

Nicotine persistently activates ventral tegmental area  
dopaminergic neurons via nicotinic acetylcholine receptors  
containing  $\alpha 4$  and  $\alpha 6$  subunits

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Abbreviations:

DA – Dopamine

nAChR – nicotinic acetylcholine receptor

VTA – ventral tegmental area

NAcc – nucleus accumbens

dh $\beta$ e – dihydro- $\beta$ -erythroidine

ACSF – artificial cerebral spinal fluid

KO – knock-out

## Abstract

Nicotine is reinforcing because it activates dopaminergic (DAergic) neurons within the ventral tegmental area (VTA) of the brain's mesocorticolimbic reward circuitry. This increase in activity can occur for a period of several minutes up to an hour and is thought to be a critical component of nicotine dependence. However, nicotine concentrations that are routinely self-administered by smokers are predicted to desensitize high affinity  $\alpha 4\beta 2$  nAChRs on the order of seconds. Thus, how physiologically relevant nicotine concentrations persistently activate VTA DAergic neurons is unknown. Here we show that nicotine can directly and robustly increase the firing frequency of VTA DAergic neurons for several minutes. In mouse midbrain slices, 300 nM nicotine elicited a persistent inward current in VTA DAergic neurons that was blocked by  $\alpha$ -conotoxin MII[H9A;L15A], a selective antagonist of nAChRs containing the  $\alpha 6$  subunit.  $\alpha$ -conotoxin MII[H9A;L15A] also significantly reduced the long-lasting increase in DAergic neuronal activity produced by low concentrations of nicotine. In addition, nicotine failed to significantly activate VTA DAergic neurons in mice that did not express either  $\alpha 4$  or  $\alpha 6$  nAChR subunits. Conversely, selective activation of nAChRs containing the  $\alpha 4$  subunit in knock-in mice expressing a hypersensitive version of these receptors yielded a biphasic response to nicotine consisting of an acute desensitizing increase in firing frequency followed by a sustained increase that lasted several minutes and was sensitive to  $\alpha$ -conotoxin MII[H9A;L15A]. These data indicate that nicotine persistently activates VTA DAergic neurons via nAChRs containing  $\alpha 4$  and  $\alpha 6$  subunits.

## Introduction

Complications from chronic exposure to cigarette smoke accounts for ~5 million deaths, making nicotine addiction the predominant preventable cause of mortality world-wide (CDC, 2010). Understanding the molecular mechanisms underlying the addictive nature of nicotine should yield new therapeutic targets aimed at facilitating smoking cessation. Nicotine, like all drugs of abuse, acts upon the mesocorticolimbic “reward” circuitry of the brain to initiate dependence (Tapper et al., 2006). Within this circuit, dopaminergic (DAergic) neurons originating in the ventral tegmental area (VTA), project to the nucleus accumbens (NAcc), as well as the prefrontal cortex and hippocampus among other regions (De Biasi and Dani, 2011; Laviolette and van der Kooy, 2004; Placzek et al., 2009). Nicotine dependence is initiated by activation of the VTA, ultimately resulting in DA release in the NAcc (Di Chiara and Imperato, 1988). Acute exposure to nicotine can increase DAergic neuron activity and DA release in NAcc for several minutes to over an hour (Brodie, 1991; De Biasi and Dani, 2011; Dong et al., 2009; Mansvelder et al., 2002; Pidoplichko et al., 2004). Dopamine release in response to nicotine can occur via at least two mechanisms: 1) An increase in baseline DAergic neuron firing frequency and 2) a switch from tonic to burst firing mode (Mameli-Engvall et al., 2006). Within the VTA, DAergic neurons receive local inhibitory input from GABAergic interneurons (Mansvelder et al., 2002). In addition cortical and thalamic projections provide glutamatergic, excitatory input into the VTA (Mansvelder and McGehee, 2000; Pidoplichko et al., 2004). Importantly, neuronal nicotinic acetylcholine receptors (nAChR) that are activated by nicotine as well as the endogenous neurotransmitter, acetylcholine, are robustly expressed in both DAergic and GABAergic

VTA neurons in addition to glutamatergic terminals (Champtiaux et al., 2002; Klink et al., 2001; Wooltorton et al., 2003).

The mechanism by which nAChR activation leads to long lasting DAergic neuronal activity is unclear. While nicotine can directly activate DAergic neurons via high affinity nAChRs (i.e., receptors containing  $\alpha 4$  and  $\beta 2$  subunits, denoted  $\alpha 4\beta 2^*$  nAChRs, \* indicates additional unidentified subunits may also be present), nAChRs containing solely  $\alpha 4$  and  $\beta 2$  subunits desensitize within seconds to a few minutes of exposure to pathologically relevant concentrations of drug (Fisher et al., 1998; Pidoplichko et al., 1997; Pidoplichko et al., 2004). In addition, the nAChR  $\alpha 6$  subunit is robustly expressed in DAergic neurons where it can potentially coassemble with both  $\alpha 4$  and  $\beta 2$  subunits, but its functional role in activation of VTA DAergic neurons by nicotine is unclear (Drenan et al., 2008; Gotti et al., 2010; Pons et al., 2008). Thus, whether the long lasting activity of DAergic neurons in response to nicotine is cell autonomous and involves nAChRs containing the  $\alpha 6$  subunit are open questions.

In this study, we investigated how exposure to pathologically relevant nicotine concentrations can induce long lasting increases in VTA DAergic neuron activity. Our data indicate that nAChR containing  $\alpha 6$  and  $\alpha 4$  subunits can directly drive increases in VTA DAergic neuron activity for extended periods of time.

## Methods

*Animals.* Adult (8-10 weeks), C57BL/6J mice (Jackson Laboratory, West Grove, PA, USA), were used in all experiments, in addition to  $\alpha 4$  KO and  $\alpha 6$  KO homozygous, and Leu9'Ala heterozygous mice as indicated. Leu9'Ala,  $\alpha 4$  KO and  $\alpha 6$  KO lines have been back-crossed to the C57BL/6J strain at least nine generations. The genetic engineering of Leu9'Ala,  $\alpha 4$  KO and  $\alpha 6$  KO lines has been described previously (Champtiaux et al., 2003; Ross et al., 2000; Tapper et al., 2004). Animals were housed four/cage up until the start of each experiment. Animals were kept on a standard 12-h light/dark cycle with lights on at 7:00 AM and off at 7:00 PM. Mice had access to food and water *ad libitum*. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National Research Council, 1996), as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

*Drugs.* Stock solutions of Nicotine hydrogen tartrate, dihydro- $\beta$ -erythroidine (DH $\beta$ E), 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate (CNQX), atropine, bicuculline methylbromide (Sigma-Aldrich), and tetrodotoxin (TTX) (Tocris) were dissolved in distilled water then diluted with artificial cerebrospinal fluid (ACSF). ACSF solution contained (in mM): 125 NaCl, 2.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O, 1.2 MgCl<sub>2</sub>•6H<sub>2</sub>O, 2.4 CaCl<sub>2</sub>•2H<sub>2</sub>O, 26 NaHCO<sub>3</sub>, 11 D-Glucose.  $\alpha$ -conotoxin MII[H9A;L15A] was synthesized as previously described (McIntosh et al., 2004).

*Slice preparation and electrophysiological recordings.* Mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and then decapitated. Brains were quickly removed and placed in an oxygenated ice-cold high sucrose artificial cerebrospinal fluid (SACSF) containing kynurenic acid (1 mM, Sigma-Aldrich). Brain slices (180 - 200  $\mu$ m) were cut using a Leica VT1200 vibratome (Leica Microsystem Inc.). The brain slices were incubated in oxygenated Earl's balanced salt solution (EBSS) supplemented with glutathione (1.5 mg/ml, Sigma), N- $\omega$ -nitro-L-arginine methyl ester hydrochloride (2.2 mg/ml, Sigma), pyruvic acid (11 mg/ml, Sigma) and kynurenic acid (1 mM) for 45 min at 34°C. Slices were transferred into oxygenated ACSF at room temperature for recording. SACSF solution contained (in mM): 250 sucrose, 2.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O, 1.2 MgCl<sub>2</sub>•6H<sub>2</sub>O, 2.4 CaCl<sub>2</sub>•2H<sub>2</sub>O, 26 NaHCO<sub>3</sub>, 11 D-Glucose. Single slices were transferred into a recording chamber continually superfused with oxygenated ACSF. The junction potential between the patch pipette and bath ACSF was nullified just prior to obtaining a seal on the neuronal membrane. Action potentials and currents were recorded at 32°C using the whole-cell configuration of a Multiclamp 700B patch-clamp amplifier (Axon Instruments, Foster City, CA). Action potentials were also measured in cell-attached mode. Action potentials were obtained using a gap-free acquisition mode and Clampex software (Axon Instruments). I<sub>h</sub> currents were elicited every 5 sec by stepping from -60 mV to a test potential of -120 mV for 1 s using Clampex. Input resistances were calculated using steady state currents elicited by 5 mV hyperpolarizing pulses. Signals were filtered at 1 kHz using the amplifier's four-pole, low-pass Bessel filter, digitized at 10 kHz with an Axon Digidata 1440A interface and stored on a personal computer. Potential DAergic neurons were selected for recording, initially

based on neuroanatomical region and soma shape. Action potential frequency ( $< 8$  hz) and  $I_h$  expression were also used for identification of neuronal identity. In addition, at the end of recording, the neuron cytoplasm was aspirated into the recording pipette and the contents were expelled into a microcentrifuge tube containing 75% ice-cold ethanol and stored at  $-20^{\circ}\text{C}$  for at least 2 h before single-cell RT-PCR experiments to verify expression of TH. Non-TH expressing neurons were excluded from analysis. DAergic neurons from the posterior portion of the VTA were recorded as previous data indicates that these are most sensitive to activation by nicotine (Zhao-Shea et al., 2011). Pipette solution contained (in mM): 121 KCl, 4  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 11 Ethylene glycol-bis(2-aminoethylether)- $N,N,N',N'$ -tetraacetic acid (EGTA), 1  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 10 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 0.2 guanosine 5'-triphosphate (GTP), and 4 adenosine 5'-triphosphate (ATP). Pipette solution was made using sterile-filtered diethyl pyrocarbonate (DEPC) treated distilled water. Drugs were applied onto slices by gravity superfusion. Action potentials were recorded in the presence of a cocktail of inhibitors including atropine ( $1 \mu\text{M}$ ) to block muscarinic receptors, bicuculline ( $20 \mu\text{M}$ ) to block  $\text{GABA}_A$  receptors, and CNQX ( $10 \mu\text{M}$ ) to block AMPA receptors. Whole-cell responses to nicotine under voltage clamp were recorded in the presence of the blockers above in addition to TTX ( $0.5 \mu\text{M}$ ).

#### *Data analysis.*

Summary data are presented as means  $\pm$  standard errors of means (SEM). For multiple comparisons, statistical significance was determined using GraphPad Prism 5 software by One-Way ANOVA followed by Tukey post hoc tests. A Paired T-test was



used to analyze differences between control and drug group. Results were considered significant at  $p < 0.05$ .

## Results

### **Nicotine directly activates VTA DAergic neurons for several minutes.**

To examine the effects of acute nicotine on VTA DAergic neuron firing rates, we recorded action potentials in current clamp mode from DAergic neurons in mesocortical slices containing the VTA at baseline and during and after 5 min. bath exposure to 1  $\mu$ M nicotine. DAergic neurons were identified by a slow firing frequency ( $< 8$  Hz, Fig. 1A), expression of a hyperpolarizing activated current  $I_h$  (Fig.1B), and expression of tyrosine hydroxylase (Fig. 1C) as described previously (Zhao-Shea et al., 2011). In addition, action potentials were recorded in the presence of a cocktail of inhibitors to block muscarinic receptors, GABA<sub>A</sub> receptors, and glutamatergic signaling (see methods) in an effort to facilitate isolation of nicotine effects directly mediated by nAChRs. A 5 min. application of nicotine elicited a long lasting (~12 min.) reversible increase in firing frequency (Fig. 2A, B). On average nicotine elicited a significant  $2.32 \pm 0.4$  fold increase in firing frequency compared to baseline ( $p < 0.01$ , paired t-test,  $n = 7$ , Fig. 2C). Nicotine exposure can elicit lasting increases on DAergic neuron firing rate for several minutes up to an hour. To test the hypothesis that nicotine concentrations experienced by smokers could directly increase DAergic neuron firing frequency, we again measured action potential frequency, but this time in response to a lower pathologically relevant nicotine concentration, 300 nM (Fig. 3A). In addition, we measured DAergic neuron activity using cell-attached recordings so as not to perturb the intracellular milieu. A 30 minute bath application of nicotine resulted in a persistent and significant increase in DAergic neuron firing that returned to baseline after washout

(Fig. 3A, B). Overall nicotine elicited a statistically significant increase in firing frequency (Repeated Measure One-way ANOVA,  $F_{(3,23)} = 6.45$ ,  $p < 0.01$ ,  $n = 6$ ). Tukey post-hoc tests revealed that DAergic neuron activity was significantly increased after 10 and 30 min nicotine exposure compared to firing frequency both at baseline and after nicotine washout ( $p < 0.05$ , Fig. 3B). Importantly, there was no statistically significant difference between firing rates regardless of nicotine exposure time. These data indicate that a sustained single bolus of nicotine at pathologically relevant concentrations can persistently activate DAergic neurons for several (i.e. 30 – 60) minutes.

#### **VTA DAergic neurons express desensitization-resistant nAChRs containing $\alpha 4$ and $\alpha 6$ subunits.**

To test the hypothesis that nicotine persistently activated DAergic neurons directly via desensitization-resistant nAChRs, we recorded whole-cell responses to 300 nM nicotine exposure in VTA DAergic neurons under voltage clamp. Nicotine elicited a significant inward current that persisted over the course of 10 min. exposure to drug (Fig. 3C, E; average current amplitude  $-21.5 \pm 8.8$  pA and  $-19.2 \pm 7.4$  pA after 5 and 10 min. nicotine exposure, respectively,  $n = 8$ ). Furthermore, there was a significant increase in RMS current noise after 10 min. nicotine compared to baseline prior to drug perfusion ( $5.48 \pm 0.64$  pA compared to  $4.26 \pm 0.25$  pA, respectively,  $p < 0.05$  paired t-test), presumably generated by an increase of randomly gating nAChR channels. As we previously had determined that nAChRs containing  $\alpha 4$  and  $\alpha 6$  subunits were expressed in VTA DAergic neurons (Zhao-Shea et al., 2011), we tested the sensitivity of nicotine-

induced current to the  $\alpha 6^*$  nAChR selective antagonist,  $\alpha$ -conotoxin MII[H9A;L15A]. During the initial 5 min. application of drug, nicotine elicited an inward current and this current was significantly attenuated during application of  $\alpha$ -conotoxin MII[H9A;L15A] (Fig. 3D, F,  $p < 0.01$ , paired t-test,  $n = 5$ ). Together, these data indicate that nicotine can persistently depolarize DAergic neurons via  $\alpha 6^*$  nAChRs.

To determine if these same receptors were responsible for our observed nicotine-induced increase in DAergic neuron firing frequency, we recorded VTA DAergic neuron activity in the cell-attached configuration in WT,  $\alpha 4$  KO, and  $\alpha 6$  KO mice. Average baseline firing frequency did not significantly differ between mouse models ( $5.0 \pm 1.8$ ,  $4.4 \pm 1.1$ , and  $4.0 \pm 1.0$  Hz in WT,  $\alpha 4$  KO, and  $\alpha 6$  KO mice, respectively,  $n = 6$  neurons/genotype). In WT animals, 300 nM nicotine persistently and significantly increased DAergic neuron firing rate (Fig. 4A,  $p < 0.05$ , paired t-test,  $n = 6$ ). Application of  $\alpha$ -conotoxin MII returned the increased firing of DAergic neurons in response to nicotine to baseline (Fig. 4A, right). Interestingly, in the presence of nicotine, 4 of 6 neurons decreased DAergic neuron firing frequency below baseline during application of  $\alpha$ -conotoxin MII[H9A;L15A] suggesting, perhaps, a role for ACh in tonic activation of DAergic neurons in our slice prep. However, when  $\alpha$ -conotoxin MII[H9A;L15A] was applied to a separate group of VTA DAergic neurons in the absence of nicotine, no significant change in baseline firing rate was observed (firing rate of DAergic neurons in the presence of  $\alpha$ -conotoxin MII[H9A;L15A] =  $103.4 \pm 0.2$  % of control,  $n = 6$ ) indicating that blockade of  $\alpha 6^*$  nAChRs unmasked an inhibitory effect of nicotine in a portion of VTA DAergic neurons. In  $\alpha 4$  KO mice, nicotine did not significantly increase DAergic

neuron activity (Fig. 4B). Similarly, nicotine did not yield a significant increase in DAergic neuron firing in  $\alpha 6$  KO mice (Fig. 4C,  $1.20 \pm 0.063$  fold compared to baseline,  $n = 6$ ) compared to WT. Together these data indicate that the persistent activation of VTA DAergic neurons arises mostly from nicotinic receptors containing  $\alpha 4$  and  $\alpha 6$  subunits.

### **Selective activation of $\alpha 4^*$ nAChRs reveals two nAChR subtypes.**

To test the hypothesis that selective activation of  $\alpha 4^*$  nAChRs was sufficient to drive nicotine-induced activation of VTA DAergic neurons, we recorded effects of nicotine on DAergic neurons in VTA slices from mice expressing an  $\alpha 4$  point mutant, Leu9'Ala, that renders  $\alpha 4^*$  nAChRs 50-fold more sensitive to nicotine compared to wild-type DAergic neurons (Tapper et al., 2004). As previously reported, we did not observe differences in baseline firing frequency between Leu9'Ala and WT VTA DAergic neurons (Tapper et al., 2004). Bath-application of 50 nM nicotine, a concentration that did not significantly increase firing of WT VTA DAergic neurons (Fig. 5C, D), dramatically altered the firing frequency of VTA DAergic neurons in Leu9'Ala mice. The response to nicotine in Leu9'Ala DAergic neurons was biphasic consisting of an early acute phase that desensitized in the presence of agonist within ~2 minutes followed by a sustained, late increase in firing frequency compared to baseline (early peak,  $p < 0.01$  compared to baseline; late sustained phase,  $p < 0.01$  compared to baseline,  $n = 7$ , Fig. 5A, D). To determine if  $\alpha 4^*$  nAChRs mediating the persistent firing phase of the nicotine response in Leu9'Ala DAergic neurons also contained the  $\alpha 6$  subunit, we co-applied  $\alpha$ -conotoxin MII[H9A;L15A] with nicotine during the sustained firing response to nicotine.  $\alpha$ -conotoxin MII[H9A;L15A] significantly reduced the sustained firing frequency of Leu9'Ala

VTA DAergic neurons ( $p < 0.05$ ,  $n = 7$ , Fig. 5A, D). As in WT VTA DAergic neurons  $\alpha$ -conotoxin MII[H9A;L15A] alone did not significantly modulate baseline firing frequency (firing rate of DAergic neurons in the presence of  $\alpha$ -conotoxin MII[H9A;L15A] =  $99.5 \pm 0.15$  % of control,  $n = 4$ ). In addition, co-application of  $\alpha$ -conotoxin MII[H9A;L15A] and the  $\beta_2^*$  competitive antagonist, DH $\beta$ E, completely blocked both acute and sustained activation of Leu9'Ala VTA DAergic neurons by nicotine (Fig. 5B, D).

## Discussion

Nicotine initiates dependence by activating the VTA ultimately resulting in an increase in DA concentration in NAcc, a phenomenon both necessary and sufficient for reinforcement (Corrigall and Coen, 1991; Corrigall et al., 1994; Corrigall et al., 1992; Tsai et al., 2009). While previous work has clearly implicated that nAChR expression and activation in the VTA is critical for nicotine reward (Picciotto et al., 1998; Tapper et al., 2004), the mechanism of how nicotine may activate DAergic neurons for extended periods of time has remained elusive. It has been proposed that low, nM concentrations of nicotine that are routinely self-administered by smokers desensitize high affinity nAChRs almost completely within seconds to a few minutes (Mansvelder et al., 2002; Pidoplichko et al., 1997). Here we show that a physiologically relevant concentration of nicotine elicited a persistent inward current in VTA DAergic neurons even in the presence of TTX and CNQX which blocks action potential and AMPA receptor-dependent signaling, respectively, indicating that the whole-cell current was carried by nAChRs expressed in VTA DAergic neurons. In addition, nicotine-induced current was blocked by an  $\alpha 6$  nAChR-selective antagonist. This inward current was sufficient to drive an increase in VTA DAergic neuron activity for over 30 minutes. Expression of  $\alpha 4^*$  nAChRs was critical for this effect of nicotine as the drug failed to elicit an increase in firing frequency in  $\alpha 4$  KO mice, further supporting our previous study that found that bath application of up to 1  $\mu$ M nicotine does not elicit an inward current in DAergic neurons from these animals (Zhao-Shea et al., 2011). Furthermore, nicotine failed to significantly increase DAergic neuron firing in  $\alpha 6$  KO mice. Interestingly, selective

activation of  $\alpha 4^*$  nAChRs in VTA DAergic neurons from Leu9'Ala mice yielded a biphasic response to nicotine consisting of a rapid, robust increase in firing frequency that desensitized quickly followed by a sustained increase in firing frequency. The sustained increase in firing frequency was sensitive to blockade by an  $\alpha 6$  selective antagonist indicating that  $\alpha 4\alpha 6^*$  nAChRs underlie the sustained increase in nicotine induced firing. Both responses could be inhibited by pre-exposure of DH $\beta$ E and  $\alpha$ -conotoxin MII[H9A;L15A] suggesting at least two distinct nAChR subtypes may contribute to nicotine's mechanism of action. While we hypothesize that the acute desensitizing nicotine response in Leu9'Ala DAergic neurons may be mediated by  $\alpha 4$ non- $\alpha 6\beta 2^*$  nAChRs, this interpretation may be confounded by the Leu9'Ala  $\alpha 4$  subunit mutation which may confer slower desensitization properties to  $\alpha 4\beta 2^*$  nAChRs compared to WT (Labarca et al., 1995). In addition, DH $\beta$ E is not truly specific for  $\alpha 4\beta 2$  nAChRs as it can also block  $\alpha 6^*$  nAChRs (Drenan et al., 2008), precluding identification of functional nAChRs containing only  $\alpha 4$  and  $\beta 2$  subunits. Previously, we have shown that nAChRs containing  $\alpha 4$  and  $\alpha 6$  subunits are critical for activation of DAergic neurons in the VTA in response to rewarding nicotine doses (i.e., doses that condition a place preference)(Zhao-Shea et al., 2011). This is also supported by studies measuring nicotine-induced DA release from striatal synaptasomes, which indicate that nAChRs containing  $\alpha 4$  and  $\alpha 6$  subunits have the highest affinity for nicotine recorded to date with an EC<sub>50</sub> of ~230 nM (Salminen et al., 2007), well within the range of nicotine blood concentrations achieved by smoking (Russell et al., 1980), as opposed to nAChRs containing (non- $\alpha 4$ ) $\alpha 6\beta 2$  nAChRs which have an EC<sub>50</sub> of 1.52  $\mu$ M nicotine.



A variety of mouse models have been used to gain insight into the potential composition of nAChRs critical for activation of DAergic neurons by nicotine and nicotine reinforcement. Mice that do not express nAChRs containing the  $\alpha 4$ ,  $\alpha 6$ , or  $\beta 2$  subunit fail to self-administer nicotine (Maskos et al., 2005; Picciotto et al., 1998; Pons et al., 2008). Re-expression of each subunit in the VTA of the respective knock-out (KO) mouse rescues nicotine self-administration indicating that activation of nicotinic receptors containing  $\alpha 4$ ,  $\alpha 6$ , and/or  $\beta 2$  subunits, specifically in the VTA, are sufficient for nicotine reinforcement (Maskos et al., 2005; Pons et al., 2008). While  $\alpha 4$  and  $\beta 2$  nAChR subunits are expressed in both VTA DAergic and GABAergic neurons, expression of  $\alpha 6$  subunits have predominantly been localized to DAergic neurons (Champtiaux et al., 2003; Drenan et al., 2008; Grady et al., 2007), although a recent report indicates they may also be expressed in GABAergic presynaptic boutons (Yang et al., 2011). More recently, Exley et al. (2011) examined intracranial nicotine self-administration in WT,  $\alpha 4$  KO, and  $\alpha 6$  KO mice. Both WT and  $\alpha 6$  KO mice self-administered high doses of nicotine (50 nL of 4 mM nicotine/infusion or 100 ng/infusion) directly into the VTA; whereas  $\alpha 4$  KO mice did not sustain nicotine self-administration for extended daily sessions (Exley et al., 2011). However,  $\alpha 6$  KO mice self-administered less nicotine than WT if the nicotine concentration was decreased 10-fold. These data indicate that  $\alpha 6^*$  nAChRs are critical for self-administration of lower nicotine doses and in combination with the present study, would indicate that nAChRs containing both  $\alpha 4$  and  $\alpha 6$  subunits are expected to mediate nicotine reward in response to nM concentrations of the drug. However, the authors suggest that nAChRs

containing both  $\alpha 4$  and  $\alpha 6$  subunits are not functionally expressed in VTA DAergic neuron soma, rather they are only expressed at presynaptic terminals. This is based on firing patterns of DAergic neurons *in vivo* in response to intravenous nicotine: Acute intravenous nicotine failed to induce an increase in the number of spikes within a burst (SWB) in  $\alpha 4$  KO VTA DAergic neurons; whereas nicotine did modestly increase SWB in both WT and  $\alpha 6$  KO mice. Re-expression of the  $\alpha 4$  subunit in the VTA rescued this effect of nicotine indicating that  $\alpha 4(\text{non-}\alpha 6)^*$  nAChRs are critical for nicotine-induced increases in SWB. However, nicotine increased the basal firing rate of VTA DAergic neurons in all three mouse lines and was only abolished in  $\alpha 4\alpha 6$  double KO animals, supporting our data that clearly indicates that these receptors can directly contribute to nicotine-induced increases in DAergic neuron activity. This is also supported by immunoprecipitation studies in rat which indicate that DAergic neurons within ventral midbrain express  $\alpha 4\alpha 6\beta 2\beta 3$  nAChRs (~21.4 % of  $\beta 2^*$  nAChRs); whereas (non- $\alpha 4$ ) $\alpha 6\beta 2\beta 3^*$  nAChRs are undetectable (Gotti et al., 2010). One limitation to our study is that it provides little insight into the contribution of nAChR subtypes that are involved in nicotine burst activity because we observe little bursting in DAergic neurons within midbrain slices. This is likely due to the fact that DAergic neuron bursting in response to concentrations of nicotine achieved by smoking requires other cholinergic and/or non-cholinergic inputs which are severed in our slice preparation, and presumably require glutamatergic signaling, which we also block in our experiments (Ishibashi et al., 2009; Lodge and Grace, 2006; Mansvelder et al., 2002).

Desensitization-resistant nAChRs are an emerging subtype of receptor. Recently, two classes of neurons have been found to express desensitization-resistant nAChRs that allow for long lasting increases in neuronal activity in response to nicotine. These include horizontally oriented interneurons in the stratum oriens/alveus and pyramidal neurons in layer VI of the prefrontal cortex (Bailey et al., 2010; Jia et al., 2009). Although the precise subunit compositions of these receptors are unknown, the  $\alpha 2$  subunit is a component of the desensitization resistant nAChR in stratum oriens horizontally oriented interneurons (Jia et al., 2009), while expression of  $\alpha 5$  nAChR subunits are required for desensitization resistant nicotine-induced currents in layer VI pyramidal neurons (Bailey et al., 2010). These nAChRs also presumably contain  $\alpha 4$  and  $\beta 2$  subunits because currents were blocked with the  $\alpha 4\beta 2$  competitive antagonist, dihydro- $\beta$ -erythroidine. Our data indicate that the assembly of  $\alpha 6$  subunits with  $\alpha 4$  subunits may be sufficient to confer nAChRs with resistance to nicotine-induced desensitization.

Previous studies have identified potential mechanisms to account for how nicotine drives activation of VTA DAergic neurons. Using a biophysical approach in midbrain slices, Mansvelder et. al. found that high affinity nAChRs in VTA GABAergic interneurons rapidly desensitize to disinhibit DAergic neurons (Mansvelder et al., 2002; Mansvelder and McGehee, 2002). In addition,  $\alpha 7$  nAChRs expressed in glutamatergic presynaptic terminals that innervate the VTA recover more rapidly from nicotine-induced desensitization allowing for persistent increases in glutamate release and excitation of VTA DAergic neurons. In the context of this model, our data provide an additional

mechanism. On the order of seconds, nicotine activates high affinity  $\alpha 4\beta 2^*$  nAChRs expressed on both GABAergic and DAergic VTA neurons. Within seconds to a few minutes, nicotine desensitizes these receptors (Mansvelder et al., 2002; Pidoplichko et al., 1997). This is followed by nicotine-induced sustained activation of  $\alpha 4\alpha 6^*$  nAChRs expressed in DAergic neurons. Together with a persistent glutamatergic drive mediated by recovery from desensitization of presynaptic  $\alpha 7$  nAChRs, these two mechanisms allow nicotine to activate VTA DAergic neurons for several minutes up to an hour.

In summary, our data provide a mechanistic explanation for how nicotine concentrations achieved by smoking can increase and maintain VTA DAergic neuron activity. VTA DAergic neurons express functional  $\alpha 4\alpha 6^*$  nAChRs that allow nicotine to depolarize and persistently activate VTA DAergic neurons for extended periods of time. Thus, this nAChR subtype is a prime candidate for therapeutics aimed at facilitating smoking cessation.

## **Authorship Contribution**

*Participated in research design:* Liu, Zhao-Shea, McIntosh, Gardner, and Tapper.

*Conducted experiments:* Liu and Zhao-Shea.

*Contributed new reagents or analytic tools:* McIntosh

*Performed data analysis:* Liu and Tapper.

*Wrote or contributed to the writing of the manuscript:* Liu, Zhao-Shea, McIntosh, Gardner, and Tapper.

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## Footnotes

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## Figure Legends

Figure 1. Characterization of VTA DAergic neurons. A) Representative cell-attached recording from a putative DAergic neuron. DAergic neurons had characteristic low baseline firing frequencies (< 8 Hz) and B) expressed the hyperpolarizing activated cation current,  $I_H$ . Currents were elicited by 20 mV hyperpolarizing steps from a holding potential of -60 mV to -120 mV. C) At the end of each recording, the content of each neuron was aspirated into the patch pipette and TH expression was verified by single-cell RT-PCR. A representative DNA agarose gel is shown illustrating a typical result for a DAergic neuron. Only neurons that clearly expressed TH (arrow) and not GAD 65/67 were included in the analysis.

Figure 2. Nicotine activation of VTA DAergic neurons. A. Representative events frequency histogram from a VTA DAergic neuron before, during, and after bath application of 1  $\mu$ M nicotine (black bar). Current clamp ( $I=0$ ) responses were recorded from visually identified VTA DAergic neurons in C57Bl/6J mesocortical coronal slices. Note that nicotine increases DAergic neuron firing frequency for several minutes during and after initial exposure. B. Representative current recordings taken from a) baseline, b) 5 min. post nicotine exposure, and c) 12 min. post nicotine exposure. C. Change in average DAergic neuron action potential frequency in response to nicotine (grey bar, 5 min. application) and after several minute wash with ACSF (white bar) compared to baseline (dotted line). \*\*  $p < 0.01$

Figure 3. Direct and persistent activation of VTA DAergic neurons by nicotine. A. Representative action potential firing frequency histogram from a VTA DAergic neuron

before, during, and after 30 min. bath application of 300 nM nicotine. Action potentials were recorded in cell attached mode. B. Change in average firing frequency compared to baseline (1 min. prior to nicotine application, dotted line) 10 min. and 30 min. after nicotine application, and after 20 min washout. \*  $p < 0.05$ , One-way ANOVA, Tukey post-hoc,  $n = 6$ . C. Representative whole-cell response to 300 nM nicotine in a VTA DAergic neuron. The neuron was voltage-clamped at -60 mV and 300 nM nicotine was bath applied for 10 minutes (grey bar). The red dotted line represents baseline current extrapolated from the recording prior to nicotine application. Responses were recorded in the presence of a cocktail of inhibitors. D. Representative whole-cell response to 300 nM nicotine (grey bar) in the presence and absence of 100 nM  $\alpha$ -conotoxin MII[H9A;L15A] (blue bar). E. Average current amplitude minus baseline during 1.5, 5, and 9 min. exposure to 300 nM nicotine.  $n = 8$  neurons. F. Average current amplitude minus baseline after a 5 min. exposure to 300 nM nicotine (white bar) and after an additional 5 minute exposure of nicotine + 100 nM  $\alpha$ -conotoxin MII[H9A;L15A]. \*\*  $p < 0.01$ , compared to nicotine-induced current,  $n = 5$  neurons/treatment.

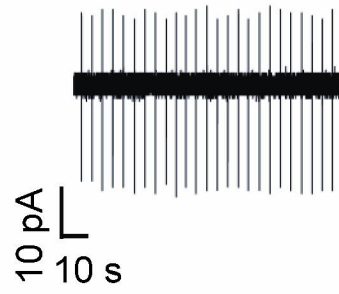
Figure 4. The contribution of  $\alpha 4$  and  $\alpha 6$  subunits to nicotine-induced increases in DAergic neuron firing frequency. A. (Left) Representative action potential firing frequency histogram from a VTA DAergic neuron in a WT midbrain slice. Nicotine (300 nM) was applied over the times indicated by the grey bar. After 5 min. of nicotine application, 100 nM  $\alpha$ -conotoxin MII[H9A;L15A] was applied concomitantly with nicotine. (Right) Change in average firing frequency compared to baseline (dotted line) 5 min. after nicotine exposure and after an additional 5 min. exposure to both nicotine and  $\alpha$ -

contoxin MII[H9A;L15A]. \*  $p < 0.05$ , compared to nicotine,  $n = 6$ . B. (Left) Representative action potential firing frequency histogram from a VTA DAergic neuron in an  $\alpha 4$  KO midbrain slice. (Right) Average firing frequency fold change compared to baseline (dotted line) 5 min. after nicotine exposure and after an additional 5 min. wash period. C. (Left) Representative action potential firing frequency histogram from a VTA DAergic neuron in an  $\alpha 6$  KO midbrain slice. (Right) Average firing frequency fold change compared to baseline (dotted line) 5 min. after nicotine exposure and after an additional 5 min. wash period.  $n = 6$  neurons/genotype.

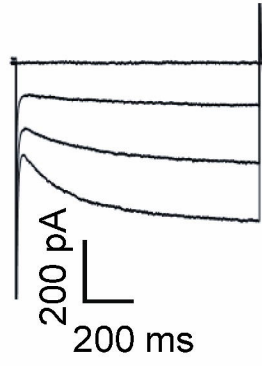
Figure 5. Selective activation of  $\alpha 4^*$  nAChRs reveals two receptor subtypes mediating nicotine-induced activation of VTA DAergic neurons. A. Representative action potential firing frequency histogram from a VTA DAergic neuron in a Leu9'Ala midbrain slice. Nicotine (50 nM) was applied over the times indicated by the grey bar. After 5 min. of nicotine application, 100 nM  $\alpha$ -contoxin MII[H9A;L15A] was applied concomitantly with nicotine (black bar). Representative cell-attached recordings corresponding to individual time points before, during, and after drug treatment are illustrated below the graph. B. Representative action potential firing frequency histogram from a VTA DAergic neuron in a Leu9'Ala midbrain slice. Nicotine was applied in the presence of 100 nM  $\alpha$ -contoxin MII[H9A;L15A] and 100 nM DH $\beta$ E. Cell-attached recordings are shown below the histogram as in panel A. C. Representative action potential firing frequency histogram from a VTA DAergic neuron from a WT animal. 50 nM nicotine was applied to the slice as in A. Cell-attached recordings are shown below the histogram as in panel A. D. Average firing frequency fold change compared to baseline

(dotted line) in response to 50 nM nicotine in DAergic neurons from WT and Leu9'Ala slices in experiments depicted in panels A-C. The early phase of the nicotine response (E) is defined as the peak fold change produced by nicotine compared to baseline within the first 2-3 min. of application; whereas the late phase of the nicotine response (L) is defined as the fold change produced by nicotine compared to baseline after 5 min. application of drug.

**A**



**B**



**C**

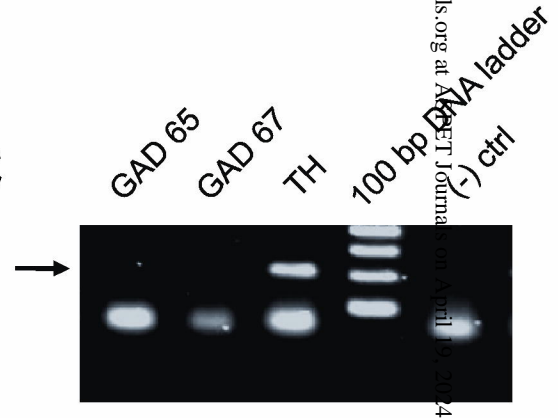


Figure 1

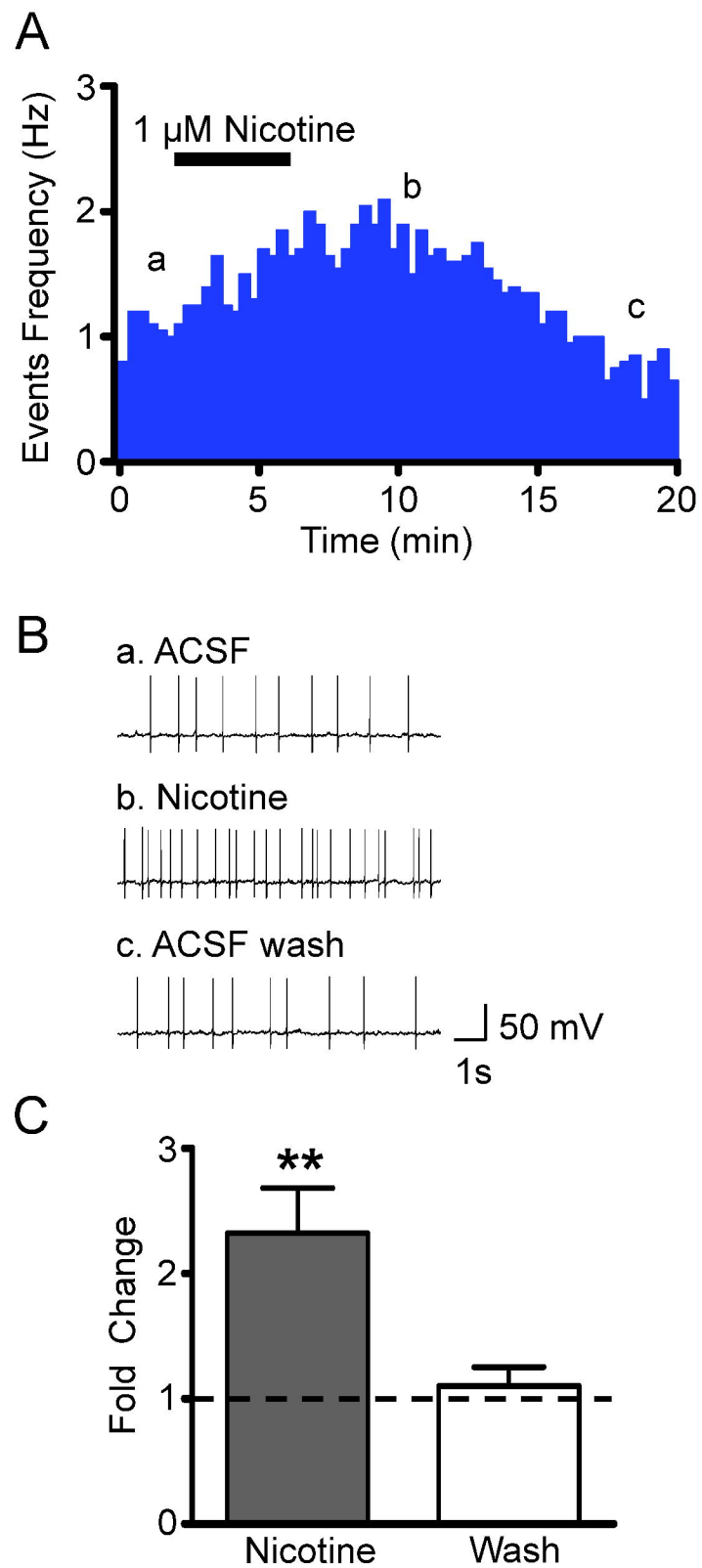


Figure 2

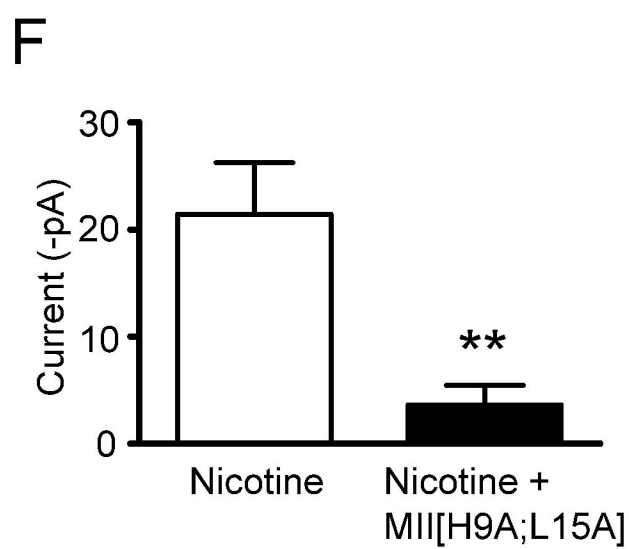
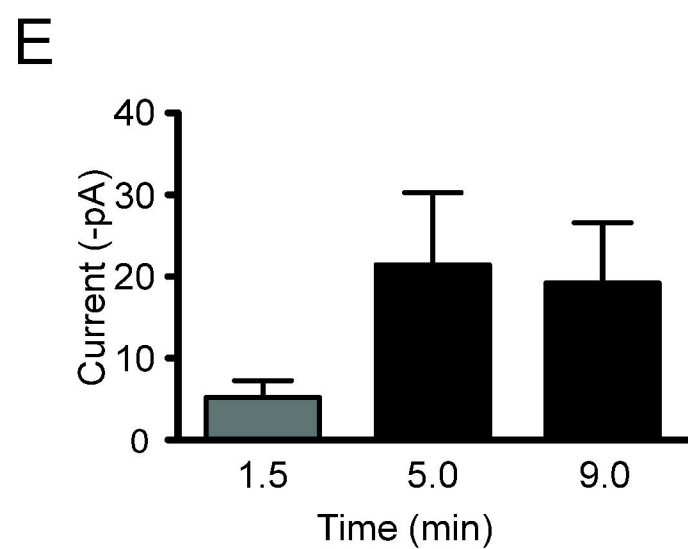
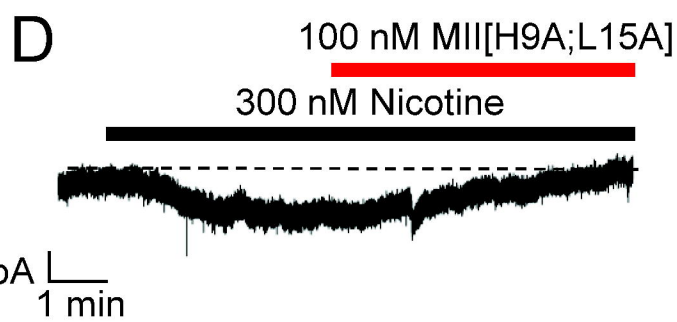
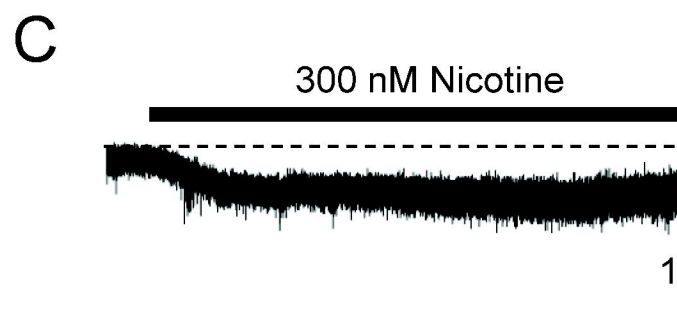
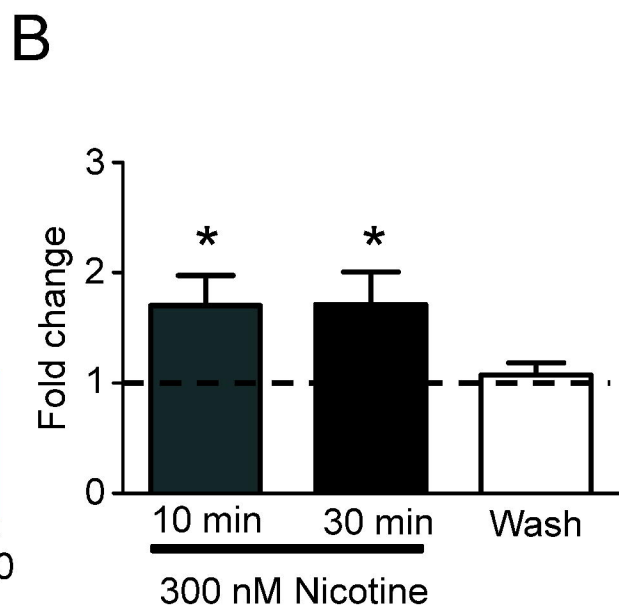
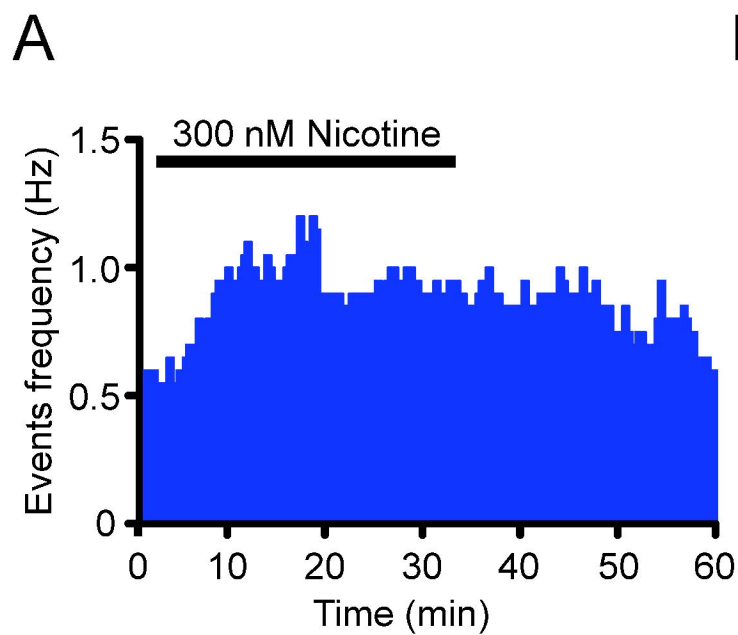
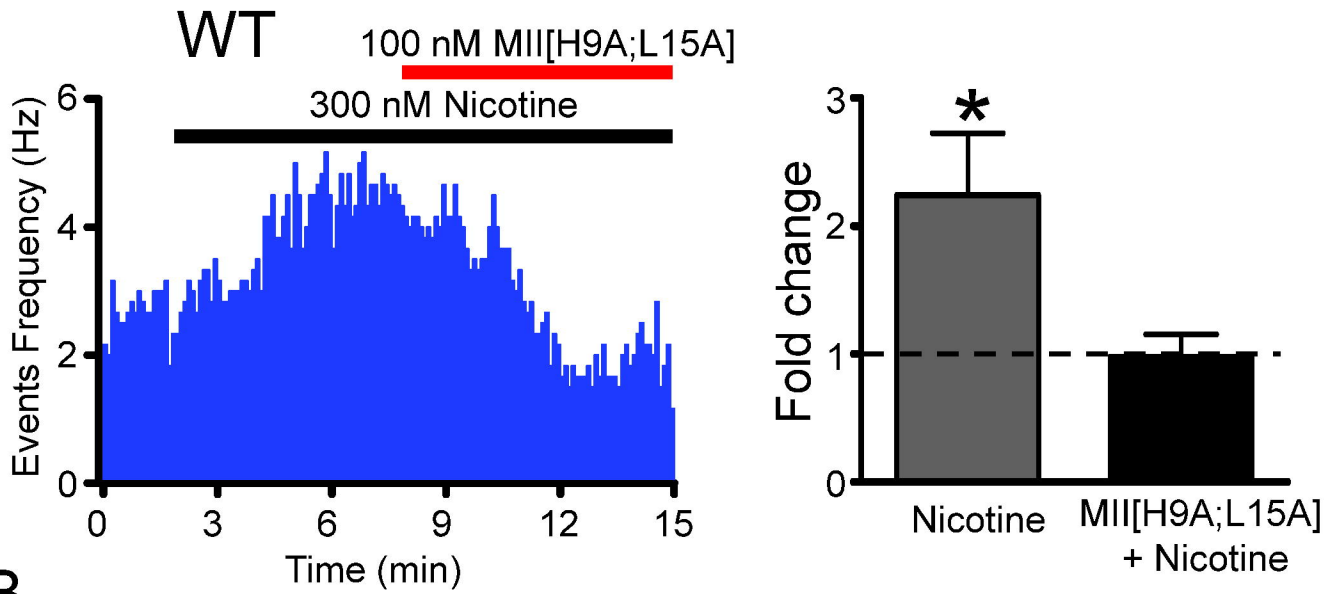


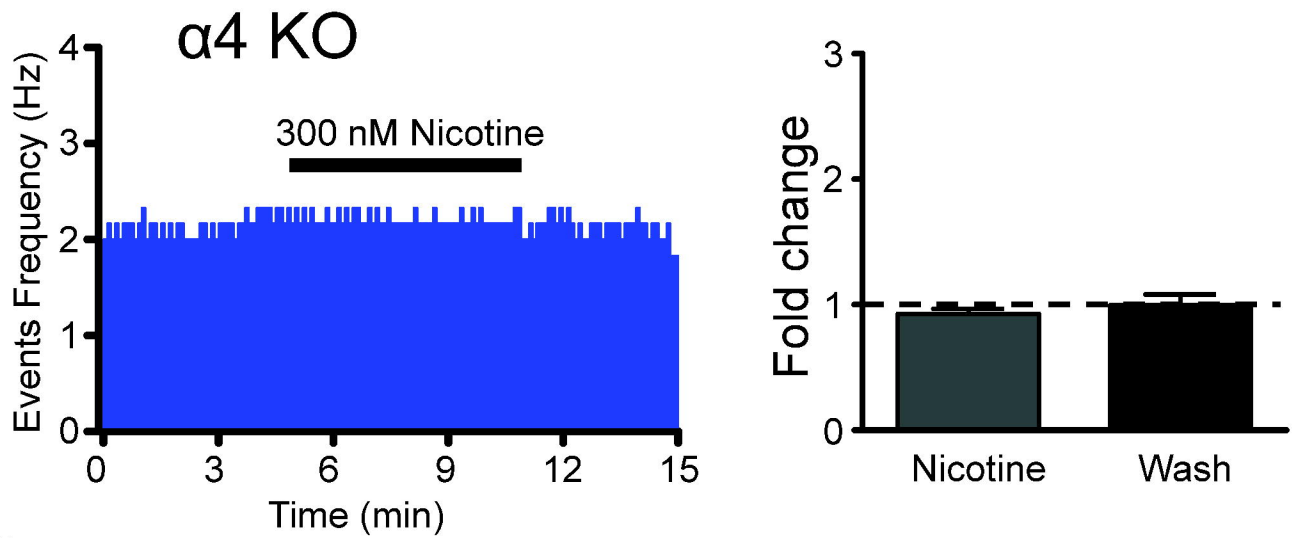
Figure 3



A



B



C

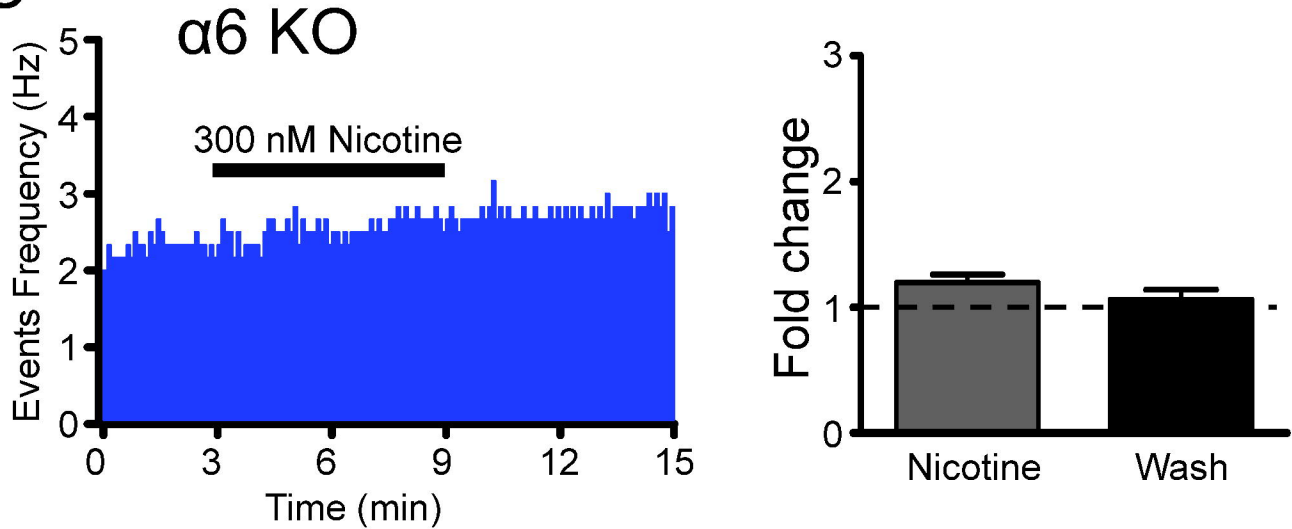


Figure 4

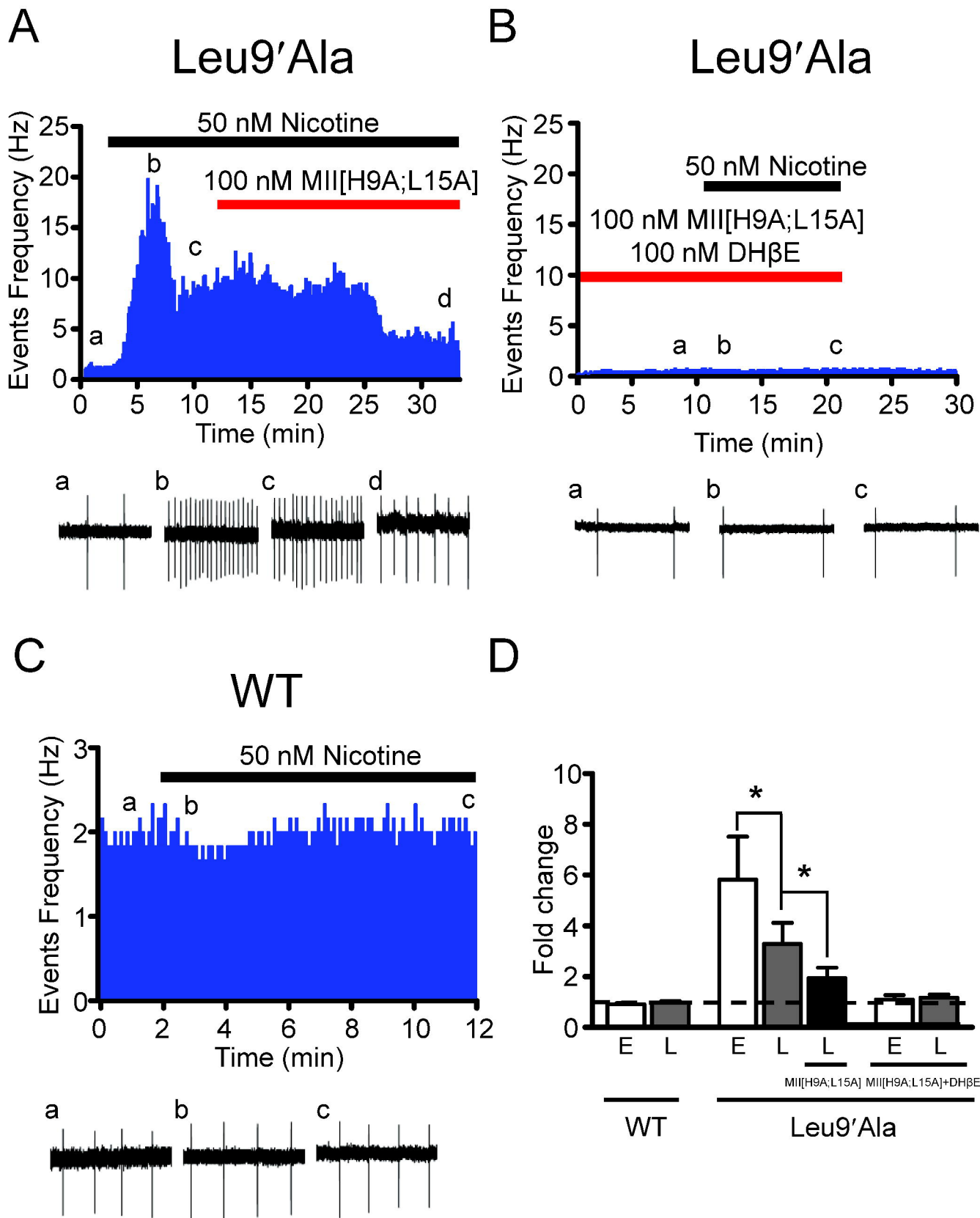


Figure 5