Functional Upregulation of $\alpha 4^*$ Nicotinic Acetylcholine Receptors in VTA GABAergic Neurons Increases Sensitivity to Nicotine Reward

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Chronic nicotine exposure increases sensitivity to nicotine reward during a withdrawal period, which may facilitate relapse in abstinent smokers, yet the molecular neuroadaptation(s) that contribute to this phenomenon are unknown. Interestingly, chronic nicotine use induces functional upregulation of nicotinic acetylcholine receptors (nAChRs) in the mesocorticolimbic reward pathway potentially linking upregulation to increased drug sensitivity. In the ventral tegmental area (VTA), functional upregulation of nAChRs containing the $\alpha 4$ subunit ($\alpha 4^*$ nAChRs) is restricted to GABAergic neurons. To test the hypothesis that increased functional expression of $\alpha 4^*$ nAChRs in these neurons modulates nicotine reward behaviors, we engineered a Cre recombinase-dependent gene expression system to selectively express $\alpha 4$ nAChR subunits harboring a “gain-of-function” mutation [a leucine mutated to a serine residue at the 9' position (Leu9'Ser)] in VTA GABAergic neurons of adult mice. In mice expressing Leu9'Ser $\alpha 4$ nAChR subunits in VTA GABAergic neurons (Gad2^{VTA}:Leu9'Ser mice), subreward threshold doses of nicotine were sufficient to selectively activate VTA GABAergic neurons and elicit acute hypolocomotion, with subsequent nicotine exposures eliciting tolerance to this effect, compared to control animals. In the conditioned place preference procedure, nicotine was sufficient to condition a significant place preference in Gad2^{VTA}:Leu9'Ser mice at low nicotine doses that failed to condition control animals. Together, these data indicate that functional upregulation of $\alpha 4^*$ nAChRs in VTA GABAergic neurons confers increased sensitivity to nicotine reward and points to nAChR subtypes specifically expressed in GABAergic VTA neurons as molecular targets for smoking cessation therapeutics.

Key words: GABA; nicotine; nicotinic receptor; reward

Introduction

Chronic exposure to tobacco smoke accounts for ~5 million deaths per year, making health complications from smoking the primary cause of preventable mortality in the world (Harris and Anthenelli, 2005). Nicotine, the addictive component of tobacco, binds to and activates neuronal nicotinic acetylcholine receptors (nAChRs), ligand-gated cation channels that are normally activated by the endogenous neurotransmitter, acetylcholine (ACh). Nicotine initiates dependence by activating neurons within the ventral tegmental area (VTA) of the mesocorticolimbic reward circuitry, ultimately driving the release of dopamine (DA) within the nucleus accumbens (NAc), a phenomenon widely associated with the rewarding or reinforcing value of nicotine (De Biasi and Dani, 2011). A large variety of nAChR subunit genes are ex-

pressed in both VTA DAergic projection neurons and GABAergic neurons (Klink et al., 2001; Wooltorton et al., 2003).

Neuronal nAChRs are pentameric receptors consisting of homologous or heterologous combinations of subunits. Twelve genes encoding 12 individual nAChR subunits have been identified, accounting for a vast array of nAChR subtypes each with distinct pharmacological and biophysical properties. A great deal of effort has focused on identifying nAChR subtype expression within the VTA to determine which subtypes, when activated, are necessary and sufficient for nicotine reinforcement and/or reward (Picciotto et al., 1998; Tapper et al., 2004; Maskos et al., 2005; Pons et al., 2008). From these studies, a general consensus is that expression of nAChRs containing $\alpha 4$ and $\beta 2$ subunits in the VTA are both necessary and sufficient for nicotine reinforcement with at least some contribution of the $\alpha 6$ subunit (Pons et al., 2008; Brunzell et al., 2010; Gotti et al., 2010). However, $\alpha 4$ and $\beta 2$ subunits are expressed in both VTA DAergic neurons and GABAergic neurons (Klink et al., 2001; Wooltorton et al., 2003). Although nicotine can directly activate VTA DAergic neurons, previous studies suggest that activation of GABAergic neurons may also modulate DAergic neuron activity and is required for nicotine reinforcement (Tolu et al., 2012).

Unlike other drugs of abuse, chronic use of nicotine leads to increased expression or “upregulation” of $\alpha 4\beta 2^*$ nAChRs (the

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asterisk denotes that nAChR subunits in addition to $\alpha 4$ and $\beta 2$ may be assembled in the nAChR complex) in the mesocortico- limbic pathways in addition to other brain regions. Although upregulation of nAChRs is a hallmark of chronic nicotine exposure, the behavioral consequence of this phenomenon and how it relates to nicotine dependence is unknown (Wonnacott, 1990). Interestingly, functional upregulation of $\alpha 4^*$ nAChRs in the mesolimbic pathway appears to be restricted to midbrain GABAergic neurons including those of the VTA (Nashmi et al., 2007; Xiao et al., 2009). In addition, the rewarding properties of nicotine have been shown to increase in chronic nicotine-exposed mice, perhaps linking upregulation and increased functional $\alpha 4^*$ nAChR expression in VTA GABAergic neurons with reward behavior (Hilario et al., 2012). We sought to test the hypothesis that increased functional expression of $\alpha 4^*$ nAChRs selectively in VTA GABAergic neurons will increase sensitivity to nicotine reward.

Materials and Methods

Mice. C57BL/6J and glutamate decarboxylase 2 (Gad2)-Cre (strain B6N.Cg-Gad2^{tmz(cre)Zjh/J}) male mice on a C57BL/6J background were used in this study (The Jackson Laboratory; Taniguchi et al., 2011). Adult (8 to 10 weeks old) Gad2-Cre mice were injected with viral particles and used for behavioral experiments 4–6 weeks after infection. All mice were kept on a 12 h light/dark cycle, with lights on at 7:00 A.M. and off at 7:00 P.M. All mice were given food and water *ad libitum*. All procedures were performed in compliance with the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs. For acute treatments, nicotine hydrogen tartrate, mecamylamine hydrochloride (Sigma-Aldrich), and dihydro- β -erythroidine hydrobromide (DH β E; Tocris Bioscience) were dissolved in sterile 0.9% PBS (Hospira). Nicotine was titrated to physiological pH (7.4) before being administered to mice. Vials containing nicotine solutions were wrapped in aluminum foil to prevent degradation by light exposure. For chronic exposure studies, nicotine dihydrate ditartrate (200 μ g/ml; Acros Organics) and L-tartaric acid (300 μ g/ml; Sigma-Aldrich) were dissolved in water. Saccharin sodium (3 mg/ml; Fisher Scientific) was added to both solutions to increase palatability. Doses for nicotine, mecamylamine, and DH β E were calculated as free base. All injections were administered subcutaneously.

Viral plasmid engineering. The mouse $\alpha 4$ -yellow fluorescent protein (YFP) nAChR subunit cDNA was obtained from Addgene (plasmid 15245) and has been described previously (Nashmi et al., 2003). Using the QuikChange site-directed mutagenesis kit (Agilent Technologies), PCR mutagenesis was done to convert the 9' leucine of the M2 pore domain to a serine (changing the codon from CTT to TCT). The resulting construct contained $\alpha 4$ -YFP cDNA with the leucine to serine mutation (Leu9'Ser $\alpha 4$ -YFP). The Leu9'Ser $\alpha 4$ -YFP cDNA was subcloned into the AAV expression vector pAAV-EF-1a-DIO-WPRE-pA (Tsai et al., 2009).

Chronic nicotine exposure. Animals were restricted to drinking either control (tartaric acid, 300 μ g/ml and 3 mg/ml saccharin) or nicotine-treated (200 μ g/ml and 3 mg/ml saccharin) water through a 250 ml opaque water bottle placed in the home cage for 6–8 weeks. To induce withdrawal, the nicotine bottle was replaced with a water bottle (Zhao-Shea et al., 2013).

Viral-mediated gene delivery. Both the pAAV-EF-1a-Leu9'Ser- $\alpha 4$ -YFP (Leu9'Ser $\alpha 4$ -YFP) and pAAV-EF-1a-DIO-hChR2(H134R)-EYFP-WPRE-pA (control) constructs were packaged into AAV2 viral particles by the University of Massachusetts Medical School Viral Vector Core. Viral titers were 10^{12} viral particles/ μ l with 1 μ l of viral supernatant bilaterally injected into the VTA. Gad2-Cre animals were anesthetized with ketamine/xylazine (0.1 ml/10 g body weight, 10 mg/ml ketamine, 1 mg/ml xylazine). To prepare the surgical area, fur was shaved, and skin was disinfected with 10% povidone iodine. The VTA was located with a stereotaxic frame (Stoelting) using the following coordinates from bregma (in mm): -3.3 AP, ± 0.3 ML, -4.0 DV. Viral particles were infused into the brain using an injection syringe (Hamilton) attached to

a syringe pump (Quintessential Stereotaxic Injector, Stoelting) at a rate of 0.25 μ l/min. The injection needle was left in place for 10 min after each injection and then slowly retracted. Mice were given 5% glucose and 15 mg/kg ketapropfen after fully regaining consciousness. Mice were given 4 weeks for recovery and to allow for expression of the viral particles before each experiment.

Immunofluorescence. For c-Fos immunolabeling, animals received subcutaneous saline injections 3 d before each experiment to reduce possible effects of stress and/or handling on neuronal activity. To assess c-Fos expression, Gad2-Cre male mice infected with either control or Leu9'Ser $\alpha 4$ -YFP were injected either with saline or 0.09 mg/kg nicotine. Ninety minutes after drug administration, brains were harvested for slice preparation. Animals were anesthetized with 200 mg/kg sodium pentobarbital (interperitoneal injection) and transcardially infused with 10 ml of chilled 0.1 M PBS following with chilled 4% (w/v) paraformaldehyde dissolved in 0.1 M PBS. The brains were harvested and placed in cold 4% paraformaldehyde for 2 h before submerging in 30% sucrose. Brains were then sectioned into 30 μ m slices using a microtome (Leica) and immunolabeled via free-floating sections. Slices were washed in PBS for 5 min, permeabilized in 0.2% (v/v) Triton X-100 (Sigma-Aldrich) for 5 min, and blocked with 2% bovine serum albumin (BSA; Fisher Scientific) for 30 min before incubation with primary antibody in 2% BSA overnight at 4 °C. Primary antibodies used were as follows: mouse anti-TH MAB318 (1:1000; lot number 2499557, Millipore), rabbit anti-Gad1/2 (1:2000; lot number 122M4761, Sigma-Aldrich), rabbit anti-c-Fos (1:1000; lot number F2510, Santa Cruz Biotechnology), rabbit anti-GFP (1:4000; lot number GR158277-1, Abcam), mouse anti-calbindin (1:3000; lot number 052M4833, Sigma-Aldrich), rabbit anti-calretinin (1:1000; lot number AB5054, Millipore), rabbit anti-parvalbumin (1:1000; lot number ab11427, Abcam), and goat anti-somatostatin (1:100; lot number sc-789, Santa Cruz Biotechnology). Secondary antibodies were Alexa Fluor 405 (lot number 1126599), 488 (lot number 1608521), and 594 (lot numbers 1431805, 1003216, and 1602780; 1:1000; Invitrogen). An AxioCam MRm camera (Carl Zeiss) attached to a Zeiss Axiovert inverted fluorescent microscope equipped with Zeiss filter sets 38HE, 49, and 20 was used to acquire fluorescent images. Zeiss objectives A-plan 10 $\times$, EC-Plan-NEOFLUAR 20 $\times$, and Plan-APOCHROMAT 63 $\times$ were used to view and capture images. Images were processed using Axiovision version 4.8.2. For quantification and colocalization analysis, images were deconvoluted and segmented using the segmentation and quantification of subcellular shapes (Squash) software plugin through ImageJ. At least 10 slices/mouse brain that spanned the entire VTA were analyzed. The VTA was located using TH staining and morphology of nearby brain regions as described previously (Zhao-Shea et al., 2011). Areas of interest for each slice were identified through TH fluorescence and measured in ImageJ using the "Analyze Particles" option to account for differences between slices.

Electrophysiological recordings. Mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and then decapitated. Slices were prepared as described previously (Zhao-Shea et al., 2011). Briefly, brains were quickly removed and placed in an oxygenated ice-cold high sucrose artificial CSF (SACSF) containing kynurenic acid (1 mM; Sigma-Aldrich). Brain slices (180–200 μ m) were cut using a Leica VT1200 vibratome. The brain slices were incubated in oxygenated Earl's balanced salt solution supplemented with glutathione (1.5 mg/ml; Sigma), N- ω -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 the following (in mM): 250 sucrose, 2.5 KCl, 1.2 NaH₂PO₄ · H₂O, 1.2 MgCl₂ · 6 H₂O, 2.4 CaCl₂ · 2 H₂O, 26 NaHCO₃, 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 before 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 (Molecular Devices). Action potentials were obtained using a gap-free acquisition mode and Clampex software (Molecular Devices). I_h currents were elicited every 5 s by stepping from -60 mV to a test potential of -120 mV for 1 s using Clampex.

Table 1. Total time spent (in seconds) in drug- and saline-paired chambers of the CPP assay before (Pre) and after (Post) training

	Sal (Pre, s)	Sal (Post, s)	Drug (Pre, s)	Drug (Post, s)
Virus (Nic, mg/kg)				
Ctrl (Sal)	379.7 $\pm$ 20.86	315.7 $\pm$ 48.44	280.2 $\pm$ 18.88	335.5 $\pm$ 45.96
Ctrl (0.09)	339 $\pm$ 9.444	309.3 $\pm$ 47.33	271.1 $\pm$ 14.35	249.5 $\pm$ 45.42
Ctrl (0.5)	334.9 $\pm$ 32.69	341.1 $\pm$ 21.41	284.9 $\pm$ 17.82	299.3 $\pm$ 24.08
Leu9'Ser (Sal)	365.6 $\pm$ 25.91	315.6 $\pm$ 53.89	261.7 $\pm$ 21.01	239.0 $\pm$ 34.97
Leu9'Ser (0.09)	347.6 $\pm$ 21.93	205.9 $\pm$ 27.43	257.1 $\pm$ 17.7	437.9 $\pm$ 37.28**,*
Leu9'Ser (0.5)	303.3 $\pm$ 24.92	240.4 $\pm$ 18.14	293 $\pm$ 13.92	319.2 $\pm$ 37.3
Leu9'Ser (0.5, chronic nicotine)	382 $\pm$ 10.11	294.1 $\pm$ 19.78	306.8 $\pm$ 16.57	376.1 $\pm$ 19.33**,*
Virus (Mec, mg/kg)				
Ctrl (1.0)	409.2 $\pm$ 28.42	352.5 $\pm$ 40.04	282.3 $\pm$ 29.93	317.6 $\pm$ 43.69
Leu9'Ser (1.0)	386.1 $\pm$ 25.30	300.1 $\pm$ 36.56	288.2 $\pm$ 23.48	354.0 $\pm$ 35.28
Virus (DHβE, mg/kg)				
Ctrl (0.5)	363.8 $\pm$ 31.05	342.9 $\pm$ 24.21	311.7 $\pm$ 30.39	315.1 $\pm$ 22.15
Ctrl (2.0)	447.6 $\pm$ 31.12	332.2 $\pm$ 38.96	226.3 $\pm$ 32.76	285.9 $\pm$ 44.42
Leu9'Ser (0.5)	396.1 $\pm$ 23.09	287.7 $\pm$ 41.97	244 $\pm$ 22.59	348.2 $\pm$ 41.85
Leu9'Ser (2.0)	383.1 $\pm$ 16.36	352 $\pm$ 55.83	309.4 $\pm$ 12.95	309.6 $\pm$ 55.02
Treatment/drug				
Control/nicotine	367 $\pm$ 20.3	328.5 $\pm$ 23.48	268.8 $\pm$ 18.67	263.7 $\pm$ 26.09
Chronic nicotine/nicotine	400.2 $\pm$ 29.79	263.2 $\pm$ 23.39	261.1 $\pm$ 15.01	379.6 $\pm$ 26.02**,*

Ctrl, Control; Nic, nicotine; Sal, saline; Mec, mecylamine.

* $p < 0.05$; ** $p < 0.01$ [post-training compared to pretraining, two-way ANOVA with repeated measures (training and nicotine/saline treatment) and Bonferroni's *post hoc* test].

$p < 0.01$ [time spent in the nicotine-paired chamber after training for nicotine-dependent animals compared to time spent in the nicotine-paired chamber for nicotine-naïve animals; two-way ANOVA with repeated measures (Pre/Post time spent in the Nic-paired chamber) and Bonferroni's *post hoc* test].

* $p < 0.001$ [time spent in the nicotine-paired chamber after training compared to time spent in the control nicotine-paired chamber, i.e., group that received saline in the "drug-paired" chamber; two-way ANOVA with repeated measures (Pre/Post time spent in the Nic-paired chamber) and Bonferroni's *post hoc* test].

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 VTA GABAergic neurons were selected for recording based on fluorescent signal and further verified by action potential frequency (~ 10 – 20 Hz) and lack of I_h expression. At the end of recording, the neuronal 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°C for at least 2 h before single-cell RT-PCR experiments to verify expression of GAD1 and GAD2. ACh chloride (Sigma) was dissolved in ACSF. Whole-cell ACh responses were recorded in the presence of TTX (0.5 μM), atropine (1 μM), bicuculline (20 μM), and CNQX (10 μM). Drugs were applied to slices by gravity superfusion.

Nicotine tolerance. Mice received subcutaneous saline injections 3 d before testing to habituate to handling. Locomotor activity was recorded as beam breaks using the Photobeam Activity System (PAS; San Diego Instruments). To determine baseline activity, mice were injected with saline and immediately placed into a novel cage inside the PAS locomotor chamber. Activity was quantified for 15 min before mice were returned to their home cage. On the second day, mice received a subcutaneous injection of nicotine and were immediately placed into a novel cage within the locomotor activity chamber. Activity was measured for 15 min. Mice were subsequently injected with nicotine each day for 6 more days, with locomotor activity recorded on the fourth and seventh days of nicotine challenge.

Conditioned place preference. A three-chamber conditioned place preference (CPP) apparatus (Med Associates) was used to measure nicotine reward. Briefly, at least 3 d before testing, mice were habituated to the test room and handling by picking them up once a day by the scruff of the neck. The testing protocol comprised three phases. In the pretraining phase, mice were placed into the CPP apparatus and allowed to freely explore all three chambers for 15 min. Time spent in each chamber was quantified using MEDPC IV software (Med Associates). The training phase lasted 4 d, where each day mice were twice confined to a chamber for 30 min. In the morning session, each mouse was given a subcutaneous injection of sterile saline and placed in the chamber assigned as the saline-paired chamber. Five hours later, each mouse was given a subcutaneous injection of drug (i.e., nicotine) and placed in the opposite, drug-paired chamber. The training phase was counterbalanced for each group in that

approximately half of the animals received nicotine paired with the white chamber, whereas the other half received nicotine paired with the black chamber. Drug was paired with the least preferred chamber. Mice that spent $>70\%$ of pretraining in any one chamber were not included in the analysis. The post-training phase was identical to the pretraining phase. Difference scores were measured by calculating the difference between the time spent in a chamber during the post-training phase and during the pretraining phase. For CPP experiments testing nicotine reward in control and Leu9'Ser $\alpha 4$ -YFP-infected mice (see Fig. 3, Table 1), the CPP procedure was initiated 4–6 weeks after infection. Mice received saline in both chambers, 0.09 mg/kg nicotine, or 0.5 mg/kg nicotine ($n = 8$ – 10 mice/group) during training. Additional groups of control and Leu9'Ser $\alpha 4$ -YFP-infected mice were tested for CPP to 1 mg/kg mecylamine ($n = 8$ – 10 mice/group) or 0.5 or 2.0 mg/kg DH β E ($n = 6$ – 8 mice/group) as described in Figure 4, A and B, and Table 1. For this set of experiments, CPP was performed exactly as described above except instead of nicotine during training, mice received mecylamine or DH β E at the indicated dose. For CPP in nicotine-dependent mice (see Fig. 3C, inset; Table 1), mice were exposed to nicotine in their drinking water for 8 weeks. Water bottles were swapped for untreated drinking water after the completion of the CPP pretraining phase, and CPP was performed as above using 0.5 mg/kg nicotine during training ($n = 11$). An additional group of control nicotine-naïve animals were also tested in the CPP procedure using 0.5 mg/kg nicotine ($n = 11$).

Statistics. Normality of data was tested before analysis. Locomotor activity data were analyzed using repeated measures one-way ANOVA. CPP data were analyzed using two-way ANOVA with drug dose and paired chamber as main factors, followed by Bonferroni's *post hoc* analysis as indicated. c-Fos data were analyzed using two-way ANOVA with virus and drug treatment or neuron subtype as main factors as indicated. Fold-change differences in inward current were analyzed using a two-tailed *t* test. Data were analyzed using GraphPad Prism (GraphPad Software).

Results

Expression of gain-of-function $\alpha 4$ nicotinic receptor subunits in VTA GABAergic neurons

To understand how increased functional expression of $\alpha 4^*$ nAChRs in VTA GABAergic neurons affects behavior, we developed a viral-mediated gene delivery system to express $\alpha 4^*$ nico-

Figure 1. Viral-mediated gene delivery of gain-of-function $\alpha 4$ nAChR subunits in VTA GABAergic neurons. **A**, Depiction of the Leu9'Ser $\alpha 4$ -YFP subunit cDNA viral plasmid. Cre-recombinase flips the Leu9'Ser $\alpha 4$ -YFP subunit cDNA in the sense orientation. The viral particles containing this plasmid were intracranially injected into the VTA of Gad2-Cre mice for selective expression in VTA GABAergic neurons. **B**, Top, Expression of Leu9'Ser $\alpha 4$ -YFP subunits in non-TH neurons of Gad2-Cre mice. Brain slices from infected mice were immunolabeled for TH to identify DAergic neurons (red, top, left). YFP fluorescence was detected, indicating Leu9'Ser $\alpha 4$ -YFP subunit expression (green, top, middle). Merged signals revealed little colocalization of Leu9'Ser $\alpha 4$ -YFP subunit expression with DAergic neurons (top, right). Photomicrographs (bottom) of a representative VTA midbrain section from Gad2-Cre mice expressing Leu9'Ser $\alpha 4$ -YFP subunits show neurons immunolabeled for Gad (left) that also express Leu9'Ser $\alpha 4$ -YFP subunits (middle). The merged photomicrograph (right) shows colocalization of Gad and YFP signals. **C**, Cell-attached (top) and whole-cell voltage-clamp (bottom) recordings from a putative VTA GABAergic neuron from a Gad2-Cre mouse midbrain slice. GABAergic neurons typically have a high frequency of firing (8–20 Hz, top) and lack of a hyperpolarizing activated current, I_h (bottom). **D**, At the end of each recording, single-neuron RT-PCR was performed to verify Gad expression in a GABAergic neuron (left lanes) or TH expression in a DAergic neuron (right lanes). **E**, Top, Whole-cell voltage-clamp recordings from a control VTA GABAergic neuron in a Gad2-Cre midbrain slice (left) and an Leu9'Ser $\alpha 4$ -YFP-expressing GABAergic neuron in a Gad2-Cre midbrain slice (right). ACh was bath applied for 3 min as indicated by the bar above each trace. Average inward current from control infected ($n = 5$) and Leu9'Ser $\alpha 4$ -YFP ($n = 8$) infected VTA GABAergic neurons. ** $p < 0.01$ (two-tailed t test). Error bars indicate SEM.

tinic receptor subunits with a gain-of-function mutation in select neuronal populations allowing for selective activation of neurons expressing this subunit with low doses of nicotine that minimally activate non- $\alpha 4^*$ nAChRs. We engineered an AAV plasmid construct containing cDNA encoding the $\alpha 4$ nAChR subunit with a single point mutation, a leucine mutated to serine, at the 9' residue of the pore-forming, M2 domain (Leu9'Ser; Fig. 1A), which renders nAChRs that incorporate this subunit significantly more sensitive to nicotine (Labarca et al., 2001). To visualize subunit expression, a YFP tag was included in the M3–M4 intracellular loop, where it does not interfere with receptor assembly or function (Leu9'Ser $\alpha 4$ -YFP; Nashmi et al., 2003; Nashmi et al., 2007). The cDNA encoding Leu9'Ser $\alpha 4$ -YFP was positioned within the AAV expression vector in the antisense orientation and flanked by two pairs of distinct Lox sites (Fig. 1A). These Lox sites regulate Leu9'Ser $\alpha 4$ -YFP expression by directing recombination of the cDNA cassette to the sense orientation in the presence of Cre recombinase (Fig. 1A) (Tsai et al., 2009).

The expression vector was packaged into AAV2, a serotype that will infect a brain region locally, and viral particles were injected into the VTA of Gad2-Cre mice (Gad2^{VTA}:Leu9'Ser) for expression of Leu9'Ser $\alpha 4$ -YFP subunits selectively in GABAergic neurons. To verify subunit expression in GABAergic neurons, Gad2^{VTA}:Leu9'Ser midbrain slices were immunolabeled with either an anti-TH or Gad1/2 antibody. VTA of infected mice exhibited robust expression of Leu9'Ser $\alpha 4$ -YFP subunits, as indicated by strong YFP fluorescent signal selectively in non-DAergic neurons (Fig. 1B). To determine functional expression of Leu9'Ser $\alpha 4$ -YFP subunits in GABAergic VTA neurons, patch-clamp recordings were made in Gad2^{VTA}:Leu9'Ser and control midbrain slices. Control Gad2-Cre animals were infected with AAV2 particles containing channelrhodopsin within the same vector so that GABAergic neurons from control mice would express a non-nAChR membrane protein insensitive to nicotinic agonists. The electrophysiological characteristics of infected neurons, as identified by YFP fluorescent signal, were analyzed to

Figure 2. Selective activation of VTA GABAergic neurons by nicotine in $Gad2^{VTA}:Leu9'Ser$ mice. **A**, Summed average number of c-Fos-immunopositive [c-Fos(+)] neurons in the VTA of control ($n = 6$ /group) and $Gad2^{VTA}:Leu9'Ser$ mice ($n = 7$ /group) after challenge with saline or 0.09 mg/kg nicotine. **B**, Representative photomicrographs illustrating c-Fos expression (red) in VTA sections taken from control (top) or $Gad2^{VTA}:Leu9'Ser$ mice (bottom) mice after 0.09 mg/kg nicotine challenge. Neurons exhibiting TH immunoreactivity are labeled in blue (left), whereas YFP expression is labeled in yellow (right). **C**, Summed average number of TH(+), c-Fos(+) and TH(-), c-Fos(+) neurons in the VTA in control ($n = 6$ mice) and $Gad2^{VTA}:Leu9'Ser$ mice ($n = 7$) after challenge with 0.09 mg/kg nicotine. $^{**}p < 0.01$ [number of TH(+), c-Fos(+) neurons compared to the number of TH(-), c-Fos(+) neurons in $Gad2^{VTA}:Leu9'Ser$ mice after nicotine challenge]; $^{***}p < 0.001$ [number of c-Fos(+) neurons in $Gad2^{VTA}:Leu9'Ser$ mice after nicotine challenge compared to saline challenge]; $^{\wedge}p < 0.05$ [number of neurons that are TH(-), c-Fos(+) in $Gad2^{VTA}:Leu9'Ser$ mice after nicotine challenge compared to the number of neurons that are TH(-), c-Fos(+) in control mice after nicotine challenge]; $^{\wedge\wedge\wedge}p < 0.001$ [number of c-Fos(+) neurons in $Gad2^{VTA}:Leu9'Ser$ mice after nicotine challenge compared to the number of c-Fos(+) neurons in control mice after nicotine challenge]. Error bars indicate SEM.

confirm incorporation of the Leu9'Ser $\alpha 4$ -YFP subunit into an AChR. Leu9'Ser $\alpha 4$ -YFP-infected neurons exhibited fast-spiking spontaneous action potentials and lacked an I_h current, both characteristics of VTA GABAergic neurons (Fig. 1C; Johnson and North, 1992). Single-cell RT-PCR from the cytoplasm of recorded neurons confirmed Gad1 and Gad2 expression in YFP-positive neurons (Fig. 1D). To test for functional expression of the Leu9'Ser $\alpha 4$ -YFP subunit, whole-cell current responses to bath application of 1 mM ACh were recorded in infected $Gad2$ -Cre midbrain slices. ACh elicited robust inward currents in Leu9'Ser $\alpha 4$ -YFP subunit-expressing VTA GABAergic neurons that were significantly larger compared to responses from neurons recorded from control slices (Fig. 1E). Together, these data suggest that $Gad2^{VTA}:Leu9'Ser$ mice express the Leu9'Ser $\alpha 4$ -YFP subunit in GABAergic neurons, and the subunit coassembles with endogenous subunits to form gain-of-function nAChRs.

Selective activation of VTA GABAergic neurons in $Gad2^{VTA}:Leu9'Ser$ mice

To test the hypothesis that a low dose of nicotine selectively increased activation of VTA neurons in nicotine-naïve $Gad2^{VTA}:Leu9'Ser$ mice compared to control animals, we challenged each group with saline or an acute dose of 0.09 mg/kg nicotine and immunolabeled VTA slices for c-Fos expression, an immediate early gene that is a marker for neuron activation (Cole et al., 1989). Two-way ANOVA indicated a significant main effect of virus expression ($F_{(1,22)} = 29.96$, $p < 0.001$) and nicotine treatment ($F_{(1,22)} = 31.62$, $p < 0.001$), and a significant virus expression by nicotine treatment interaction ($F_{(1,22)} = 22.23$, $p < 0.001$; Fig. 2A). *Post hoc* analysis indicated that there was a statistically significant increase of c-Fos-immunopositive VTA neurons from $Gad2^{VTA}:Leu9'Ser$ mice receiving 0.09 mg/kg nicotine compared to $Gad2^{VTA}:Leu9'Ser$ mice receiving saline injection ($p < 0.0001$). In addition, the number of c-Fos-immunopositive VTA neurons in $Gad2^{VTA}:Leu9'Ser$ mice was also significantly greater than the number of c-Fos-immunopositive VTA neurons in nicotine-treated control animals. In control mice, 0.09 mg/kg nicotine did not significantly increase the number of c-Fos-immunopositive VTA neurons compared to saline. To determine whether nicotine-activated neurons were predominantly non-

DAergic, we challenged control and $Gad2^{VTA}:Leu9'Ser$ mice with 0.09 mg/kg nicotine and double immunolabeled for c-Fos and TH (Fig. 2B,C). Two-way ANOVA revealed a significant main effect of virus expression ($F_{(1,22)} = 5.3$, $p < 0.05$) and neuron subpopulation ($F_{(1,22)} = 10.0$, $p < 0.01$), and a virus expression by nicotine treatment interaction ($F_{(1,22)} = 4.73$, $p < 0.05$). *Post hoc* analysis indicated that the number of TH-immunonegative, c-Fos-immunopositive neurons in $Gad2^{VTA}:Leu9'Ser$ was significantly larger than TH-immunopositive, c-Fos-immunopositive neurons after nicotine challenge ($p < 0.01$). The number of TH-immunonegative, c-Fos-immunopositive neurons in $Gad2^{VTA}:Leu9'Ser$ was also larger than the number of TH-immunonegative, c-Fos-immunopositive neurons in control mice after nicotine challenge ($p < 0.05$). The number of TH-immunopositive, c-Fos-immunopositive neurons in $Gad2^{VTA}:Leu9'Ser$ mice after nicotine challenge was small and not significantly different from control mice. In addition, YFP signal could be detected in c-Fos-immunopositive neurons in $Gad2^{VTA}:Leu9'Ser$ mice, but not in control mice (Fig. 2B). Together, these data indicate that 0.09 mg/kg nicotine selectively activates non-DAergic (i.e., GABAergic) neurons in $Gad2^{VTA}:Leu9'Ser$ mice.

Nicotine activation of $\alpha 4^*$ nicotinic receptors in VTA GABAergic neurons: locomotor effects

To test the hypothesis that functional upregulation of nAChRs in GABAergic neurons may be involved in nicotine tolerance (Nashmi et al., 2007), we measured nicotine-induced locomotor activity in response to single daily injections of the drug for 7 d in $Gad2^{VTA}:Leu9'Ser$ and control animals. Mice were challenged with 0.09 mg/kg nicotine delivered subcutaneously, a dose that activated GABAergic neurons in $Gad2^{VTA}:Leu9'Ser$ but had little effect on neuronal activation in control mice. In control mice, 0.09 mg/kg nicotine did not significantly modulate locomotor activity compared to saline injection (Fig. 3A). In $Gad2^{VTA}:Leu9'Ser$ mice, one-way ANOVA revealed a significant main effect of nicotine injections on locomotor activity ($F_{(3,18)} = 7.86$, $p < 0.01$). *Post hoc* analysis revealed that nicotine significantly depressed locomotor activity upon first injection compared to saline ($p < 0.01$), and tolerance to this hypolocomotor response

Figure 3. Selective activation of VTA GABAergic neurons by nicotine is sufficient for nicotine-induced hypolocomotion and reward. **A**, Summed 15 min total locomotor activity after saline injection or daily injection of 0.09 mg/kg nicotine in control mice ($n = 7$). **B**, Summed 15 min total locomotor activity after saline injection or daily injection of 0.09 mg/kg nicotine in $Gad2^{VTA}$:Leu9^{Ser} mice ($n = 7$). ** $p < 0.01$, compared to saline challenge. **C, D**, Difference scores indicate the time spent in the nicotine- or saline-paired chamber after training minus the time spent in the nicotine- or saline-paired chamber before training in control (**C**) or $Gad2^{VTA}$:Leu9^{Ser} mice (**D**) in response to 0 mg/kg (saline; $n = 10$ and 8, respectively), 0.09 mg/kg ($n = 9$ /group), and 0.5 mg/kg nicotine ($n = 8$ /group). **C**, Inset, Difference scores in the CPP assay in response to 0.5 mg/kg nicotine from WT mice previously exposed to 6 weeks of nicotine or vehicle ($n = 11$ /group). *** $p < 0.001$ (nicotine-paired chamber compared to saline-paired chamber); $\wedge p < 0.05$ (nicotine-paired chamber in dependent mice compared to nicotine-paired chamber in nicotine-naïve mice); $\wedge\wedge p < 0.01$ (nicotine-paired chamber compared to saline in the drug-paired chamber); two-way ANOVA, Bonferroni's *post hoc* test. Error bars indicate SEM.

Figure 4. nAChR antagonists do not condition a place preference in $Gad2^{VTA}$:Leu9^{Ser} mice. **A**, Difference scores in the CPP assay in response to 1.0 mg/kg mecamylamine in control and $Gad2^{VTA}$:Leu9^{Ser} mice ($n = 10$ /group). **B**, Difference scores in the CPP assay in response to 0.5 or 2.0 mg/kg DHβE in control mice ($n = 7$ /group) and $Gad2^{VTA}$:Leu9^{Ser} mice ($n = 8$ and 6, respectively). Error bars indicate SEM.

occurred with subsequent injections. Thus, acute activation of nAChRs in VTA GABAergic neurons induces hypolocomotor activity, which triggers tolerance after subsequent nicotine exposures.

Sensitivity to nicotine reward is modulated by activation of VTA GABAergic $\alpha 4^*$ nicotinic receptors

To test the hypothesis that functional upregulation of VTA GABAergic neurons modulates nicotine reward, the ability of

nicotine to condition a place preference in $Gad2^{VTA}$:Leu9^{Ser} and control mice was measured using the CPP procedure. During training, mice were challenged with saline or 0.09 or 0.5 mg/kg nicotine (Fig. 3C,D, Table 1), delivered subcutaneously. In control animals, nicotine did not condition a significant place preference in response to either dose (Fig. 3C), similar to previous reports delivering subcutaneous nicotine injections with the CPP procedure in C57BL/6J mice (Hilario et al., 2012), the background strain of the $Gad2$ -Cre mice. However, a significant CPP in response to 0.5 mg/kg was observed in this strain during withdrawal from 6 weeks chronic nicotine treatment (Fig. 3C, inset; significant main effect of drug-paired chamber, $F_{(1,20)} = 5.97$, $p < 0.05$; significant drug-paired chamber by chronic treatment interaction, $F_{(1,20)} = 10.10$, $p < 0.01$; two-way ANOVA on difference scores, significant increase in difference score in the nicotine-paired chamber between nicotine-dependent and nicotine-naïve mice, $p < 0.01$; Table 1). In $Gad2^{VTA}$:Leu9^{Ser} mice, two-way ANOVA of difference scores (Fig. 3D) indicated a significant main effect of drug ($F_{(1,26)} = 18.64$, $p < 0.001$) but not training chamber ($F_{(2,46)} = 0.6807$, $p > 0.05$), and a significant drug by training chamber interaction ($F_{(2,46)} = 7.086$, $p < 0.01$). *Post hoc* analysis revealed a significant difference between difference scores in the nicotine-paired chamber at the dose of 0.09 mg/kg, but not 0.5 mg/kg, nicotine in $Gad2^{VTA}$:Leu9^{Ser} mice compared to saline (Fig. 3D). Repeated measures two-way ANOVA of time spent in the drug and saline-paired chamber before and after training with 0.09 mg/kg nicotine (Table 1) did not indicate significant main effects of training or time spent in either chamber, but did reveal a significant training by chamber interaction ($F_{(1,8)} = 25.48$, $p < 0.001$). A *post hoc* test indicated a significant increase in time spent in the nicotine-paired chamber after training compared to before training. Finally, two-way ANOVA of time spent in the drug-paired chamber before and after training in $Gad2^{VTA}$:Leu9^{Ser} mice that received 0.09 mg/kg nicotine during training compared to mice that received saline in the drug-paired chamber (Table 1) indicated a significant main effect of drug ($F_{(1,15)} = 10.32$, $p < 0.01$) and time spent in the drug-paired chamber ($F_{(1,15)} = 7.97$, $p < 0.05$), and a significant interaction ($F_{(1,15)} = 13.19$, $p < 0.01$). *Post hoc* analysis indicated a significant increase in time spent in the drug-paired chamber after training with 0.09 mg/kg nicotine compared to saline. Interestingly, after chronic nicotine exposure, $Gad2^{VTA}$:Leu9^{Ser} mice did develop a modest CPP to 0.5 mg/kg nicotine (repeated measures two-way ANOVA; significant

Figure 5. GABAergic neurons mediating reward in $Gad2^{VTA};Leu9'Ser$ mice include neurons that project to the lateral habenula. **A**, Representative photomicrographs illustrating calretinin (top left), calbindin (bottom left), parvalbumin (top right), and somatostatin (bottom right) immunolabeling (red) in C57BL/6J midbrain sections. Insets depict distinct localization of calretinin or calbindin (red) compared to YFP expression (yellow) in $Gad2^{VTA};Leu9'Ser$ mice. **B**, Depiction of a coronal section from a $Gad2^{VTA};Leu9'Ser$ mouse used for analysis of GABAergic projections. The photomicrograph illustrates YFP signal in the VTA. **C**, Representative photomicrographs from cortical (top), striatal (middle), and epithalamic (bottom) sections from the $Gad2^{VTA};Leu9'Ser$ mouse in **B**. IPN, Interpeduncular nucleus; ml, mammillary nucleus; aca, anterior commissure; MHb, medial habenula.

training by chamber interaction, $F_{(1,9)} = 27.61$, $p < 0.001$; significant increase in time spent in the nicotine-paired chamber after training, $p < 0.05$; Table 1).

Whereas a low dose of nicotine may elicit a CPP in $Gad2^{VTA};Leu9'Ser$ mice by activating GABAergic neurons, it is also possible that nicotine may be desensitizing $Leu9'Ser$ $\alpha 4$ -YFP nAChRs in VTA GABAergic interneurons, reducing endogenous ACh activation of the mutant nAChRs (i.e., blocking GABAergic interneuron activity) and thereby disinhibiting DAergic neurons to promote reward (Mansvelder et al., 2002). We hypothesized that if this were occurring, then an nAChR antagonist would elicit a similar effect by blocking endogenous activity through the $Leu9'Ser$ $\alpha 4$ -YFP nAChRs. Thus, we measured the ability of the noncompetitive nAChR antagonist mecamylamine to condition a place preference in $Gad2^{VTA};Leu9'Ser$ and control, nicotine-naïve animals. In both groups, 1.0 mg/kg mecamylamine failed to significantly condition a place preference (Fig. 4A). In an additional group of $Gad2^{VTA};Leu9'Ser$ and control nicotine-naïve animals, we tested whether DH β E, a selective $\alpha 4\beta 2$ competitive antagonist, would be sufficient to disinhibit DAergic neurons and condition a place preference. Training with 0.5 or 2.0 mg/kg DH β E, subcutaneously, in control and $Gad2^{VTA};Leu9'Ser$ mice failed to condition a place preference (Fig. 4B). These data indicate that selective activation of $\alpha 4^*$ nAChRs in GABAergic neurons is sufficient for nicotine reward.

$Gad2^{VTA};Leu9'Ser$ mice $\alpha 4^*$ nAChRs are expressed in VTA GABAergic neurons that project to the lateral habenula

To gain insight into the neuronal subpopulation(s) of VTA GABAergic neurons that may express the $Leu9'Ser$ $\alpha 4$ -YFP subunit and elicit nicotine-induced reward in $Gad2^{VTA};Leu9'Ser$ animals, we immunolabeled C57BL/6J midbrain slices with potential GABAergic neuronal markers calretinin, calbindin, somatostatin, and parvalbumin (Fig. 5A). Interestingly, we did not detect somatostatin nor parvalbumin expression in the area of the VTA that was targeted with our virus expression system (that is, the posterior VTA), although somatostatin expression was observed in the rostral interpeduncular nucleus as described previously (Zhao-Shea et al., 2013). Both calretinin- and calbindin-immunopositive cells were detected (Fig. 5A). However, analysis

of YFP signal in $Gad2^{VTA};Leu9'Ser$ mice revealed that calretinin- and calbindin-immunopositive neurons did not express YFP (Fig. 5A, insets), consistent with previous reports that the majority of calretinin- and calbindin-expressing VTA neurons are actually DAergic (Olson and Nestler, 2007). We next analyzed YFP signal in known VTA efferent brain regions of $Gad2^{VTA};Leu9'Ser$ mice including the NAc, prefrontal cortex (PFC), and lateral habenula (LHb), for YFP fluorescence (Fig. 5B,C). Interestingly, YFP signal was not detected in the PFC or NAc. However, fluorescence was observed in the LHb.

Discussion

We expressed $Leu9'Ser$ $\alpha 4$ -YFP nAChR subunits in VTA GABAergic neurons in an effort to understand how functional upregulation of $\alpha 4^*$ nAChRs in this neuronal subpopulation may contribute to behaviors associated with nicotine dependence. Chronic nicotine upregulates $\alpha 4^*$ nAChRs selectively in GABAergic neurons of the VTA, and this is accompanied by an increase in functional expression as measured by an increase in nicotine activation of these neurons (Nashmi et al., 2007). It is important to note that if chronic nicotine merely upregulated the $\alpha 4$ nAChR subunit and not the $\beta 2$ subunit, then this would result in a change in $\alpha 4\beta 2$ nAChR stoichiometry to the low sensitivity ($\alpha 4$) $3(\beta 2)$ subtype (Eaton et al., 2014). However, a functional increase in activation is observed in chronic nicotine-treated animals, suggesting that upregulation of both $\alpha 4$ and $\beta 2$ subunits occurs (Srinivasan et al., 2011), leading to the observed increase in nAChR function in GABAergic VTA neurons. To mimic this phenomenon, we chose to express gain-of-function $\alpha 4$ nAChR subunits in GABAergic VTA neurons instead of wild-type (WT) subunits, which would have likely changed the $\alpha 4\beta 2$ nAChR stoichiometry to the low sensitivity ($\alpha 4$) $3(\beta 2)$ subtype resulting in a loss-of-function phenotype.

In $Gad2^{VTA};Leu9'Ser$ mice, a low dose of 0.09 mg/kg nicotine was sufficient to activate GABAergic neurons. This same dose failed to significantly activate neurons in control animals. Interestingly, there were few DAergic neurons activated in both control and $Gad2^{VTA};Leu9'Ser$ mice after low-dose nicotine challenge. Importantly, nicotine was delivered subcutaneously in these experiments, whereas this same dose has been shown to

elicit reward in mice when delivered intraperitoneally, highlighting that routes of nicotine administration yield differences in bioavailability of the drug (Brunzell et al., 2009; Alcantara et al., 2014).

Acute nicotine activation of VTA GABAergic neurons induces hypolocomotion

We assessed how functional upregulation of VTA GABAergic neurons may contribute to nicotine tolerance and reward. Acute nicotine induces hypolocomotion in rodents, which is alleviated with multiple nicotine exposures, providing a behavioral measure of tolerance (Tapper et al., 2007). Typically, locomotor suppression has been observed in C57BL/6 mice given a dose of ~ 0.5 mg/kg nicotine in a novel environment or open field (Salas et al., 2004). A single injection of 0.09 mg/kg nicotine in a novel cage was sufficient to decrease locomotor activity in $Gad2^{VTA}$:Leu9'Ser mice, but had little effect on locomotor activity in control mice. Interestingly, $Gad2^{VTA}$:Leu9'Ser mice developed a tolerance to this effect with daily low-dose nicotine injections, indicating that acute activation of VTA GABAergic neurons induces hypolocomotion, with subsequent exposures eliciting tolerance to this effect. The mechanism underlying nicotine-induced hypolocomotor activity is unknown. Our data indicate that activation of VTA GABAergic neurons may cause the initial nicotine-induced decrease in locomotor activity perhaps by inhibiting DA release into the striatum. However, additional experiments will be needed to determine whether the tolerance to this hypolocomotion involves $\alpha 4^*$ nAChRs in GABAergic neurons or, alternatively, triggers a non-nAChR mechanism that opposes hypolocomotion.

Selective activation of VTA GABAergic neurons by nicotine is sufficient for reward

Previous studies using optogenetic stimulation have shown that activation of VTA GABAergic neurons can lead to disruption of reward and induce aversion (Tan et al., 2012; van Zessen et al., 2012). Surprisingly, selective activation of VTA GABAergic neurons in $Gad2^{VTA}$:Leu9'Ser mice using a low dose of nicotine conditioned a robust place preference in these animals, suggesting that nicotine activation of these neurons may be sufficient for reward. Conversely, a more typical "rewarding" dose of 0.5 mg/kg subcutaneous nicotine (Hilario et al., 2012; Smith et al., 2012) failed to elicit a place preference in $Gad2^{VTA}$:Leu9'Ser mice, consistent with a shift in the inverted-U-shaped dose–response curve often seen with nicotine reward and reinforcement (Picciotto, 2003). In control mice, nicotine failed to condition a place preference at any of the doses tested. While stress could be a contributing factor to lack of CPP in control animals, this is rendered unlikely because mice were habituated to handling before the beginning of the CPP assay. Our results are similar to those of Hilario et al. (2012), who demonstrated that withdrawal from chronic nicotine exposure was necessary for the expression of nicotine reward and that this is correlated with nAChR upregulation. Verifying these data, we confirmed that control mice withdrawn from chronic nicotine also exhibit a place preference with 0.5 mg/kg nicotine compared to nicotine-naïve mice. Based on data indicating that (1) increased sensitivity to nicotine reward occurs after chronic nicotine exposure and withdrawal, (2) sensitivity to nicotine reward correlates with nAChR upregulation (Hilario et al., 2012), (3) upregulation of $\alpha 4^*$ nAChRs occurs selectively in VTA GABAergic neurons (Nashmi et al., 2007), and (4) selective activation of functionally upregulated $\alpha 4^*$ nAChRs in VTA GABAergic neurons elicits reward, we suggest that upregulation of $\alpha 4^*$ nAChRs specifically in VTA GABAergic neurons increases sensitivity to nicotine reward.

How might activation of GABAergic neurons by nicotine elicit reward? One possibility is that nicotine desensitizes GABAergic nAChRs, reducing GABAergic neuron activity and disinhibiting DAergic neurons (Mansvelder et al., 2002). Our data indicate that, at least using our expression system, this possibility is unlikely because (1) we did not observe increased activation of DAergic neurons (as measured by c-Fos induction) after low-dose nicotine challenge in $Gad2^{VTA}$:Leu9'Ser neurons, and (2) mecamylamine and DH β E failed to elicit reward in $Gad2^{VTA}$:Leu9'Ser mice. One caveat to these results is that disinhibition of DAergic neurons by nAChR desensitization in GABAergic neurons would require that Leu9'Ser $\alpha 4$ -YFP nAChRs are predominantly expressed in GABAergic interneurons. In analyzing VTA neuron subpopulations in the injection area of $Gad2^{VTA}$:Leu9'Ser mice, which was focused on the posterior VTA, we failed to detect parvalbumin- or somatostatin-immunopositive neurons, whereas calbindin and calretinin neurons were detected, but did not colocalize with Leu9'Ser $\alpha 4$ -YFP expression, consistent with previous studies indicating that these two populations of neurons are largely DAergic in the VTA (Gerfen et al., 1987; Olson and Nestler, 2007). A more recent study indicated that activation of GABAergic neurons via $\beta 2^*$ nAChRs is required for DAergic neuron burst activity and nicotine self-administration (Tolu et al., 2012). Thus, nicotine activation of GABAergic neurons in $Gad2^{VTA}$:Leu9'Ser mice could lead to increased DAergic neuron bursting and reward. However, as indicated above, increased activation of DAergic neurons, at least with acute nicotine injections, was not observed in these animals. A third and more likely possibility is that a portion of VTA GABAergic neurons expressing Leu9'Ser $\alpha 4$ -YFP nAChRs project to brain regions that, when inhibited, promote reward behavior. Indeed, upon analysis of known VTA projection regions, we found Leu9'Ser $\alpha 4$ -YFP expression in the LHB. GABAergic neurons make up $\sim 35\%$ of VTA neurons, and although little is known about their function within the VTA (Nair-Roberts et al., 2008), previous studies indicate that a portion of VTA GABAergic neurons innervate the LHB and, when activated, elicit reward (Stamatakis et al., 2013; Lammel et al., 2015). This is accomplished by inhibiting LHB glutamatergic inputs to the rostromedial tegmental nucleus (RMTg), which, in turn, disinhibits VTA DAergic neurons to promote reward (Hong et al., 2011; Lecca et al., 2011). Thus, one mechanism by which nicotine activation of VTA GABAergic neurons could elicit nicotine reward is through inhibiting these LHB inputs to the RMTg. Future studies should focus on how VTA GABAergic neuron activation alters the excitability of these downstream brain regions in the context of nicotine-induced reward. Our data suggest that activation of functionally upregulated $\alpha 4^*$ nAChRs in VTA GABAergic neurons confers increased sensitivity to nicotine reward. These data indicate that nAChR subtypes specifically expressed in VTA GABAergic neurons may be good molecular targets for therapeutics to aid in smoking cessation.

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