$\alpha 4\alpha 6\beta 2^*$ 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 $\alpha 6$ subunits are sufficient to activate VTA DA neurons, we studied mice expressing hypersensitive, gain-of-function $\alpha 6$ nAChRs ($\alpha 6L9'S$ 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 $\alpha 6\beta 2^*$ nAChRs. In addition,

we found that low concentrations of nicotine could act selectively through $\alpha 6\beta 2^*$ 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, $\alpha 6\beta 2^*$ activation did not enhance *N*-methyl-D-aspartic acid receptor function. Finally, AMPA receptor (AMPA) function was not similarly enhanced in brain slices from $\alpha 6L9'S$ mice lacking $\alpha 4$ nAChR subunits, suggesting that $\alpha 4\alpha 6\beta 2^*$ nAChRs are important for enhancing AMPAR function in VTA DA neurons. Together, these data suggest that activation of $\alpha 4\alpha 6\beta 2^*$ nAChRs in VTA DA neurons is sufficient to support the initiation of cellular changes that play a role in addiction to nicotine. $\alpha 4\alpha 6\beta 2^*$ 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 (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

This research was supported by the National Institutes of Health National Institute on Drug Abuse [Grant R00 DA030396]; the National Institutes of Health National Institute of General Medical Sciences [Grants P01 GM48677 and R01 GM103801]; and the Ralph W. and Grace M. Showalter Research Trust. S.E.E. was supported by a Purdue University Frederick N. Andrews Fellowship.

dx.doi.org/10.1124/mol.113.087346.

ABBREVIATIONS: ACh, acetylcholine; α CtxMII, α -conotoxin MII; AMPA, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid; AMPAR, AMPA receptor; AP-5, D-2-amino-5-phosphonopentanoate; BAC, bacterial artificial chromosome; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DA, dopamine; EPSC, excitatory postsynaptic current; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GluR, AMPA-type ionotropic glutamate receptor; KO, knockout; LTP, long-term potentiation; MLA, methyllycaconitine A; nAChR, nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartic acid; NMDG, *N*-methyl-D-glucamine; non-Tg, nontransgenic; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; SCH23390, 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol; TBS, Tris-buffered saline; TH, tyrosine hydroxylase; TTL, transistor-transistor logic; VTA, ventral tegmental area; WT, wild-type.

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 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 β subunits ($\beta 2$ and/or $\beta 4$) and two or more α subunits ($\alpha 2$ – $\alpha 6$) (Itier and Bertrand, 2001). “Auxiliary” subunits $\alpha 5$ or $\beta 3$ 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). $\alpha 4\beta 2^*$ (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 $\alpha 7$ 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 $\alpha 4\beta 2^*$ nAChRs on the soma of these cells (Mansvelder et al., 2002).

Interest in nAChRs containing $\alpha 6$ 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). $\alpha 6^*$ nAChRs require $\beta 2$ 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 $\alpha 4\beta 2^*$ nAChRs in VTA DA neurons induces prolonged depolarization of these cells, an effect that was sensitive to an $\alpha 6^*$ 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 $\alpha 6^*$ nAChRs (Yang et al., 2011). These approaches relied on pharmacological blockade to discern the role of $\alpha 6^*$ nAChRs, and the results indicate that more experiments are needed to better understand $\alpha 6^*$ nAChRs in the VTA.

In the present study, we studied transgenic mice expressing $\alpha 6$ 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 activate $\alpha 6^*$ nAChRs but do not activate (non- $\alpha 6$) * nAChRs, we tested the hypothesis that $\alpha 6^*$ 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 $\alpha 4$ 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).

$\alpha 6L9/S$ mice were generated as described (Drenan et al., 2008a). Briefly, a mouse bacterial artificial chromosome (BAC) containing the *Chrna6* gene was obtained and an L9/S mutation was introduced by codon replacement using a BAC recombineering approach. Mutant BAC DNA was introduced into FVB/N embryos, which were then implanted into pseudopregnant 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 $\alpha 6L9/S$ 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. $\alpha 6L9/S$ mice are thus transgenic and express mutant (L9/S) and wild-type (WT) $\alpha 6$ nAChR subunits (Cohen et al., 2012). $\alpha 6^*$ nAChR function is sensitized in these mice, producing a 10- to 100-fold leftward shift in concentration-response relationships involving $\alpha 6^*$ nAChRs, depending on the assay being used (Drenan et al., 2008a, 2010; Cohen et al., 2012). We previously confirmed that $\alpha 6^*$ nAChRs in $\alpha 6L9/S$ mice are not overexpressed or misexpressed in ectopic brain locations (Drenan et al., 2008a, 2010).

$\alpha 6L9/S$ mice lacking $\alpha 4$ nAChR subunits were generated as previously described (Drenan et al., 2010). $\alpha 4$ 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 $\alpha 4KO$ allele. Briefly, $\alpha 6L9/S$ mice, in which the mutant allele is maintained in a heterozygous fashion, were crossed to homozygous $\alpha 4KO$ mice to produce mice that are heterozygous for both the $\alpha 6L9/S$ allele and the $\alpha 4KO$ allele. These mice were subsequently crossed to homozygous $\alpha 4KO$ mice to produce mice heterozygous for the $\alpha 6L9/S$ allele and homozygous for the $\alpha 4KO$ allele. $\alpha 6$ green fluorescent protein (GFP) mice were generated as previously described (Mackey et al., 2012). To create $\alpha 6GFP$ mice lacking $\alpha 4$ subunits, $\alpha 4KO$ mice were crossed to $\alpha 6GFP$ mice, generating $\alpha 6GFP$ mice heterozygous for the $\alpha 4KO$ allele. These mice were crossed again to mice homozygous for the $\alpha 4KO$ allele, yielding $\alpha 6GFP$ mice that were also homozygous for the $\alpha 4KO$ 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: D-2-amino-5-phosphonopentanoate (AP-5), tetrodotoxin, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). α -Conotoxin MII (αCtx II) 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). $\alpha 6L9/S$ 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% O_2 /5% CO_2), 4°C *N*-methyl-D-glucamine (NMDG) recovery solution containing the following: 93 mM NMDG, 2.5 mM KCl, 1.2 mM NaH_2PO_4 , 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 5 mM Na^+ ascorbate, 2 mM thiourea, 3 mM Na^+ pyruvate, 10 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mM $CaCl_2 \cdot 2H_2O$ (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 NaH_2PO_4 , 30 mM NaHCO_3 , 20 mM HEPES, 25 mM glucose, 5 mM Na^+ ascorbate, 2 mM thiourea, 3 mM Na^+ pyruvate, 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (300–310 mOsm, 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-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 NaH_2PO_4 , 24 mM NaHCO_3 , 12.5 mM glucose, 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (300–310 mOsm, 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 M Ω when filled with internal pipette solution (pH adjusted to 7.25 with Tris base, osmolarity 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 CaCl_2 , 2 mM MgCl_2 , 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 CsCH_3SO_3 , 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 (I_h). 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). Atropine ($1 \mu\text{M}$) was present in the bath solution when administering ACh to avoid activation of muscarinic receptors. A drug-filled micropipette, identical to a typical recording pipette, was mounted onto a single-dimension piezoelectric translator (PA-100/12; Piezosystem Jena, Inc., Hopedale, MA), which was fixed to a micromanipulator (Sutter Instrument Company). Between drug applications, the drug-filled pipette was maintained $\geq 100 \mu\text{m}$ from the recorded cell. To apply drugs to the recorded cell, pClamp software triggered the piezoelectric translator to advance the drug-filled pipette to a predetermined position adjacent to the recorded cell (20 – $40 \mu\text{m}$ from the cell), drug was applied to the cell using a 250-millisecond pressure (12 psi) ejection, and the piezoelectric translator subsequently retracted the drug-filled pipette. This setup allowed for better reproducibility and reduced receptor desensitization compared with manual control of the drug-filled pipette (Engle et al., 2012). The first application to a cell was typically with the drug-filled tip approximately $40 \mu\text{m}$ 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 $> 1 \text{ G}\Omega$ 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) (Sensiscript 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 (CAGTGATGCCAAGGACAAGC), TH_R2 (GAGAAGGGGCTGGGAACCTTT), GAPDH_F2 (AACTTGGCATTGTGGAAGG), and GAPDH_R2 (CCCTGTTGCTGTAGCCGTAT). Subsequently, TH and GAPDH signals were further amplified (1 cycle: 94°C for 2 minutes; 36 cycles: 94°C for 1 minutes, 56°C for 1 minutes, 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 TTC 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 $\alpha 6^*$ nAChR subunits fused in-frame with GFP ($\alpha 6\text{GFP}$ mice; $n = 3$), along with $\alpha 6\text{GFP}$ mice homozygous for the $\alpha 4\text{KO}$ 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 ($50 \mu\text{m}$) were cut on a micro slicer and collected into PBS. Sections were permeabilized (20 mM HEPES, pH 7.4, 0.5% Triton X-100, 50 mM NaCl, 3 mM MgCl_2 , 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 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 $10\times$ air and $60\times$ oil objectives were used. Alexa 488 was excited with an argon laser at 488 nm. VTA DA neurons were imaged at $60\times$, and mean pixel intensity per cell was measured for > 100 cells in both $\alpha 6\text{GFP}$ and $\alpha 6\text{GFP}\alpha 4\text{KO}$ slices.

Statistical Analysis. Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Data are reported as the mean $\pm$ 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_{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 $\alpha 6^*$ nAChRs is sufficient to elicit inward currents in VTA DA neurons by recording from VTA DA neurons from adult $\alpha 6L9/S$ 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 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 $\alpha 6L9/S$ 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 $\alpha CtxMII$ (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 $\alpha 6^*$ nAChRs mediate inward currents in response to 100 nM nicotine. To determine whether responses to 100 nM nicotine were selective for $\alpha 6^*$ nAChRs in $\alpha 6L9/S$ 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.

Fig. 2. A low concentration of nicotine is sufficient to increase inward currents in VTA DA neurons. (A) $\alpha 6L9'S$ 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 $\alpha CtxMII$ (100 nM). A representative experiment from a $\alpha 6L9'S$ neuron is shown. Expanded recordings from time points (i), (ii), and (iii) are shown in B–D. (B–D) Voltage-clamp recording segments from $\alpha 6L9'S$ (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 $\alpha CtxMII$. (E) Summary showing mean holding current (–pA) change from baseline in response to nicotine, and nicotine plus $\alpha CtxMII$ in the indicated mouse strain ($\alpha 6L9'S$ and non-Tg littermate). * $P < 0.05$.

pre-nicotine baseline = -3.1 ± 0.8 pA; Fig. 2, C and E), but the same concentration of nicotine significantly increased inward currents in $\alpha 6L9'S$ neurons (Mann–Whitney test, $P < 0.05$; Fig. 2E). $\alpha CtxMII$ 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 pre-nicotine baseline = -8.7 ± 2.2 pA; Fig. 2, D and E), consistent with a previous report (Liu et al., 2012). $\alpha CtxMII$ did not block these responses (mean change in holding current value relative to pre-nicotine baseline = -7.0 ± 1.0 pA; Fig. 2,

D and E), presumably because responses in non-Tg cells are mediated by both $\alpha 6^*$ and non- $\alpha 6^*$ ($\alpha 4\beta 2$) nAChRs. Together, these results demonstrate that selective activation of $\alpha 6^*$ nAChRs is sufficient to increase inward currents in VTA DA neurons. Application of 100 nM nicotine to $\alpha 6$ L9'S slices was used in subsequent experiments to study the effects of selectively activating $\alpha 6^*$ 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 (AMPA) 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 $\alpha 6^*$ nAChRs are expressed only in DA neurons in VTA (Mackey et al., 2012). We hypothesized that selective activation of $\alpha 6^*$ 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 $\alpha 6^*$ nAChRs is sufficient to enhance AMPAR function, we prepared coronal slices from $\alpha 6$ L9'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 $\alpha 6$ L9'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 $\alpha 6$ L9'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. 4, 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 $\alpha 6$ L9'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 $\alpha 6$ L9'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 $\alpha 6^*$ nAChRs is sufficient to enhance AMPAR function on the surface of VTA DA neurons. (A) Slice treatment procedure. Brain slices from adult $\alpha 6L9'S$ 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 μ M) at holding potentials of -60 , 0 , and $+40$ mV. Representative recordings from incubation of slices in control and nicotine solutions are shown for $\alpha 6L9'S$ and non-Tg littermate mice. (C and D) Summary showing mean AMPA-evoked currents ([AMPA] = 100 μ M) in non-Tg littermate (C) and $\alpha 6L9'S$ (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, $n = 12$); $\alpha 6L9'S$ control (-60 mV, $n = 14$; 0 mV, $n = 13$; $+40$ mV, $n = 13$); and $\alpha 6L9'S$ 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 $\alpha 6L9'S$ neurons. AMPA concentrations and number of observations at each data point are as follows: non-Tg (1 μ M, $n = 2$; 10 μ M, $n = 6$; 50 μ M, $n = 5$; 100 μ M, $n = 10$; 250 μ M, $n = 5$; 500 μ M, $n = 14$; 1000 μ M, $n = 11$), and $\alpha 6L9'S$ (1 μ M, $n = 2$; 10 μ M, $n = 4$; 50 μ M, $n = 4$; 100 μ M, $n = 14$; 250 μ M, $n = 5$; 500 μ M, $n = 4$; 1000 μ M, $n = 5$; 3000 μ M, $n = 2$). Data (mean $\pm$ 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 $\alpha 6L9'S$ VTA DA neurons. AMPA-evoked currents were measured in $\alpha 6L9'S$ control slices or slices incubated in 100 nM nicotine for 60 minutes followed by >60 minutes washout prior to recording. Control treated $\alpha 6L9'S$ data from (E) are replotted here for reference. AMPA concentrations and number of observations at each data point for $\alpha 6L9'S$ slices treated with nicotine are as follows: $\alpha 6L9'S$ (1 μ M, $n = 2$; 10 μ M, $n = 3$; 50 μ M, $n = 2$; 100 μ M, $n = 11$; 300 μ M, $n = 3$; 1000 μ M, $n = 3$). Data (mean $\pm$ 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 $\alpha 6L9'S$ and non-Tg neurons, and the data were fitted to the Hill equation (non-Tg: $R^2 = 0.9467$; $\alpha 6L9'S$: $R^2 = 0.9819$). There was no substantial difference in AMPA EC_{50} in $\alpha 6L9'S$ VTA DA neurons compared with non-Tg neurons ($EC_{50} = 174 \mu M$ for non-Tg, and $EC_{50} = 182 \mu M$ for $\alpha 6L9'S$; 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 $\alpha 6L9'S$ 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 ($\alpha 6L9'S$ nicotine: $R^2 = 0.9942$). The EC_{50} for AMPA-evoked currents in nicotine-exposed $\alpha 6L9'S$ slices was shifted to the left compared with $\alpha 6L9'S$ slices not exposed to nicotine ($EC_{50} = 37 \mu M$; 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 $\alpha 6L9'S$ 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 ± 29.4 pA; 10-minute nicotine incubation/washout 60–240 minutes = -213.5 ± 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 ± 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 ± 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 $\alpha 6L9'S$ VTA DA neurons. (A) Slice treatment procedure. Brain slices from $\alpha 6L9'S$ 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 ($[AMPA] = 100 \mu M$) at $+40$ and -60 mV in VTA DA neurons in response to treatment detailed in (A). (C) Summary showing mean $\pm$ S.E.M. AMPA-evoked ($[AMPA] = 100 \mu M$) current in $\alpha 6L9'S$ 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 $\alpha 6L9'S$ 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 $\alpha CtxMII$ eliminated the enhanced AMPA-evoked currents seen in $\alpha 6L9'S$ 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) did not appear to substantially reduce nicotine-mediated enhancement in AMPA-evoked currents (SCH23390 = -325.0 ± 44.7 pA; Fig. 6, B and C), but a Kruskal–Wallis test comparing AMPA-evoked currents from $\alpha 6L9'S$ SCH23390-treated slices and untreated control $\alpha 6L9'S$ slices did not reveal a statistical difference. Several previous reports indicate that homomeric $\alpha 7$ nAChRs play a role in nicotine-elicited AMPAR upregulation (Gao et al., 2010; Jin

et al., 2011). However, pretreatment of slices with MLA (10 nM) did not reduce nicotine-elicited AMPAR upregulation in $\alpha 6L9'S$ VTA DA neurons (MLA = -497.8 ± 119.8 pA; Kruskal–Wallis test, $P < 0.05$; Fig. 6, B and C).

Elimination of $\alpha 4$ nAChR subunits via gene knockout has been shown to significantly reduce $\alpha 6^*$ 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 $\alpha 4$ nAChR subunits are important for $\alpha 6^*$ nAChR-mediated enhancement of AMPAR function in VTA DA neurons, we crossed $\alpha 6L9'S$ mice with $\alpha 4KO$ animals to eliminate $\alpha 4$ nAChR subunits while still retaining gain-of-function $\alpha 6$ 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 $\alpha 6L9'S$ slices leads to enhanced AMPAR function, identical treatment of slices from $\alpha 6L9'S$ mice lacking $\alpha 4$ subunits did not increase AMPA-evoked currents ($\alpha 6L9'S$: control = -173.5 ± 29.4 pA, nicotine = -358.4 ± 48.5 pA; $\alpha 6L9'S\alpha 4KO$: control = -205.9 ± 23.3 pA, nicotine = -280.3 ± 45.3 pA; Kruskal–Wallis test, $P < 0.05$ for $\alpha 6L9'S$ control versus nicotine and $P > 0.05$ for $\alpha 6L9'S\alpha 4KO$ control versus nicotine; Fig. 7, B and C). To determine whether these results were due to reduced $\alpha 6$

Fig. 6. Pharmacology of AMPA-evoked current induction in $\alpha 6L9'S$ VTA DA neurons. (A) Slice treatment procedure. $\alpha 6L9'S$ brain slices were cut and recovered for 60 minutes. Slices were pretreated 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 ($[AMPA] = 100 \mu M$) at $+40$ and -60 mV in VTA DA neurons from $\alpha 6L9'S$ 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: $\alpha CtxMII$ (MI), SCH23390 (SCH), AP-5, and MLA. (C) Summary showing mean $\pm$ S.E.M. AMPA-evoked currents ($[AMPA] = 100 \mu M$) in $\alpha 6L9'S$ 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 $\alpha 4$ KO animals. First, we crossed $\alpha 4$ KO mice with transgenic mice expressing $\alpha 6$ subunits fused with GFP (Fig. 8A). This manipulation results in the production of only (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs (Fig. 8B). We used anti-GFP immunohistochemistry and confocal microscopy, as previously described in these mice (Mackey et al., 2012), to quantify $\alpha 6^*$ nAChR expression in VTA neurons in $\alpha 6$ GFP mice and $\alpha 6$ GFP mice crossed to $\alpha 4$ KO mice. We found a small but significant reduction in $\alpha 6$ GFP expression in VTA neurons in $\alpha 6$ GFP mice lacking $\alpha 4$ subunits compared with $\alpha 6$ GFP with intact $\alpha 4$ nAChR subunit expression ($\alpha 4$ WT = $17,921 \pm 698$ arbitrary units, $\alpha 4$ KO = $14,507 \pm 816$ arbitrary units; Mann-Whitney test, $P = 0.0011$; Fig. 8C). Next, we measured $\alpha 6^*$ nAChR function directly by comparing nicotine- and ACh-evoked currents in $\alpha 6$ L9'S mice and $\alpha 6$ L9'S mice lacking $\alpha 4$ subunits (Fig. 8D). In contrast to ACh-evoked responses in $\alpha 6$ L9'S VTA DA neurons with intact $\alpha 4$ subunits, responses from VTA DA neurons in $\alpha 6$ L9'S slices lacking $\alpha 4$ subunits were smaller (Fig. 8E). Inward current amplitudes after puff-application of both 1 and 100 μ M ACh were smaller in $\alpha 6$ L9'S/ $\alpha 4$ KO neurons relative to $\alpha 6$ L9'S neurons ($\alpha 4$ WT 1 μ M ACh = -171 ± 30.3 pA, $\alpha 4$ KO 1 μ M ACh = -57.8 ± 21.8 pA, and $\alpha 4$ KO 100 μ M ACh = -77.6 ± 20.2 pA; Fig. 8, E and F). Similarly, $\alpha 6$ L9'S VTA DA neurons lacking $\alpha 4$ subunits were less sensitive to nicotine compared with $\alpha 6$ L9'S cells expressing $\alpha 4$ subunits (Fig. 8G). Whereas 1 μ M nicotine evoked large inward currents in $\alpha 6$ L9'S VTA DA neurons that express $\alpha 4$ subunits, 30 μ M

nicotine was required to elicit inward currents of the same amplitude in $\alpha 6$ L9'S slices lacking $\alpha 4$ subunits ($\alpha 4$ WT 1 μ M nicotine = -198.4 ± 25.8 pA, $\alpha 4$ KO 1 μ M nicotine = -58.3 ± 11.5 pA, and $\alpha 4$ KO 30 μ M nicotine = -189.6 ± 45.4 pA; Fig. 8, G and H). Together, these experiments suggest that activation of $\alpha 4\alpha 6\beta 2^*$ nAChRs are responsible for enhanced AMPA-evoked currents in $\alpha 6$ L9'S VTA DA neurons.

Finally, we tested whether nicotine (100 nM), acting through $\alpha 6^*$ 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 $\alpha 6$ L9'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 $\alpha 6$ L9'S = 172.8 ± 32.6 pA; nicotine: non-Tg = 185.2 ± 28.5 pA and $\alpha 6$ L9'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 $\alpha 6\beta 2^*$ nAChRs by nicotine is sufficient

Fig. 7. Enhanced AMPA-evoked currents in $\alpha 6$ L9'S VTA DA neurons are mediated, in part, by $\alpha 4$ nAChR subunits. (A) Slice treatment procedure. Brain slices from adult $\alpha 6$ L9'S and $\alpha 6$ L9'S/ $\alpha 4$ KO 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 AMPA-evoked currents ([AMPA] = 100 μ M) at +40 and -60 mV in VTA DA neurons from $\alpha 6$ L9'S and $\alpha 6$ L9'S/ $\alpha 4$ KO brain slices after incubation in 100 nM nicotine for 60 minutes. (C) Summary showing mean $\pm$ S.E.M. AMPA-evoked currents ([AMPA] = 100 μ M) in $\alpha 6$ L9'S and $\alpha 6$ L9'S/ $\alpha 4$ KO VTA DA neurons in response to the conditions described in A. *** $P < 0.001$.

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. VTA DA neurons from both genotypes were patch clamped and ACh was puff-applied (250 milliseconds) at the indicated concentration. (F) Summary showing mean $\pm$ S.E.M. ACh-evoked current 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. (H) Summary showing mean $\pm$ S.E.M. nicotine-evoked current in $\alpha 6$ L9'S and $\alpha 6$ L9'S $\alpha 4$ KO VTA DA neurons in response to the indicated concentration of nicotine. ** $P < 0.01$.

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 $\alpha 6\beta 2^*$ 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 (Fig. 2). Other reports studying the role of VTA $\alpha 6\beta 2^*$ 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 $\alpha 6L9'S$ and $\alpha 6L9'S\alpha 4KO$ brain slices (Fig. 8) provide evidence that $\alpha 4$ subunits play an important role in $\alpha 6$ -mediated neuronal activation. In VTA, $\alpha 4\beta 2^*$ nAChRs are found in DAergic neurons and in GABAergic neurons and/or terminals (Nashmi et al., 2007). Nicotine may act through VTA $\alpha 4\beta 2^*$ nAChRs via two mechanisms: 1) direct activation at $\alpha 4\beta 2^*$ nAChRs on

DA neurons, and/or 2) desensitization of $\alpha 4\beta 2^*$ nAChRs in GABAergic neurons leading to DA neuron disinhibition (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 $\alpha 4\alpha 6L9'S\beta 2^*$ 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 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 $\beta 2^*$ nAChRs

Fig. 9. NMDA-evoked currents are not changed by nicotine in $\alpha 6L9'S$ VTA DA neurons. (A) Slice treatment procedure. Brain slices from adult $\alpha 6L9'S$ 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 ($[NMDA] = 100 \mu M$) at +40 mV in VTA DA neurons from $\alpha 6L9'S$ 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 $\alpha 6L9'S$ and non-Tg littermate VTA DA neurons in response to the conditions described in A.

(but not $\alpha 7$ 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 $\beta 2^*$ or $\alpha 7$ nAChRs (Gao et al., 2010; Jin et al., 2011). Our results using naïve or nicotine-exposed slices from adult non-Tg or $\alpha 6L9/S$ mice are more consistent with the former, because we find no necessary role for $\alpha 7$ 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 $\alpha 6L9/S$ 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 Ca^{2+} -impermeable AMPARs containing AMPA-type ionotropic glutamate receptor (GluR) 2 subunits for high-conductance, Ca^{2+} -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 $\alpha 6\beta 2^*$ activation (Fig. 4F) support a number of possible mechanisms, including increased AMPAR conductance—a hallmark of GluR2-lacking AMPARs. Future pharmacological studies in $\alpha 6L9/S$ 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 $\alpha 6\beta 2^*$ 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 $\alpha 6\beta 2^*$ 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 (Schilström 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 $\alpha 6\beta 2^*$ nAChR-mediated increases in AMPAR function (Fig. 6C). Together with previous studies on nicotine and other drugs of abuse, our data studying $\alpha 6\beta 2^*$ 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 $\alpha 4\alpha 6\beta 2^*$ 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 $\alpha 6\beta 2^*$ -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 $\alpha 6\beta 2^*$ 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 $\alpha 4\alpha 6\beta 2^*$ 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

- Azam L, Maskos U, Changeux JP, Dowell CD, Christensen S, De Biasi M, and McIntosh JM (2010) α -Conotoxin BuIA[TSa;P6O]: a novel ligand that discriminates between $\alpha 6\beta 4$ and $\alpha 6\beta 2$ nicotinic acetylcholine receptors and blocks nicotine-stimulated norepinephrine release. *FASEB J* 24:5113–5123.
- Baker LK, Mao D, Chi H, Govind AP, Vallejo YF, Iacoviello M, Herrera S, Cortright JJ, Green WN, and McGehee DS et al. (2013) Intermittent nicotine exposure upregulates nAChRs in VTA dopamine neurons and sensitizes locomotor responding to the drug. *Eur J Neurosci* 37:1004–1011.
- Bellone C and Lüscher C (2006) Cocaine triggered AMPA receptor redistribution is reversed in vivo by mGluR-dependent long-term depression. *Nat Neurosci* 9: 636–641.
- Brown MT, Bellone C, Mameli M, Labouëbe G, Bocklisch C, Balland B, Dahan L, Luján R, Deisseroth K, and Lüscher C (2010) Drug-driven AMPA receptor redistribution mimicked by selective dopamine neuron stimulation. *PLoS ONE* 5: e15870.
- Calabresi P, Lacey MG, and North RA (1989) Nicotinic excitation of rat ventral tegmental neurones in vitro studied by intracellular recording. *Br J Pharmacol* 98: 135–140.
- Champtiaux N, Gotti C, Cordero-Erausquin M, David DJ, Przybylski C, Léna C, Clementi F, Moretti M, Rossi FM, and Le Novère N et al. (2003) Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J Neurosci* 23:7820–7829.
- Champtiaux N, Han ZY, Bessis A, Rossi FM, Zoli M, Marubio L, McIntosh JM, and Changeux JP (2002) Distribution and pharmacology of $\alpha 6$ -containing nicotinic acetylcholine receptors analyzed with mutant mice. *J Neurosci* 22:1208–1217.
- Cohen BN, Mackey ED, Grady SR, McKinney S, Patzlaff NE, Wageman CR, McIntosh JM, Marks MJ, Lester HA, and Drenan RM (2012) Nicotinic cholinergic

- mechanisms causing elevated dopamine release and abnormal locomotor behavior. *Neuroscience* **200**:31–41.
- Cui C, Booker TK, Allen RS, Grady SR, Whiteaker P, Marks MJ, Salminen O, Tritto T, Butt CM, and Allen WR et al. (2003) The $\beta 3$ nicotinic receptor subunit: a component of α -conotoxin MII-binding nicotinic acetylcholine receptors that modulate dopamine release and related behaviors. *J Neurosci* **23**:11045–11053.
- Di Chiara G and Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci USA* **85**:5274–5278.
- Drenan RM, Grady SR, Steele AD, McKinney S, Patzlaff NE, McIntosh JM, Marks MJ, Miwa JM, and Lester HA (2010) Cholinergic modulation of locomotion and striatal dopamine release is mediated by $\alpha 6\alpha 4^*$ nicotinic acetylcholine receptors. *J Neurosci* **30**:9877–9889.
- Drenan RM, Grady SR, Whiteaker P, McClure-Begley T, McKinney S, Miwa JM, Bupp S, Heintz N, McIntosh JM, and Bencherif M et al. (2008a) In vivo activation of midbrain dopamine neurons via sensitized, high-affinity $\alpha 6$ nicotinic acetylcholine receptors. *Neuron* **60**:123–136.
- Drenan RM and Lester HA (2012) Insights into the neurobiology of the nicotinic cholinergic system and nicotine addiction from mice expressing nicotinic receptors harboring gain-of-function mutations. *Pharmacol Rev* **64**:869–879.
- Drenan RM, Nashmi R, Imoukhuede P, Just H, McKinney S, and Lester HA (2008b) Subcellular trafficking, pentameric assembly, and subunit stoichiometry of neuronal nicotinic acetylcholine receptors containing fluorescently labeled $\alpha 6$ and $\beta 3$ subunits. *Mol Pharmacol* **73**:27–41.
- Engle SE, Broderick HJ, and Drenan RM (2012) Local application of drugs to study nicotinic acetylcholine receptor function in mouse brain slices. *J Vis Exp* (68): e50034.
- Fowler CD, Lu Q, Johnson PM, Marks MJ, and Kenny PJ (2011) Habenular $\alpha 5$ nicotinic receptor subunit signalling controls nicotine intake. *Nature* **471**:597–601.
- Gao C and Wolf ME (2007) Dopamine alters AMPA receptor synaptic expression and subunit composition in dopamine neurons of the ventral tegmental area cultured with prefrontal cortex neurons. *J Neurosci* **27**:14275–14285.
- Gao M, Jin Y, Yang K, Zhang D, Lukas RJ, and Wu J (2010) Mechanisms involved in systemic nicotine-induced glutamatergic synaptic plasticity on dopamine neurons in the ventral tegmental area. *J Neurosci* **30**:13814–13825.
- Gotti C, Guiducci S, Tedesco V, Corbioli S, Zanetti L, Moretti M, Zanardi A, Rimondini R, Mugnaini M, and Clementi F et al. (2010) Nicotinic acetylcholine receptors in the mesolimbic pathway: primary role of ventral tegmental area $\alpha 6\beta 2^*$ receptors in mediating systemic nicotine effects on dopamine release, locomotion, and reinforcement. *J Neurosci* **30**:5311–5325.
- Grady SR, Murphy KL, Cao J, Marks MJ, McIntosh JM, and Collins AC (2002) Characterization of nicotinic agonist-induced [3 H]dopamine release from synaptosomes prepared from four mouse brain regions. *J Pharmacol Exp Ther* **301**: 651–660.
- Isaac JT, Ashby MC, and McBain CJ (2007) The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. *Neuron* **54**:859–871.
- Itier V and Bertrand D (2001) Neuronal nicotinic receptors: from protein structure to function. *FEBS Lett* **504**:118–125.
- Jin Y, Yang K, Wang H, and Wu J (2011) Exposure of nicotine to ventral tegmental area slices induces glutamatergic synaptic plasticity on dopamine neurons. *Synapse* **65**:332–338.
- Kauer JA and Malenka RC (2007) Synaptic plasticity and addiction. *Nat Rev Neurosci* **8**:844–858.
- Kobayashi M, Kojima M, Koyanagi Y, Adachi K, Imamura K, and Koshikawa N (2009) Presynaptic and postsynaptic modulation of glutamatergic synaptic transmission by activation of $\alpha 1$ - and β -adrenoceptors in layer V pyramidal neurons of rat cerebral cortex. *Synapse* **63**:269–281.
- Kuryatov A and Lindstrom J (2011) Expression of functional human $\alpha 6\beta 2\beta 3^*$ acetylcholine receptors in *Xenopus laevis* oocytes achieved through subunit chimeras and concatamers. *Mol Pharmacol* **79**:126–140.
- Labarca C, Nowak MW, Zhang H, Tang L, Deshpande P, and Lester HA (1995) Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors. *Nature* **376**:514–516.
- Lammel S, Hetzel A, Häckel O, Jones I, Liss B, and Roeper J (2008) Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. *Neuron* **57**:760–773.
- Lammel S, Ion DI, Roeper J, and Malenka RC (2011) Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. *Neuron* **70**: 855–862.
- Lavolette SR and van der Kooy D (2004) The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat Rev Neurosci* **5**:55–65.
- Le Novère N, Zoli M, and Changeux JP (1996) Neuronal nicotinic receptor $\alpha 6$ subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. *Eur J Neurosci* **8**:2428–2439.
- Léna C, de Kerchove D'Exaerde A, Cordero-Erausquin M, Le Novère N, del Mar Arroyo-Jimenez M, and Changeux JP (1999) Diversity and distribution of nicotinic acetylcholine receptors in the locus ceruleus neurons. *Proc Natl Acad Sci USA* **96**: 12126–12131.
- Li DP, Yang Q, Pan HM, and Pan HL (2008) Pre- and postsynaptic plasticity underlying augmented glutamatergic inputs to hypothalamic presympathetic neurons in spontaneously hypertensive rats. *J Physiol* **586**:1637–1647.
- Liu L, Zhao-Shea R, McIntosh JM, Gardner PD, and Tapper AR (2012) Nicotine persistently activates ventral tegmental area dopaminergic neurons via nicotinic acetylcholine receptors containing $\alpha 4$ and $\alpha 6$ subunits. *Mol Pharmacol* **81**:541–548.
- Liu SJ and Zukin RS (2007) Ca^{2+} -permeable AMPA receptors in synaptic plasticity and neuronal death. *Trends Neurosci* **30**:126–134.
- Lüscher C and Malenka RC (2011) Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. *Neuron* **69**:650–663.
- Mackey ED, Engle SE, Kim MR, O'Neill HC, Wageman CR, Patzlaff NE, Wang Y, Grady SR, McIntosh JM, and Marks MJ et al. (2012) $\alpha 6^*$ nicotinic acetylcholine receptor expression and function in a visual salience circuit. *J Neurosci* **32**: 10226–10237.
- Mansvelder HD, Keath JR, and McGehee DS (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron* **33**:905–919.
- Mansvelder HD and McGehee DS (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron* **27**:349–357.
- Mao D, Gallagher K, and McGehee DS (2011) Nicotine potentiation of excitatory inputs to ventral tegmental area dopamine neurons. *J Neurosci* **31**:6710–6720.
- Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, Evrard A, Cazala P, Cormier A, and Mameli-Engvall M et al. (2005) Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. *Nature* **436**: 103–107.
- Nashmi R, Xiao C, Deshpande P, McKinney S, Grady SR, Whiteaker P, Huang Q, McClure-Begley T, Lindstrom JM, and Labarca C et al. (2007) Chronic nicotine cell specifically upregulates functional $\alpha 4^*$ nicotinic receptors: basis for both tolerance in midbrain and enhanced long-term potentiation in perforant path. *J Neurosci* **27**: 8202–8218.
- Peto R and Lopez AD (2001) Future worldwide health effects of current smoking patterns, in *Critical Issues in Global Health* (Koop CE, Pearson CE, and Schwarz MR eds) pp 154–161, Jossey-Bass, San Francisco.
- Picciotto MR, 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.
- Picciotto MR, Zoli M, Rimondini R, Léna C, Marubio LM, Pich EM, Fuxe K, and Changeux JP (1998) Acetylcholine receptors containing the $\beta 2$ subunit are involved in the reinforcing properties of nicotine. *Nature* **391**:173–177.
- Pidoplichko VI, DeBiasi M, Williams JT, and Dani JA (1997) Nicotine activates and desensitizes midbrain dopamine neurons. *Nature* **390**:401–404.
- Pons S, Fattore L, Cossu G, Tolu S, Porcu E, McIntosh JM, Changeux JP, Maskos U, and Fratta W (2008) Crucial role of $\alpha 4$ and $\alpha 6$ nicotinic acetylcholine receptor subunits from ventral tegmental area in systemic nicotine self-administration. *J Neurosci* **28**:12318–12327.
- Revah F, Bertrand D, Galzi JL, Devillers-Thiéry A, Mulle C, Hussy N, Bertrand S, Ballivet M, and Changeux JP (1991) Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. *Nature* **353**:846–849.
- Saal D, Dong Y, Bonci A, and Malenka RC (2003) Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. *Neuron* **37**:577–582.
- Salminen O, Drapeau JA, McIntosh JM, Collins AC, Marks MJ, and Grady SR (2007) Pharmacology of α -conotoxin MII-sensitive subtypes of nicotinic acetylcholine receptors isolated by breeding of null mutant mice. *Mol Pharmacol* **71**:1563–1571.
- Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, and Grady SR (2004) Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol Pharmacol* **65**:1526–1535.
- Sanchez JT, Wang Y, Rubel EW, and Barria A (2010) Development of glutamatergic synaptic transmission in binaural auditory neurons. *J Neurophysiol* **104**:1774–1789.
- Schilström B, Yaka R, Argilli E, Suvarna N, Schumann J, Chen BT, Carman M, Singh V, Maillard WS, and Ron D et al. (2006) Cocaine enhances NMDA receptor-mediated currents in ventral tegmental area cells via dopamine D5 receptor-dependent redistribution of NMDA receptors. *J Neurosci* **26**:8549–8558.
- Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, Whiteaker P, Marks MJ, Collins AC, and Lester HA (2004) Nicotine activation of $\alpha 4^*$ receptors: sufficient for reward, tolerance, and sensitization. *Science* **306**:1029–1032.
- Ungless MA, Whistler JL, Malenka RC, and Bonci A (2001) Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. *Nature* **411**:583–587.
- Wolf ME, Sun X, Mangiavacchi S, and Chao SZ (2004) Psychomotor stimulants and neuronal plasticity. *Neuropharmacology* **47** (Suppl 1):61–79.
- Wooltorton JR, Pidoplichko VI, Broide RS, and Dani JA (2003) Differential desensitization and distribution of nicotinic acetylcholine receptor subtypes in mid-brain dopamine areas. *J Neurosci* **23**:3176–3185.
- Yang K, Buhlman L, Khan GM, Nichols RA, Jin G, McIntosh JM, Whiteaker P, Lukas RJ, and Wu J (2011) Functional nicotinic acetylcholine receptors containing $\alpha 6$ subunits are on GABAergic neuronal boutons adherent to ventral tegmental area dopamine neurons. *J Neurosci* **31**:2537–2548.
- Zhang TA, Placzek AN, and Dani JA (2010) In vitro identification and electrophysiological characterization of dopamine neurons in the ventral tegmental area. *Neuropharmacology* **59**:431–436.
- Zhao-Shea R, Liu L, Soll LG, Improgo MR, Meyers EE, McIntosh JM, Grady SR, Marks MJ, Gardner PD, and Tapper AR (2011) Nicotine-mediated activation of dopaminergic neurons in distinct regions of the ventral tegmental area. *Neuropsychopharmacology* **36**:1021–1032.

Address correspondence to: Ryan M. Drenan, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, 575 Stadium Mall Dr., West Lafayette, IN 47907. E-mail: drenan@purdue.edu