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

Staci E. Engle, Pei-Yu Shih, J. Michael McIntosh, and Ryan M. Drenan

Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana (S.E.E., P.Y.S., R.M.D.); George E. Wahlen Veterans Affairs Medical Center, Salt Lake City, Utah (J.M.M.); and Departments of Psychiatry and Biology, University of Utah, Salt Lake City, Utah (J.M.M.)

Received May 13, 2013; accepted June 20, 2013

**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 α6 nAChRs (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—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 (Pilditchko 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 (Ungless et al., 2001; Saal et al., 2003). In particular, nicotine acts through VTA nAChRs on DA neuron somata, as well as presynaptic nAChRs, to depolarize...
these cells, facilitate N-methyl-D-aspartic acid (NMDA) receptor activation, and enhance glutamate-induced excitatory postsynaptic currents (EPSCs) (Saal et al., 2003; Gao et al., 2010; Jin et al., 2011; Mao et al., 2011). Understanding which proteins—including which nAChR subtypesmediate 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 β subunits (β2 and/or β4) and two or more α subunits (α2–α6) (Itié and Bertrand, 2001). “Auxiliary” subunits α5 or β3 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). α4β2* (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 α7 nAChRs expressed on glutamatergic axon terminals that synapse onto VTA neurons can enhance glutamatergic excitation of VTA neurons (Mansvelder and McGeehe, 2000), thereby potentiating nicotine’s direct action at α4β2* nAChRs on the soma of these cells (Mansvelder et al., 2002).

Interest in nAChRs containing α6 subunits is strong due to their high sensitivity to nicotine (Salminen et al., 2007), and their selective expression in DA and noradrenergic-producing cells (Le Noèvre et al., 1996; Léna et al., 1999; Champtiaux et al., 2002; Mackey et al., 2012). α6* nAChRs require β2 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 α4β2* nAChRs in VTA DA neurons induces prolonged depolarization of these cells, an effect that was sensitive to an α6* nAChR antagonist (Liu et al., 2012). Using a similar antagonist, Wu and colleagues reported that GABAAergic transmission onto VTA DA neurons may be mediated by α6* nAChRs (Yang et al., 2011). These approaches relied on pharmacological blockade to discern the role of α6* nAChRs, and the results indicate that more experiments are needed to better understand α6* nAChRs in the VTA.

In the present study, we studied transgenic mice expressing α6 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 activate α6* nAChRs but do not activate (non-α6*) nAChRs, we tested the hypothesis that α6* nAChR activation in VTA DA neurons is sufficient to elicit slow inward currents, and to enhance 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptor function. Furthermore, we also assessed whether α4 nAChR subunits were permissive in any or all of these measurements.

Materials and Methods

Mice. All experiments were conducted in accordance with the guidelines for the care and use of animals provided by the National Institutes of Health Office of Laboratory Animal Welfare, and protocols were approved by the Institutional Animal Care and Use Committee at Purdue University. Mice were kept on a standard 12-hour light/dark cycle at 22°C and given food and water ad libitum. On postnatal day 21, mice were weaned and housed with same-sex littermates. Tail biopsies were taken for genotype analysis by polymerase chain reaction (PCR) as previously described (Drenan et al., 2010).

α6L9/S mice were generated as described (Drenan et al., 2008a). Briefly, a mouse bacterial artificial chromosome (BAC) containing the Chnαδ gene was obtained and an L9/S mutation was introduced by codon replacement using a BAC recombinase approach. Mutant BAC DNA was introduced into FVB/N embryos, which were then implanted into pseudogene Swiss-Webster surrogates. The BAC insertion site in the mouse genome is unknown. Founder animals were isolated and have been continuously back-crossed to C57BL/6 for >12 generations. Over 90% of the α6L9/S strain genome is expected to contain C57BL/6 alleles, but FVB/N allelic DNA close to the insertion site is likely to remain in place in this strain. α6L9/S mice are thus transgenic and express mutant (L9/S) and wild-type (WT) α6 nAChR subunits (Cohen et al., 2012). α6* nAChR function is sensitized in these mice, producing a 10- to 100-fold leftward shift in concentration-response relationships involving α6* nAChRs, depending on the assay being used (Drenan et al., 2008a, 2010; Cohen et al., 2012). We previously confirmed that α6* nAChRs in α6L9/S mice are not overexpressed or misexpressed in ectopic brain locations (Drenan et al., 2008a, 2010).

α6L9/S mice lacking α4 nAChR subunits were generated as previously described (Drenan et al., 2010). α4 Knockout (KO) mice were a generous gift of Dr. Michael Marks (University of Colorado, Boulder, CO), and were produced by mating mice heterozygous for the α4KO allele. Briefly, α6L9/S mice, in which the mutant allele is maintained in a heterozygous fashion, were crossed to homozygous α4KO mice to produce mice that are heterozygous for both the α6L9/S allele and the α4KO allele. These mice were subsequently crossed to homozygous α4KO mice to produce mice heterozygous for the α6L9/S allele and homozygous for the α4KO allele. α6 green fluorescent protein (GFP) mice were generated as previously described (Mackey et al., 2012). To create α6GFP mice lacking α4 subunits, α4KO mice were crossed α6GFP mice, generating α6GFP mice heterozygous for the α4KO allele. These mice were crossed again to mice homozygous for the α4KO allele, yielding α6GFP mice that were also homozygous for the α4KO allele. All groups of mice in this study contained approximately equal numbers of male and female mice.

Materials. All chemicals were from Sigma-Aldrich (St. Louis, MO). Sigma-Aldrich was also the source for the following compounds: atropine sulfate, (−)-nicotine tartrate, acetylcholine (ACh) HCl, AMPA, NMDA, methyllycaconitine A (MLA), 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol (SCH23390), and picrotoxin. Tocris Biosciences (Ellisville, MO) was the source of the following compounds: 3,4-dihydroxy-6-(2-hydroxy-4-nitrophenyl) benzonic acid (a-Conotoxin MII) (CtxMII) was synthesized by previously described methods (Azam et al., 2010).

Brain Slice Preparation for Electrophysiology. Brain slices were prepared as previously described (Engle et al., 2012). α6L9/S and nontransgenic (non-Tg) mice were genotyped at 21–28 days after birth. Mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.) followed by cardiac perfusion with oxygenated (95% O2/5% CO2), 4°C 2-methyl-5-norvaleraldehyde (NMDG) recovery solution containing the following: 93 mM NMDG, 2.5 mM KCl, 1.2 mM NaHPO4, 30 mM NaHCO3, 20 mM HEPES, 25 mM glucose, 3 mM Na+ ascorbate, 2 mM thiourea, 3 mM Na+ pyruvate, 10 mM MgSO4•7H2O, and 0.5 mM CaCl2•2H2O (300–310 mOsm, pH 7.3–7.4). Brains were removed and retained in 4°C NMDG recovery solution for 1 minute. Coronal slices (200 μm) were cut with a microslicer (DTK-Zero 1; Ted Pella, Redding, CA). Brain slices recovered for 12 minutes at 33°C in oxygenated NMDG recovery solution, after which they were held until recording in HEPES holding solution containing the following: 92 mM NaCl, 2.5 mM KCl,
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 98% 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-6D 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, osmolality adjusted to 290 mOsM with sucrose). Two internal pipette solutions were used. The following solution was used when recording nicotine- or ACh-evoked 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 mM GTP. The following solution was used when recording AMPA- or NMDA-evoked currents: 117 mM CaCl₂·2H₂O, 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 Digidata 1440A A/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 nulled immediately prior to gigaseal formation. Series resistance was uncompensated.

DA neurons in VTA were identified according to previously published methods (Wooltorton 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 a Picospritzer III (General Valve, Fairfield, NJ) as previously described (Engle et al., 2012). Between drug applications, the drug-filled pipette was maintained ~40 μm from the cell, and we subsequently moved the position of the drug-filled pipette closer to the cell to achieve a more rapid response. Responses to AMPA were deemed acceptable based on two criteria: 1) the pressure application 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 seal 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 (CATGTAGTGGCAAGGCAAGC), TH_R2 (GAAGAGUGCT-GGGAACCTT), GAPDH_F2 (AATTTGGCATTGGAAGG), and GAPDH_R2 (CCCTGTTGCTGTAGGCGTAT). 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 (CTG GTG GTG GTG ACC CTA CTG), GAPDH_F1 (GTG TCC CTA CCT CCA ATG TG), and GAPDH_R1 (GGT CCG 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 α6nAChR subunits fused in-frame with GFP (α6GFp mice; n = 3), along with α6GFp 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) 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 (A11122; 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 (A11008; 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 Quinacrine 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× air and 60× 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
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 Dunn’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\textsubscript{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’s 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 \( \mu \)M), picrotoxin (75 \( \mu \)M), and tetrodotoxin (0.5 \( \mu \)M) 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’s VTA DA neurons (mean change in holding current value relative to prenicotine baseline = \(-18.0 \pm 3.0 \) 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 = \(-2.7 \pm 4.1 \) 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’s 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

---

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.
A low concentration of nicotine is sufficient to increase inward currents in VTA DA neurons. (A) α6L9'S or non-Tg control neurons were voltage clamped in whole-cell mode. Inhibitor cocktail [10 μM CNQX, 75 μM picrotoxin, 0.5 μM tetrodotoxin (TTX)] was superfused, followed by nicotine (100 nM) and then αCtxMII (100 nM). A representative experiment from a α6L9'S neuron is shown. Expanded recordings from time points (i), (ii), and (iii) are shown in B–D. (B–D) Voltage-clamp recording segments from α6L9'S (B; 100 nM nicotine), non-Tg (C; 100 nM nicotine), and non-Tg (D; 300 nM nicotine) VTA DA neurons at (i) baseline with inhibitor cocktail present, (ii) inhibitor cocktail plus nicotine, and (iii) inhibitor cocktail/nicotine plus αCtxMII. (E) Summary showing mean holding current (pA) change from baseline in response to nicotine, and nicotine plus αCtxMII in the indicated mouse strain (α6L9'S and non-Tg littermate). *P < 0.05.
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., 2008; Kobayashi et al., 2009; Sanchez et al., 2010) and could be activated by a TTL pulse (5 V, 250-millisecond duration) that triggered a response to 100 nM nicotine (100 nM) exposed to 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 = −14.3 ± 18.3 pA; 100 nM nicotine = −283.4 ± 35.8 pA; Mann–Whitney test, P = 0.0487) (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 = −298.4 ± 35.8 pA; unpaired t test, P = 0.0087) (Fig. 4, B and C), consistent with previous 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 were applied to each slice, and the resulting inward currents elicited by AMPA were measured. The data were analyzed using a pharmacokinetic model to determine the potency and efficacy of AMPA-evoked currents.

**Fig. 3.** AMPA-evoked current methodology. (A) A drug-filled pipette is positioned above/next to the cell being recorded. A piezoelectric translator allows the pipette to be moved away from the cell during recording. (B) Representative recording showing the movement of the piezoelectric translator, the TTL pulse, and a response to 100 μM AMPA in a VTA DA neuron.
Fig. 4. Activation of $\alpha6^\* \ \text{nAChR}$ is sufficient to enhance AMPAR function on the surface of VTA DA neurons. (A) Slice treatment procedure. Brain slices from adult $\alpha6L9^\*$ 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 $\mu$M) at holding potentials of −60, 0, and +40 mV. Representative recordings from incubation of slices in control and nicotine solutions are shown for $\alpha6L9^\*$ and non-Tg littermate mice. (C and D) Summary showing mean AMPA-evoked currents ([AMPA] = 100 $\mu$M) in non-Tg littermate (C) and $\alpha6L9^\*$ (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), $\alpha6L9^\*$ control (−60 mV, n = 14; 0 mV, n = 13; +40 mV, n = 13), and $\alpha6L9^\*$ 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 $\alpha6L9^\*$ neurons. AMPA concentrations and number of observations at each data point are as follows: non-Tg (1 $\mu$M, n = 2; 10 $\mu$M, n = 6; 50 $\mu$M, n = 5; 100 $\mu$M, n = 10; 250 $\mu$M, n = 5; 500 $\mu$M, n = 14; 1000 $\mu$M, n = 11), and $\alpha6L9^\*$ (1 $\mu$M, n = 2; 10 $\mu$M, n = 4; 50 $\mu$M, n = 4; 100 $\mu$M, n = 14; 250 $\mu$M, n = 5; 500 $\mu$M, n = 4; 1000 $\mu$M, n = 5; 3000 $\mu$M, n = 2). Data (mean ± S.E.M.) were fitted to the Hill equation, and the EC$_{50}$ ($\pm$ 95% confidence interval) for each curve is plotted. (F) AMPA concentration-response curve in $\alpha6L9^\*$ VTA DA neurons. AMPA-evoked currents were measured in $\alpha6L9^\*$ control slices or slices incubated in 100 nM nicotine for 60 minutes followed by 60 minutes washout prior to recording. Control treated $\alpha6L9^\*$ data from (E) are replotted here for reference. AMPA concentrations and number of observations at each data point for $\alpha6L9^\*$ slices treated with nicotine are as follows: $\alpha6L9^\*$ (1 $\mu$M, n = 2; 10 $\mu$M, n = 3; 50 $\mu$M, n = 2; 100 $\mu$M, n = 11; 300 $\mu$M, n = 3; 1000 $\mu$M, n = 3). Data (mean ± S.E.M.) were fitted to the Hill equation and the EC$_{50}$ ($\pm$ 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 ($R^2 = 0.9942$). 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.](image-url)
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 MLA (10 μM) prior to nicotine treatment eliminated enhanced AMPA-evoked currents (MLA = −479.7 ± 119.8 pA; Kruskal–Wallis test, P < 0.05; Fig. 6, B and C).

Elimination of α4 nAChR subunits via gene knockout has been shown to significantly reduce α6* nAChR function in synaptosomal DA release experiments (Salminen et al., 2004, 2007; Drenan et al., 2010), direct assays of striatal nAChR function in brain slices (Drenan et al., 2010), and in behavioral experiments (Drenan et al., 2010). To test the hypothesis that α4 nAChR subunits are important for α6* nAChR-mediated enhancement of AMPAR function in VTA DA neurons, we crossed α6L9’S mice with α4KO animals to eliminate 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’S: control = −173.5 ± 29.4 pA, nicotine = −358.4 ± 48.5 pA; α6L9’Sα4KO: control = −205.9 ± 23.3 pA, nicotine = −280.3 ± 45.3 pA; Kruskal–Wallis test, P < 0.05 for α6L9’S control versus nicotine and P > 0.05 for α6L9’Sα4KO 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’S VTA DA neurons. (A) Slice treatment procedure. α6L9’S 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 cotreatment 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’S 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’S 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 α6L9'S VTA DA neurons with intact α4 subunits, responses from VTA DA neurons in α6L9'S slices lacking α4 subunits were smaller (Fig. 8E). Inward current amplitudes after puff-application of nicotine compared with α6KO neurons with intact current testing, 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 α6L9'S VTA DA neurons with intact α4 subunits, responses from VTA DA neurons in α6L9'S slices lacking α4 subunits were smaller (Fig. 8E). Inward current amplitudes after puff-application of both 1 and 100 μM ACh were smaller in α6L9'S neurons relative to α6L9'S neurons (α4WT 1 μM ACh = −171 ± 30.3 pA, α4KO 1 μM ACh = −57.8 ± 21.8 pA, and α4KO 100 μM ACh = −77.6 ± 20.2 pA; Fig. 8, E and F). Similarly, α6L9'S VTA DA neurons lacking α4 subunits were less sensitive to nicotine compared with α6L9'S cells expressing α4 subunits (Fig. 8G). Whereas 1 μM nicotine evoked large inward currents in α6L9'S VTA DA neurons that express α4 subunits, 30 μM nicotine was required to elicit inward currents of the same amplitude in α6L9'S slices lacking α4 subunits (α4WT 1 μM nicotine = −198.4 ± 25.8 pA, α4KO 1 μM nicotine = −58.3 ± 11.5 pA, and α4KO 30 μM nicotine = −189.6 ± 45.4 pA; Fig. 8, G and H). Together, these experiments suggest that activation of α4α6β2* nAChRs is responsible for enhanced AMPA-evoked currents in α6L9'S VTA DA neurons.

Finally, we tested whether nicotine (100 nM), acting through α6* nAChRs, can increase or decrease NMDA receptor function on the surface of VTA DA neurons (Ungless et al., 2001). Whole-cell voltage-clamp recordings were established in VTA DA neurons, and NMDA currents were evoked via puff-application of NMDA at a holding potential of +40 mV. Incubation of α6L9'S and non-Tg slices in nicotine (100 nM) for 60 minutes (Fig. 9A) did not result in changes in NMDA-evoked currents relative to control treatments (control: non-Tg = 167.8 ± 17.8 pA and α6L9'S = 172.8 ± 32.6 pA; nicotine: non-Tg = 185.2 ± 28.5 pA and α6L9'S = 167.3 ± 18.7 pA; Kruskal–Wallis test, P = 0.7893; Fig. 9, B and C). 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.
VTA DA Neuron Activation by α6β2* 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 α6β2* 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). β2* 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 α4β2* 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 α6β2* nAChR antagonist, implicating α4α6β2* nAChRs. This report is consistent with our study, which suggests that α6β2* nAChR activation can increase inward currents in VTA DA neurons (Fig. 2). Other reports studying the role of VTA α6β2* nAChRs in nicotine self-administration (Pons et al., 2008; Gotti et al., 2010) and DA release (Gotti et al., 2010) support the data we present here. Furthermore, our experiments employing puff-application of nicotine and ACh in 6L9’S and α6L9’S/e4KO brain slices (Fig. 8) provide evidence that α4 subunits play an important role in α6-mediated neuronal activation. In VTA, α4β2* nAChRs are found in DAergic neurons and in GABAergic neurons and/or terminals (Nashmi et al., 2007). Nicotine may act through VTA α4β2* nAChRs via two mechanisms: 1) direct activation at α4β2* nAChRs on DA neurons, and/or 2) desensitization of α4β2* nAChRs in GABAergic neurons leading to DA neuron disinhibition (Mansvelder et al., 2002; Nashmi et al., 2007). Because α6* nAChRs are restricted to DAergic cells in VTA (Mackey et al., 2012), our results suggest that direct action by nicotine on somatodendritic α6* 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 α6β2* 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 α4α6L9’Sβ2* nAChRs via concatamers (Kuryatov and Lindstrom, 2011) will be useful in exploring the latter possibility, whereas development of α6β2*-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 α6β2* 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 McGehee, 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, McGehee and colleagues report that β2* nAChRs

![Diagram A](image1)

**Fig. 9.** NMDA-evoked currents are not changed by nicotine in α6L9’S VTA DA neurons. (A) Slice 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) Representative NMDA-evoked currents (NMDA) = 100 μM) at +40 mV in VTA DA neurons from α6L9’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 ± S.E.M. NMDA-evoked currents (NMDA) = 100 μM) in α6L9’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-elicted increases in AMPAR function can proceed either through β2* or α7 nAChRs (Gao et al., 2010; Jin et al., 2011). Our results using naive 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 outward 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 (Schilstrom 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 α6α6β2* 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 α6α6β2* nAChRs are emerging as a key target for smoking cessation pharmacotherapy.

**Acknowledgments**

The authors thank members of the Drenan laboratory for helpful advice and discussions. They also especially thank Hilary Broderick, Gyeon Oh, and Karen Wethington for technical assistance.

**Authorship Contributions**

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

**Conducted experiments:** Engle, Shih, Drenan.

**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.

**References**


Cohen BN, Mackey ED, Grady SR, McKinney S, Patzalaff NE, Wageman CR, McIntosh JM, Marks MJ, Lester HA, and Drenan RM (2012) Nicotinic cholinergic....


Pietrini PE, Addy NA, Mineur YS, and Brunzell DH (2008) It is not “either/or”: activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood. Prog Neurobiol 84:329–342.


