated for 60 minutes in nicotine (100 nM) followed by a washout period of 60–240 minutes prior to recording, 3) incubated for 10 minutes in nicotine (100 nM) followed by a washout period of 60–240 minutes prior to recording, and 4) incubated for 60 minutes in nicotine (100 nM) followed by a washout period of greater than 240 minutes prior to recording (Fig. 5A). Exposure of α6L9’S slices to 100 nM nicotine for 10 minutes was insufficient to augment AMPA-evoked currents above control levels (control incubation/washout 60–240 minutes = $-173.5 \pm 29.4$ pA; 10-minute nicotine incubation/washout 60–240 minutes = $-213.5 \pm 27.9$ pA; Fig. 5, B and C). However, a 60-minute exposure to nicotine was sufficient to augment AMPA-evoked currents over control (60-minute nicotine incubation/washout 60–240 minutes = $-351.1 \pm 64.9$ pA; Kruskal–Wallis test, $P < 0.05$; Fig. 5, B and C). The effect of a 60-minute nicotine exposure was prolonged, as AMPA-evoked currents were still enhanced after a washout period of >240 minutes (60-minute nicotine incubation/washout 60–240 minutes = $-411.4 \pm 75.5$ pA; Kruskal–Wallis test, $P < 0.05$; Fig. 5, B and C).

**Fig. 5.** Time dependence for enhancement of AMPA-evoked currents in α6L9’S VTA DA neurons. (A) Slice treatment procedure. Brain slices from α6L9’S mice were cut and recovered for 60 minutes. Slices were then incubated in nicotine (100 nM) for either 10 or 60 minutes, followed in either case by a washout period of ≥60 minutes. Some slices treated with nicotine for 60 minutes were allowed >240 minutes of washout prior to recording. (B) Representative AMPA-evoked currents ([AMPA] = 100 μM) at +40 and −60 mV in VTA DA neurons in response to treatment detailed in (A). (C) Summary showing mean ± S.E.M. AMPA-evoked (f[AMPA] = 100 μM) current in α6L9’S VTA DA neurons in response to the conditions described in (A). *$P < 0.05$. 
To better understand the mechanism within VTA DA neurons that leads to enhanced AMPA-evoked currents, we pretreated α6L9′s slices for 10 minutes with several pharmacological agents prior to 60 minutes nicotine (100 nM) exposure, washout, and subsequent AMPA-evoked current measurements (Fig. 6A). Pretreatment of slices with αCtxMII eliminated the enhanced AMPA-evoked currents seen in α6L9′s slices exposed to a control pretreatment prior to nicotine exposure (control = −173.5 ± 29.4 pA, nicotine = −358.4 ± 48.5 pA, and MII = −221.5 ± 45.8 pA; Kruskal–Wallis test, P < 0.05; Fig. 6, B and C). Similarly, blockade of NMDA receptors with AP-5 (10 μM) prior to nicotine treatment eliminated enhanced AMPA-evoked currents (AP-5 = −194.4 ± 32.9 pA; Fig. 6, B and C). Previous studies indicate that DA D1/D5 receptors in VTA may play a role in altered synaptic plasticity after exposure to drugs of abuse (Gao and Wolf, 2007; Mao et al., 2011). Blockade of DA D1/D5 receptors with SCH23390 (10 μM) prior to nicotine exposure (control = −280.3 ± 45.3 pA; Kruskal–Wallis test, P < 0.05 for SCH23390-treated slices and untreated control versus nicotine and MLA pretreatment; Fig. 7, B and C). To determine whether these results were due to reduced α4 nAChR subunits while still retaining gain-of-function α6 subunits (Drenan et al., 2010). Slice treatment in this experiment (Fig. 7A) was identical to experiments reported in Fig. 4. Whereas nicotine (100 nM) treatment of α6L9′s slices leads to enhanced AMPAR function, identical treatment of slices from α6L9′s mice lacking α4 subunits did not increase AMPA-evoked currents (α6L9′: control = −173.5 ± 29.4 pA, nicotine = −358.4 ± 48.5 pA; α6L9′α4KO: control = −205.9 ± 23.3 pA, nicotine = −280.3 ± 45.3 pA; Kruskal–Wallis test, P > 0.05 for α6L9′ control versus nicotine and P < 0.05 for α6L9′α4KO control versus nicotine; Fig. 7, B and C). To determine whether these results were due to reduced α6

**Fig. 6.** Pharmacology of AMPA-evoked current induction in α6L9′ VTA DA neurons. (A) Slice treatment procedure. α6L9′ brain slices were cut and recovered for 60 minutes. Slices were pre-treated for 10 minutes with one of the drugs indicated in B, followed by treatment with the drug plus nicotine (100 nM) for 60 minutes. Slices were washed out for >60 minutes prior to recording. (B) Representative AMPA-evoked currents ([AMPA] = 100 μM) at +40 and −60 mV in VTA DA neurons from α6L9′ brain slices pre-exposed for 10 minutes to either control recording solution or the following drugs followed by incubation in 100 nM nicotine for 60 minutes: αCtxMII (MII), SCH23390 (SCH), AP-5, and MLA. (C) Summary showing mean ± S.E.M. AMPA-evoked currents ([AMPA] = 100 μM) in α6L9′ VTA DA neurons in response to the conditions described in (A). *P < 0.05; **P < 0.01.
expression and/or function, we performed a series of controls using α4KO animals. First, we crossed α4KO mice with transgenic mice expressing α6 subunits fused with GFP (Fig. 8A). This manipulation results in the production of only non-α4α6β2* nAChRs (Fig. 8B). We used anti-GFP immunohistochemistry and confocal microscopy, as previously described in these mice (Mackey et al., 2012), to quantify α6* nAChR expression in VTA neurons in α6GFP mice and α6GFP mice crossed to α4KO mice. We found a small but significant reduction in α6GFP expression in VTA neurons in α6GFP mice lacking α4 subunits compared with α6GFP with intact α4 nAChR subunit expression (α4WT = 17,921 ± 698 arbitrary units, α4KO = 14,507 ± 816 arbitrary units; Mann–Whitney test, P = 0.0011; Fig. 8C). Next, we measured α6* nAChR function directly by comparing nicotine- and ACh-evoked currents in α6L9'S mice and α6L9'S mice lacking α4 subunits (Fig. 8D). In contrast to ACh-evoked responses in nicotine compared with VTA DA neurons lacking αδ test, function directly by comparing nicotine- and ACh-evoked currents, α6L9'S VTA DA neurons that express α4 subunits, responses from VTA DA neurons (Fig. 6), activation of nAChRs does not significantly alter NMDA function after 60 minutes of exposure to nicotine. These results suggest that although NMDA activation is required for upregulation of AMPAR function on VTA DA neurons (Fig. 6), activation of nAChRs does not significantly alter NMDA function after 60 minutes of exposure to nicotine.

Discussion

Our recordings in isolated brain slices demonstrate that selective activation of α6β2* nAChRs by nicotine is sufficient
to increase slow inward currents in VTA DA neurons (Fig. 2) and enhance the function of AMPARs (Fig. 4). Our finding that greater than 10 minutes of exposure to nicotine is required to enhance AMPAR function (Fig. 5) suggests that multiple signal transduction events and/or ionic conductances are involved. Whereas $\alpha 7$ nAChR activation is not required (Fig. 6), NMDA receptor activation is necessary for $\alpha 6\beta 2^*$-mediated enhanced AMPAR function (Fig. 6). Interestingly, $\alpha 6\beta 2^*$-mediated AMPAR enhancement requires midbrain $\alpha 4$ nAChR subunits (Fig. 7), suggesting that pentamers containing both $\alpha 4$ and $\alpha 6$ subunits are responsible. These data, together with previous findings showing that $\alpha 6\beta 2^*$ nAChRs are selectively expressed in DA neurons within the VTA (Mackey et al., 2012), suggest that nicotine can act exclusively in a postsynaptic manner on VTA DA neurons to sensitize these cells to excitatory input.

**Fig. 8.** $\alpha 6^*$ nAChR function is reduced in $\alpha 4$KO mice. (A) Schematic of $\alpha 6$GFP transgenic mice and $\alpha 6$GFP nAChRs. (B) The resulting $\alpha 6^*$ nAChR that remains after crossing $\alpha 6$GFP mice to $\alpha 4$KO mice is shown. (C) $\alpha 6^*$ nAChRs were quantified in $\alpha 6$GFP and $\alpha 6$GFP/$\alpha 4$KO VTA DA neurons using anti-GFP immunohistochemistry and confocal microscopy. Mean per-cell pixel intensity for each genotype is shown. (D) The resulting $\alpha 6^*$ nAChR that remains after crossing $\alpha 6$L9*S mice to $\alpha 4$KO mice is shown. (E) Representative ACh-evoked currents in $\alpha 6$L9*S and $\alpha 6$L9*S/$\alpha 4$KO VTA DA neurons in response to the indicated concentration of ACh. (G) Representative nicotine-evoked currents in $\alpha 6$L9*S and $\alpha 6$L9*S/$\alpha 4$KO VTA DA neurons. VTA DA neurons from both genotypes were patch clamped and nicotine was puff-applied at the indicated concentration. **P \leq 0.01.
VTA DA Neuron Activation by \( \alpha 6 \beta 2^* \) nAChRs. Understanding which nAChR subtypes are necessary and sufficient to mediate nicotine’s complex action on VTA neurons is a challenge (Drenan and Lester, 2012), and our data provide new information. We show that nicotine-elicited activation of somatodendritic \( \alpha 6 \beta 2^* \) nAChRs in VTA DA neurons is sufficient to stimulate an inward conductance that could, under physiologic conditions, support prolonged depolarization of these cells (Fig. 2). \( \beta 2^* \) nAChRs are absolutely required for nicotine-induced increases in VTA DA neuron firing (Picciotto et al., 1998; Maskos et al., 2005), and Tapper and colleagues recently reported that activation of \( \alpha 4 \beta 2^* \) nAChRs in VTA DA neurons by smoking-relevant concentrations of nicotine can support depolarization and action potential firing (Liu et al., 2012). These actions were sensitive to a \( \alpha 6 \beta 2^* \) nAChR antagonist, implicating \( \alpha 4 \alpha 6 \beta 2^* \) nAChRs. This report is consistent with our study, which suggests that \( \alpha 6 \beta 2^* \) nAChR activation can increase inward currents in VTA DA neurons (Mansvelder et al., 2002; Nashmi et al., 2007). Because \( \alpha 6 \) nAChRs are restricted to DAergic cells in VTA (Mackey et al., 2012), our results suggest that direct action by nicotine on somatodendritic \( \alpha 6^* \) nAChRs may be sufficient to depolarize these cells. In human brain, there may be redundant mechanisms in the VTA that allow nicotine to activate the mesolimbic DA system. Although our previous work indicates no evidence for overexpression of \( \alpha 6 \beta 2^* \) nAChRs (Drenan et al., 2010), the TM2 pore-lining mutation used to sensitize these receptors may alter their pharmacological properties (Revah et al., 1991; Labarca et al., 1995). Future studies using restricted expression of \( \alpha 4 \alpha 6 \beta 2^* \) nAChRs via concatamers (Kuryatov and Lindstrom, 2011) will be useful in exploring the latter possibility, whereas development of \( \alpha 6 \beta 2^* \)-selective ligands will be useful in addressing the importance of the former possibility.

Nicotine-Induced Changes in AMPAR Function. To our knowledge, this study is the first to implicate \( \alpha 6 \beta 2^* \) nAChRs in nicotine-induced changes in AMPAR function in VTA DA neurons. A single exposure to nicotine or other drugs of abuse enhances AMPAR-mediated EPSCs in VTA DA neurons (Saal et al., 2003), which strongly suggests LTP of excitatory inputs to these cells (Mansvelder and McGeehee, 2000; Ungless et al., 2001; Luscher and Malenka, 2011). Subsequent studies addressing which nAChR subtypes mediate this effect are not completely consistent. In slice experiments, McGeehee and colleagues report that \( \beta 2^* \) nAChRs

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**Fig. 9.** NMDA-evoked currents are not changed by nicotine in \( \alpha 6 \beta 9^\prime S \) VTA DA neurons. (A) Slice treatment procedure. Brain slices from adult \( \alpha 6 \beta 9^\prime S \) and non-Tg littermate mice were cut, recovered for 60 minutes, and incubated for 60 minutes in control recording solution or recording solution plus nicotine (100 nM). Nicotine was washed out for \( \geq 60 \) minutes, and whole-cell recordings were established in VTA DA neurons. (B) Representative NMDA-evoked currents (NMDA) = 100 \( \mu \)M) at +40 mV in VTA DA neurons from \( \alpha 6 \beta 9^\prime S \) and non-Tg littermate brain slices in response to control incubation or incubation in 100 nM nicotine for 60 minutes. (C) Summary showing mean \( \pm \) S.E.M. NMDA-evoked currents (NMDA) = 100 \( \mu \)M) in \( \alpha 6 \beta 9^\prime S \) and non-Tg littermate VTA DA neurons in response to the conditions described in A.
(but not α7 nAChRs) are necessary for increased AMPAR function in synapses after nicotine exposure (Mao et al., 2011), whereas in studies with animals injected with nicotine prior to slice preparation, Wu and colleagues suggest that nicotine-elicited increases in AMPAR function can proceed either through β2* or α7 nAChRs (Gao et al., 2010; Jin et al., 2011). Our results using naïve or nicotine-exposed slices from adult non-Tg or α6L9/S mice are more consistent with the former, because we find no necessary role for α7 nAChRs in AMPAR functional enhancement (Fig. 6). As in any comparison between scientific studies, differences in experimental details may account for disparate results. Similar to previous approaches (Ungless et al., 2001; Kobayashi et al., 2009; Sanchez et al., 2010), our experiments used direct application of AMPA to VTA cell bodies. Thus, our results likely include a contribution from nonsynaptic AMPAR pools on the plasma membrane of VTA DA neurons. However, the fact that incubating non-Tg slices in 500 nM nicotine led to a significant increase in whole-cell AMPA-evoked currents gave us confidence that we are studying a similar increase in AMPAR function compared with the phenomenon seen in other reports that used electrically evoked EPSCs as an endpoint. Future studies probing LTP in α6L9/S neurons will address the relative role of synaptic versus nonsynaptic AMPAR pools in the response to nicotine.

In VTA DA neurons, changes in both AMPAR distribution and/or composition are proposed to occur after exposure to nicotine and other drugs of abuse. Several reports suggest that drug exposure (including nicotine) leads to signal transduction events that promote exchange of Ca2+-impermeable AMPARs containing AMPA-type ionotropic glutamate receptor (GluR) 2 subunits for high-conductance, Ca2+-permeable AMPARs lacking GluR2 subunits (Bellone and Lüscher, 2006; Lüscher and Malenka, 2011). This GluR2-lacking receptor pool typically displays inward rectification (Isaac et al., 2007; Liu and Zukin, 2007), and one study confirms the appearance of this type of AMPAR after a single exposure to nicotine (Gao et al., 2010). Another study on nicotine (Baker et al., 2013) exposure to VTA DA neurons, however, demonstrated increases in AMPA/NMDA ratios but no appearance of an AMPAR pool displaying inward rectification. We find no appearance of inward rectification in AMPA-evoked currents (Fig. 4, B and D), which is more consistent with enhancement in numbers of GluR2-containing AMPARs rather than production of a significant amount of GluR2-lacking AMPARs. However, our data showing an increase in AMPAR sensitivity in response to α6β2* activation (Fig. 4F) support a number of possible mechanisms, including increased AMPAR conductance—a hallmark of GluR2-lacking AMPARs. Future pharmacological studies in α6L9/S and WT slices exposed to nicotine are needed to characterize AMPAR sensitivity changes.

What circuit and/or molecular signal transduction events after nicotine exposure are necessary and/or sufficient to enhance AMPAR function in VTA DA neurons? At the circuit level, an approach utilizing optogenetics demonstrated conclusively that in vivo activation of VTA DA neurons was sufficient to promote AMPAR redistribution (Brown et al., 2010). Because α6β2* nAChRs are selectively expressed in DA neurons in VTA (Drenan et al., 2008a; Mackey et al., 2012), our results lead us to favor a similar conclusion for nicotine: activation of α6β2* nAChRs on VTA DA neurons is sufficient to promote enhanced AMPAR function. Two other molecular events have been shown to be important for induction of synaptic plasticity in VTA DA neurons: D1/D5 DA receptor activation (Schilstöm et al., 2006; Brown et al., 2010; Mao et al., 2011), and NMDA receptor activation (Ungless et al., 2001; Saal et al., 2003). Although our SCH23390 results are inconclusive, NMDA receptor activation is necessary for α6β2* nAChR-mediated increases in AMPAR function (Fig. 6C). Together with previous studies on nicotine and other drugs of abuse, our data studying α6β2* nAChRs support the contention that there may be multiple mechanisms in place that nicotine can use to enhance the responsiveness of VTA DA neurons, ultimately leading to a heightened behavioral response to nicotine.

**Future Studies.** Our data show for the first time that activation of α6αβ2* nAChRs by nicotine is sufficient to stimulate a depolarizing conductance in VTA DA neurons as well as enhance AMPAR function on the cell surface. Future studies should include determining the contribution of synaptic versus extrasynaptic AMPARs, as well as studying whether acute exposure of intact animals to α6β2*-specific concentrations of nicotine is sufficient to drive changes in AMPAR function. Most importantly, it will be very important to report whether selective activation of α6β2* nAChRs is sufficient to support nicotine reward and/or reinforcement, and whether AMPAR activation plays a role in such behaviors. Such studies are ongoing. Together, our data show that α6αβ2* nAChRs are emerging as a key target for smoking cessation pharmacotherapy.

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**Authorship Contributions**

**Participated in research design:** Engle, Shih, Drenan.

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**Contributed new reagents or analytic tools:** McIntosh.

**Performed data analysis:** Engle, Shih, Drenan.

**Wrote or contributed to the writing of the manuscript:** Engle, McIntosh, Drenan.

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