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# CHRNA5 Gene Variation Affects the Response of VTA Dopaminergic Neurons During Chronic Nicotine Exposure and Withdrawal

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## Abstract

Nicotine is the principal psychoactive component in tobacco that drives addiction through its action on neuronal nicotinic acetylcholine receptors (nAChR). The nicotinic receptor gene CHRNA5, which encodes the α5 subunit, is associated with nicotine use and dependence. In humans, the CHRNA5 missense variant rs16969968 (G>A) is associated with increased risk for nicotine dependence and other smoking-related phenotypes. In rodents, α5-containing nAChRs in dopamine (DA) neurons within the ventral tegmental area (VTA) powerfully modulate nicotine reward and reinforcement. Although the neuroadaptations caused by long-term nicotine exposure are being actively delineated at both the synaptic and behavioral levels, the contribution of α5-containing nAChRs to the cellular adaptations associated with long-term nicotine exposure remain largely unknown. To gain insight into the mechanisms behind the influence of α5-containing nAChRs and the rs16969968 polymorphism on nicotine use and dependence, we used electrophysiological approaches to examine changes in nAChR function arising in VTA neurons during chronic nicotine exposure and multiple stages of nicotine withdrawal. Our results

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demonstrate that CHRNA5 mutation leads to profound changes in VTA nAChR function at baseline, during chronic nicotine exposure, and during short-term and prolonged withdrawal. Whereas nAChR function was suppressed in DA neurons from WT mice undergoing withdrawal relative to drug-naive or nicotine-drinking mice, α5-null mice exhibited an increase in nAChR function during nicotine exposure that persisted throughout 5-10 weeks of withdrawal. Re-expressing the hypofunctional rs16969968 *CHRNA5* variant in α5-null VTA DA neurons did not rescue the phenotype, with α5-SNP neurons displaying a similar increased response to ACh during nicotine exposure and early stages of withdrawal. These results demonstrate the importance of VTA α5-nAChRs in the response to nicotine and implicate them in the time course of withdrawal.

**Keywords**

α5 nicotinic subunit; nicotine; withdrawal; VTA; dopamine; CHRNA5; rs16969968 SNP

## Introduction

Tobacco use is a major public health problem, leading to hundreds of thousands of preventable deaths each year in the United States (United States. Public Health Service. Office of the Surgeon General., 2014). Establishing and maintaining abstinence from tobacco is difficult. In the United States, nearly 70% of active smokers express interest in quitting, but less than 10% report past-year success (Babb et al., 2017). Moreover, those who successfully quit typically require multiple attempts (Chaiton et al., 2016). This phenomenon seems to arise, at least in part, from the withdrawal syndrome that accompanies cessation, which includes physical, affective, and cognitive symptoms (McLaughlin et al., 2015; Paolini and De Biasi, 2011). Intriguingly, the risk of relapse remains high even after symptoms of withdrawal have abated (Garcia-Rodriguez et al., 2013), suggesting long-term adaptations that increase the difficulty of smoking cessation.

Nicotine, the principal addictive component of tobacco, acutely increases dopamine (DA) neuron activity in the ventral tegmental area (VTA) (Forget et al., 2018; Grenhoff et al., 1986; Pidoplichko et al., 1997) and—like other addictive drugs—increases DA release in the nucleus accumbens (NAc) (Di Chiara and Imperato, 1988) to exert its rewarding and reinforcing effects (Dani and Heinemann, 1996; De Biasi and Dani, 2011). In addition to reinforcement, DA in the NAc is also associated with nicotine withdrawal. Animals show significantly reduced NAc DA concentrations during the first few days of abstinence (Hildebrand et al., 1998; Rada et al., 2001; Zhang et al., 2012), and enhancing NAc DA activity has been suggested to ameliorate affective signs and symptoms of nicotine withdrawal (Paterson et al., 2007; Radke and Gewirtz, 2012). Nicotine modulates DA release in the brain through complex interactions with nicotinic acetylcholine receptors (nAChRs) (Dani and Bertrand, 2007; Dani and Heinemann, 1996). These pentameric receptors comprise five α subunits, alone or in combination with β subunits, with the subunit composition and position determining receptor pharmacology and function (see (Dani, 2015; Dani and Bertrand, 2007) for reviews).

Receptors containing the α5 subunit are expressed on neurons within the VTA and on DA terminals in the dorsal striatum (Azam et al., 2002; Exley et al., 2012; Grady et al., 2007; Klink et al., 2001), contributing to the importance of α5-containing receptors during the study of nicotine dependence. Distinguished by its role as an obligate accessory subunit, the α5 subunit does not contribute to the agonist binding site (Dani, 2015). However, several studies have consistently shown that its genetic deletion significantly reduces nAChR function (Chatterjee et al., 2013; Morel et al., 2014; Sciaccaluga et al., 2015). Moreover, the non-synonymous single nucleotide polymorphism (SNP) rs16969968 in *CHRNA5* encodes a change in amino acid (Asp398Asn) in the α5 subunit that results in a partial loss of function (Bierut et al., 2008; Kuryatov et al., 2011; Sciaccaluga et al., 2015). Inclusion of the α5 subunit modulates nicotine-induced nAChR desensitization, which depends upon the broader receptor composition (Bailey et al., 2010; Gerzanich et al., 1998; Grady et al., 2012 Marks 2012) and prevents the subsequent upregulation of high-affinity α4β2-nAChRs (Mao et al., 2008).

Expression of α5-containing nAChRs in VTA DA neurons powerfully modulates nicotine intake in rodents (Morel et al., 2014). Additionally, a highly replicated genetic association of habitual smoking and nicotine dependence links the rs16969968 SNP with heavy smoking in humans (Bierut et al., 2008; Lips et al., 2010; Sarginson et al., 2011; Sherva et al., 2008; Stevens et al., 2008). Fewer studies have addressed the role of the α5 subunit in nicotine withdrawal and relapse. However, mice lacking the α5 subunit do not display physical signs of nicotine withdrawal (Salas et al., 2009) or withdrawal-induced hyperalgesia (Jackson et al., 2008), suggesting that the α5 subunit plays an important role during withdrawal from chronic nicotine intake. Overall, the contribution of the α5 subunit to the cellular adaptations associated with long-term nicotine exposure remain largely unknown. To shed light on the mechanisms behind the influence of α5-containing nAChRs and the rs16969968 SNP on nicotine use and dependence, we examined changes in nAChR function arising from chronic nicotine exposure and during multiple stages of nicotine withdrawal.

## Methods

### Subjects

We studied 149 α5-null mice and 162 wild-type (WT) littermate controls (male and female, aged 4-8 months). We also crossed α5-null mice with mice expressing Cre recombinase under the control of the DA transporter (DAT-Cre) to be able to express the rs16969968 SNP exclusively in midbrain DA neurons (see below). All mice were on a C57BL/6J background and were maintained in a 12h light:dark cycle, temperature- and humidity-controlled vivarium. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania and in accordance with the guidelines provided by the National Institutes of Health Guide for care and use of laboratory animals. Our animal care facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

### Mesolimbic α5-SNP re-expression

Fifty-one adult α5-null x DAT-Cre mice (age ~2 months), which from here on are referred to as SNP mice, were deeply anesthetized with isoflurane (1-2% in $O_2$) and mounted in a stereotactic apparatus. The α5-SNP virus (AAVDJ-DIO-α5SNP-copGFP) was infused bilaterally into the VTA (AP: −3.2 to −3.25 cm; ML: ± 0.05 to 0.12; DV: −4.2 to −4.35) at a rate of 0.01 μL/min for a total of 1.7-2.0 μL using a 10 μL syringe (Hamilton, Reno, Nevada) coupled to a microinfusion pump (KdScientific, Hollistin, MA). Meloxicam was administered to minimize post-operative pain. The vector was designed to express cop-GFP to aid identification of infected neurons expressing the α5-SNP.

### Drugs

Nicotine hydrogen tartrate salt was purchased from Glentham Life Sciences (Corsham, United Kingdom). Neurobiotin Tracer was purchased from Vector Laboratories (Burlingame, California, USA). All other chemicals were procured from Sigma-Aldrich (St. Louis, MO, USA).

### Nicotine Treatment and Withdrawal

Mice were randomly assigned to one of five treatment groups: saccharin control (SAC), chronic nicotine (NIC), short-term/early withdrawal (1-4 days), intermediate withdrawal (2 weeks), or long-term/late withdrawal (5-10 weeks). Mice received 200 mg/L nicotine + 0.2 % saccharin or 0.2% saccharin alone in the drinking water for at least 8 weeks. Mice used to evaluate the effect of nicotine vs. saccharin were studied during nicotine exposure, while those used to evaluate the effects of withdrawal were provided with plain drinking water until reaching their assigned endpoint.

### Slice Preparation

Mice were deeply anesthetized using intraperitoneal ketamine/xylazine followed by exsanguination via transcardial perfusion, performed as previously described (Broussard et al., 2016; Yang et al., 2017) using ice-cold N-methyl-D-glucamine (NMDG) based artificial cerebrospinal fluid (ACSF, in mM): 92 NMDG, 2.5 KCl, 1.2 $NaH_2PO_4$, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$, and 10 $MgSO_4$, pH 7.3-7.4 with concentrated HCl (Ting et al., 2014). After perfusion, the brain was rapidly removed and placed in ice-cold, oxygenated NMDG solution. Horizontal slices containing the VTA (230 μm) were obtained using a Leica VT1200S vibratome and allowed to recover in 32°C NMDG for 13 min. Slices were then transferred to a HEPES-based holding ACSF solution (in mM): 92 NaCl, 2.5 KCl, 1.2 $NaH_2PO_4$, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2$, and 2 $MgSO_4$. Slices were kept in the ACSF holding solution at room temperature for at least 1 hour prior to recording.

### Electrophysiological Recordings

Slices were placed in a home-made recording chamber and were continuously bathed in well-oxygenated standard recording ACSF (in mM): 124 NaCl, 2.5 KCl, 1.2 $NaH_2PO_4$, 24 $NaHCO_3$, 5 HEPES, 12.5 glucose, 2 $CaCl_2$, and 2 $MgSO_4$, maintained at 32–34 °C using an inline heater system (TC-324B, Warner Instrument Corp, Hamden, CT).

Responses were recorded using glass recording electrodes (~2-3 MΩ), which were pulled from borosilicate glass capillaries (TW 150-4, World Precision Instruments, Inc, Sarasota, FL) using a micropipette puller (Narishige PC-10, Tokyo, Japan) and were filled with a K-gluconate-based intracellular solution (in mM): 140 K-gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP, 0.3 $Na_2GTP$, and 10 $Na_2$-phosphocreatine, pH 7.3 with KOH. The tight-seal patch-clamp recording configuration was first achieved by applying a brief gentle suction. Under this configuration, spontaneous action potential firing was observed in most recorded neurons which could be blocked by puff-applied quinpirole (2 μM), a D2-receptor agonist (Fig 1A). Whole-cell mode configuration was then achieved by briefly applying strong suction. Only access resistance (Ra) < 10 MΩ was accepted, and the Ra was monitored throughout the experiment. Putative DA neurons were then identified by the presence of hyperpolarization-activated cationic currents (H-current, Fig 1C), a typical electrophysiological property of DA neurons of the lateral VTA (Zhang et al., 2010). A subset of recorded putative DA neurons was labeled with neurobiotin during patch-clamp recording to confirm that they were dopaminergic (as verified by positivity to tyrosine hydroxylase (TH) staining) and were located in the VTA (Fig 1B & D). To fully activate nAChRs in the recorded neurons, 1 mM acetylcholine (ACh) was pressure-applied (100 ms at 20 psi) by Picospritzer II (Parker Instrumentation, Fairfield, NJ) every 2 min via a puffer pipette. The puffer pipette was identical to those used for electrical recording. During the ACh puff, the recorded neurons were clamped at −60 mV (VH = −60 mV) under voltage-clamp mode. Atropine (1 μM) was added to recording ACSF to block muscarinic receptors, ensuring that the recorded response to ACh was mediated entirely by nAChRs (Yang et al., 2011; Yang et al., 2009). The data presented were collected from a minimum of 6 mice for each genotype within each experimental condition.

## Immunohistochemistry

Slices used for TH staining were fixed overnight and washed with PBS before incubation in a blocking solution comprising 3% normal goat serum (NGS) and 0.3% Triton-X in PBS for 2 hours. Slices were then incubated in primary mouse anti-TH (1:200) at 4°C overnight, and in secondary goat anti-mouse Alexa Fluor 594 (1:200) and streptavidin DyLight 488 (1:1000) for 7 hours. Slices were imaged on an epifluorescence microscope to confirm TH expression in filled neurons.

## Statistical Analyses

All values are expressed as mean ± SEM, and the number of cells analyzed is denoted by “n”. Variance in the peak ACh-induced currents in WT SAC mice was >30 times higher than in both α5-SNP and α5-null mice (see Fig. 2B). The baseline current differences between drug-naïve/SAC-treated groups were therefore determined using one-way ANOVA with Welch’s correction for unequal variances and multiple comparisons were performed using Games-Howell post-hoc. Planned within-group comparisons were then conducted using one-way Welch’s ANOVA with Games-Howell post-hoc for multiple comparisons between timepoints. Statistical tests were performed using SPSS v27 (IBM, Armonk, NY).

## Results

### The α5 subunit is necessary for baseline nAChR currents in DA neurons

To better understand the role of the α5 subunit in nAChR modulation of DA neuron activity, we first evaluated the response to ACh in drug-naive/saccharin-treated WT, α5-null, and SNP mice. Whole-cell currents were elicited through pressure puff-application of 1 mM ACh every two minutes, evoking stable and repeatable responses in all groups (Fig 2A). Disruption of the α5 subunit resulted in significant differences in the response to ACh [Welch's $F_{(2, 18.663)}$=32.591, p<0.001] (Fig 2B). Recordings from α5-null mice showed very limited function, demonstrated by the small peak amplitude of inward-currents (9.66 ± 1.17 pA, n=20 from 16 mice) in VTA DA neurons relative to recordings from WT mice (99.34 ± 11.59 pA, n=17 from 13 mice) [p<0.001]. These results corroborate previous work demonstrating that α5-containing nAChRs are prominent contributors to baseline nAChR currents in VTA DA neurons in both juvenile (Chatterjee et al., 2013; Sciaccaluga et al., 2015) and adult (Morel et al., 2014) mice. A reduction in nAChR function was also observed when the α5 subunit encoding the rs16969968 polymorphism was expressed in VTA DA neurons (22.51 ± 2.76 pA, n=10 from 6 mice; p<0.001). The response to ACh was significantly larger in SNP-expressing neurons compared to α5-null cells [p<0.005], but the SNP-currents were still significantly smaller than WT, suggesting partial diminution of nAChR function when the α5 subunit is present but mutated.

### Dramatic, long-lasting reduction in nAChR currents after withdrawal in WT mice

There are very few patch-clamp studies in VTA DA neurons of adult animals that examine the dynamic changes in nAChR-expressing VTA DA neurons following prolonged nicotine exposure (≥ 2 months). We first measured nAChR-mediated whole-cell currents in VTA DA neurons in WT mice to determine the effects of long-term nicotine exposure and withdrawal on nAChR function (Fig 3A). One-way Welch's ANOVA revealed a significant difference between time points associated with withdrawal [ $F_{(4,26.441)}$=5.969, p<0.01] in WT animals. ACh-induced whole-cell currents were comparable between saccharine-treated (99.34± 11.59 pA, n=17) and nicotine-treated (89.47 ± 10.15 pA, n=16 from 12 mice) groups [p=0.999], consistent with previous findings indicating that chronic nicotine does not functionally alter nAChRs in midbrain DA neurons (Nashmi et al., 2007; Xiao et al., 2009) (Fig 3B).

However, ACh-induced whole-cell currents became significantly smaller following 1-4 days of nicotine withdrawal [WD 1-4 days: 54.40 ± 4.96 pA, n=10 from 8 mice, p<0.05 vs SAC/NIC], indicating reduced nAChR function resulting from nicotine withdrawal. The suppressed ACh-mediated currents did not recover after 2-4 weeks of withdrawal [WD 2-4 weeks: 50.14 ± 4.06 pA, n=7 from 6 mice, p<0.05 vs SAC/NIC, p=0.96 vs WD 1-4 days] and, surprisingly, remained similarly depressed even after 10 weeks of withdrawal [WD 5-10 weeks: 48.40 ± 4.97 pA, n=10 from 9 mice]. ACh-induced currents from mice following 5-10 weeks of withdrawal were significantly lower than saccharin only- or nicotine-drinking mice [p<0.05] but did not significantly differ from earlier time points during withdrawal [p=0.91 vs WD 1-4 days; p=0.99 vs WD 2-4 weeks]. These results suggest that chronic

nicotine does not change nAChR function in WT mice, but that nicotine withdrawal results in long-lasting down-regulation of nAChR function (Gould et al., 2012).

### Nicotine-induced increase in nAChR currents in α5-null mice is long-lasting

We evaluated the effect of chronic nicotine exposure and withdrawal in mice lacking the α5 subunit. One-way ANOVA revealed a significant difference across treatment conditions [Welch's $F_{(4,26.165)}$=14.262, p<0.001] in α5-null animals (Fig 4 A,B). In contrast to the observations in WT mice, VTA DA neurons in α5-null mice chronically treated with nicotine were significantly more responsive to ACh [26.34 ± 3.39 pA, n=15 from 12 mice; p<0.005 vs SAC] than those from SAC-treated mice [9.66 ± 1.17pA, n=20], implying that chronic nicotine treatment leads to enhanced nAChR function in the absence of the α5 subunit. This increase in nAChR function persisted beyond cessation of nicotine intake. The peak amplitude of ACh-induced currents in α5-null mice in the first 1-4 days of withdrawal (42.65 ± 7.9, n=13 from 12 mice) remained significantly higher than SAC-treated controls [p<0.01] but did not significantly differ from α5-nulls drinking nicotine [p=0.355]. The enhanced response to ACh persisted after 2-4 weeks [36.91 ± 6.67 pA, n=13 from 11 mice, p<0.05 vs SAC] and 5-10 weeks [37.08 ± 7.33 pA n=12 from 10 mice, p<0.05 vs SAC] of withdrawal from chronic nicotine treatment.

### The effects of nicotine in α5-SNP mice are complex, with some similarities to α5-null mice

Neurons from α5-SNP mice displayed an interesting response to nicotine and withdrawal. As in WT and α5-null mice, one-way ANOVA revealed a significant effect of treatment condition on ACh-induced currents [Welch's $F_{(4,\ 21.883)}$=6.335, p<0.005] in α5-SNP mice (Fig 5A,B). The peak nAChR current amplitude in the α5-SNP mice was significantly higher during nicotine-drinking (60.03 ± 10.22 pA, n=11 from 8 mice) than SAC-treated controls (22.51 ±2.8 pA, n=10; p<0.05 NIC vs SAC), demonstrating that chronic nicotine exposure enhances nAChR function in mice expressing the rs16969968 SNP in VTA DA neurons. Although the currents in SAC-treated controls are smaller in the α5-SNP mice than the WT mice, the ACh-induced currents in α5-SNP mice approached amplitudes closer to those observed in WT mice before and during nicotine treatment. Enhanced nAChR function in α5-SNP mice persisted in the days immediately following cessation of nicotine treatment (WD 1-4 days: 72.59 ± 12.22 pA, n=12 from 9 mice; p<0.05 vs SAC). However, unlike the WT and α5 null mice, the peak amplitude of ACh-induced currents was no longer significantly different from SAC controls at either 2-4 weeks (35.19 ± 9.65 pA, n= 8 from 5 mice; p=0.718 vs. SAC) or 5-10 weeks after nicotine cessation (28.15 ± 3.24 pA, n= 14 from 11 mice; p=0.68 vs. SAC), with a trend towards reduced function compared to nicotine-drinking α5-SNP mice [p=0.072, WD 5-10 weeks vs. NIC].

## Discussion

Our study addressed the long-term changes in nAChR function that take place in DA neurons both during and after prolonged exposure to nicotine. We demonstrated that α5-containing nAChRs are essential for the adaptations in nAChR function that occur during nicotine exposure and withdrawal. We also showed that expression of the hypofunctional rs16969968 *CHRNA5* variant in VTA neurons leads to abnormal nAChR responses during

both long-term nicotine use and the early phases of withdrawal, shedding light on the role of this human polymorphism in nicotine use and abuse.

## The α5 subunit governs basal nAChR responsivity in VTA DA Neurons

Studies using α5-null animals have consistently demonstrated reduced nAChR functionality when the α5 subunit is absent (Chatterjee et al., 2013; Morel et al., 2014; Sciaccaluga et al., 2015). Corroborating these observations, we found that ACh-induced whole-cell currents were significantly reduced in DA neurons from α5-null mice.

Recent studies suggest that, under baseline conditions, observed differences in the neuronal response to ACh between WT neurons and neurons with disrupted α5 subunits are balanced by other factors, resulting in a similar level of functionality at a systems level. For example, although the absence of the α5 subunit decreases nAChR function (Chatterjee et al., 2013; Morel et al., 2014; Sciaccaluga et al., 2015), baseline firing frequency and bursting in VTA DA neurons of α5-null and α5-SNP mice and rats did not significantly differ from WT controls (Forget et al., 2018; Morel et al., 2014). Similarly, basal levels of DA in the NAc—a primary target of the VTA—did not differ between α5-null and WT mice (Besson et al., 2016). When challenged with acute nicotine, however, disruptions in DA neuron function are unmasked. α5-null mice show blunted DA release at nicotine doses that reliably increase DA in WT mice (Besson et al., 2016), and VTA DA neurons lacking α5 fail to respond to doses that increase firing in WT cells (Forget et al., 2018; Morel et al., 2014).

Cholinergic inputs to VTA DA neurons are thought to promote fast, “phasic” firing (Floresco et al., 2003), which is believed to be a critical component of reward and reinforcement (Dautan et al., 2016; Grace et al., 2007). Because α5-null mice demonstrate a hypofunctional response to ACh, it seems reasonable to hypothesize that initial exposure to nicotine might be less rewarding/reinforcing when mutated CHRNA5 is expressed in VTA neurons. Indeed, both α5 null and α5 SNP mice required a dose of nicotine at least twice as high as WT mice to self-administer intravenous nicotine in a model of initiation of drug-taking behavior (Morel et al., 2014). This result suggests that the dose of nicotine required to elicit a sufficient DA response to support reinforcement during early use is higher when CHRNA5 is mutated.

## α5 regulates nAChR changes in response to chronic nicotine exposure

Adaptations to chronic nicotine are different when α5 is absent or mutated. Consistent with previous reports suggesting that homeostatic regulation masked differences in nAChR number and function in WT animals (Besson et al., 2007), we found that responsivity to ACh in cells from WT mice undergoing chronic nicotine consumption was similar to that of cells from drug-naïve mice. In contrast to neurons from WT mice, we observed a significant increase in the response to ACh in α5-null VTA DA neurons from mice undergoing chronic nicotine treatment compared to drug-naïve mice. Therefore, chronic nicotine exposure enhanced nAChR function, thereby shifting nAChR responsivity closer to the higher response level observed in drug-naïve WT mice.

The effects of chronic nicotine on nAChR expression and function are complex, with regional and subunit-specific variability. Chronic nicotine exposure is believed to trigger

a homeostatic response to nAChR desensitization, including subunit-specific increases in nAChR binding that are primarily attributable to an upregulation of β2-containing nAChRs (Besson et al., 2007; Dani and Heinemann, 1996; Mao et al., 2008)—particularly in mesolimbic areas (Nashmi et al., 2007; Nguyen et al., 2003; Renda and Nashmi, 2014)—in both rodents (Nashmi et al., 2007; Renda and Nashmi, 2014) and humans (Staley et al., 2006). Nicotine-induced upregulation of α4β2-nAChRs accounts for a large proportion of the increase in nAChRs (Marks et al., 2014; Nguyen et al., 2003), whereas evidence suggests that α6β2-nAChRs are downregulated (Perez et al., 2008; Perry et al., 2007). The presence of the α5 subunit modulates these dynamics: expression of α5 in α4α5β2-nAChRs reduces desensitization and speeds subsequent recovery in response to nicotine (Grady et al., 2012) and prevents upregulation following chronic nicotine administration (Mao et al., 2008). α4β2-nAChR upregulation in the VTA has been primarily reported in GABA neurons in WT animals, where α5 is found in fewer than 20% of those neurons compared to approximately 80% in VTA DA neurons (Klink et al., 2001). Because α5-null mice do not express any α4α5β2 nAChRs, the observed increased responsivity to ACh in VTA DA neurons may be the result of an overall upregulation of high-affinity α4β2-nAChRs that, under normal circumstances, is selective to GABA neurons.

As previously mentioned, α5-null and α5-SNP mice require nicotine doses at least twice as high as WT mice to reach the same levels of self-administration (Morel et al., 2014). Furthermore, while WT mice titrate the amount of nicotine they self-administer—maintaining a preferred level of intake despite increasing doses—α5-null and α5-SNP mice increase the amount of nicotine taken (Fowler et al., 2011; Morel et al., 2014) when receiving doses that would normally be aversive (Fowler et al., 2011) or even toxic (Salas et al., 2003) to WT mice. This ‘loss of control’ over nicotine consumption at high doses was corrected by re-expression of α5 in VTA DA cells (Morel et al., 2014), suggesting a direct involvement of the VTA in this behavior.

Although the mesolimbic DA system plays a role in the dose-dependent effects of nicotine on both reinforcement and aversion (Morel et al., 2014; Wills et al., 2022), other brain areas expressing CHRNA5 might also contribute to these effects. Besides the VTA, α5-containing nAChRs are highly expressed in few other brain areas, with the interpeduncular nucleus (IPN) representing the region most enriched in those receptors (Beiranvand et al., 2014; Forget et al., 2018; Salas et al., 2003; Salas et al., 2009). Although stimulation of α5-expressing IPN GABAergic neurons does not induce withdrawal, it does trigger aversion-like behavior in animals exposed to nicotine (Morton et al., 2018). Interestingly, a subpopulation of IPN GABA neurons implicated in nicotine aversion sends projections to the laterodorsal tegmentum (LDTg) (Wolfman et al., 2018), which in turn mediates reward (Lammel et al., 2012) and promotes DA-neuron burst firing (Lodge and Grace, 2006; Omelchenko and Sesack, 2005) via projections to the VTA. Therefore, altered nAChR function in both VTA DA neurons and IPN GABA neurons could contribute to the escalation of nicotine consumption observed in α5-null and α5-SNP mice.

### α5 regulates nAChR changes in response during withdrawal from chronic nicotine

Nicotinic responses during withdrawal from nicotine differ from WT mice when α5 is absent or mutated. Although WT nAChR peak currents were unchanged during nicotine exposure, we observed a dramatic reduction in nAChR function during nicotine withdrawal that persisted for at least 10 weeks in tested mice (Fig 3B). The plastic changes that maintain homeostasis during chronic nicotine intake become inappropriate during abstinence, leading to a withdrawal syndrome that includes physical, affective, and cognitive symptoms (McLaughlin et al., 2015; Shiffman et al., 2004). Withdrawal severity is believed to contribute to the risk of relapse (Zhou et al., 2009) and relies on multiple areas of the brain in addition to the VTA, particularly the medial habenula and interpeduncular nucleus, where α5-containing nAChRs are fundamental to nicotine withdrawal (Salas et al., 2009). The mechanisms underlying the dramatic, long-lasting reduction in nAChR function observed in DA neurons from WT mice during nicotine withdrawal are unclear but appear to rely on the α5 subunit. In stark contrast to WT mice, the increased nAChR function observed in neurons from nicotine-treated α5-null mice persisted throughout withdrawal, remaining significantly elevated above ACh-induced currents in drug-naive controls. Because α5-null mice do not display physical signs of withdrawal (Salas et al., 2009), it is tempting to speculate that the effects we describe in this study might contribute to the behavioral phenotype of α5-null mice.

Nicotine withdrawal is associated with a reduction in tonic and phasic DA release in the nucleus accumbens shell (Zhang et al., 2012). There are many factors controlling VTA cell function and DA release (Kramer et al., 2022; Liu and Kaeser, 2019), and nAChRs are particularly important contributors. Nicotine exposure enhances DA release through a dynamic balance of activation and desensitization of nAChRs in the VTA and the striatum (Mansvelder and McGehee, 2000; Picciotto et al., 2008; Pidoplichko et al., 2004). That nAChR function does not decrease during withdrawal in α5 null mice, remaining elevated above drug-naïve levels, might result in persistent changes in phasic and tonic DA signaling (Zhang et al., 2012). Furthermore, nicotine withdrawal is associated with increased activity of IPN GABAergic neurons (Klenowski et al., 2022), many of which contain α5 nAChRs. Therefore, mutated α5 might alter VTA and IPN cellular and circuit-level adaptations that normally lead to the manifestation of nicotine withdrawal.

### Similar effect of the rs16969968 SNP and the a5 null mutation.

The human polymorphism rs16969968 produces nAChRs with hypofunctional α5 subunits, resulting in significantly lower calcium permeability and diminished α4β2α5-nAChR function in heterologous expression systems and neurons expressing the α5 SNP in an α5 null background (Bierut et al., 2008; Sciaccaluga et al., 2015). We used a viral vector to re-express the SNP variant of the α5 subunit in DA neurons within the VTA of α5-null mice to determine how the SNP modifies nAChR function at baseline and across nicotine use and withdrawal. Similar to nicotine-naive α5-null mice, VTA nAChR currents from nicotine-naïve α5-SNP mice are significantly smaller than those recorded from WT neurons, but nAChR currents from α5-SNP neurons were over ≈33% larger than those from α5-null neurons. We also showed that, similar to the α5-null mutation, α5-SNP nAChR currents increase after chronic nicotine exposure.

Functional alterations produced by CHRNA5 mutations—such as the rs16969968—are relevant when considering VTA neuronal adaptations to chronic nicotine exposure. Smokers with the risk allele encoding the D398N CHRNA5 mutation alter their smoking behavior to obtain more nicotine (Macqueen et al., 2014) and display lower nicotine aversion than subjects carrying the common allele (Jensen et al., 2015). Interestingly, smokers with high-risk genetic variants in the *CHRNA5-CHRNA3-CHRNB4* region, including rs16969968, are at increased risk of nicotine cessation failure, a phenomenon that may reflect the fact that, in subjects carrying the mutation, continued nicotine exposure might partially ameliorate the decreased nAChR function, making it more difficult to quit (Chen et al., 2012).

Neither α5-null nor α5-SNP mice demonstrated the dramatic and persistent reduction in ACh-induced currents observed in WT mice during nicotine withdrawal. During the early stages of withdrawal, neurons from α5-SNP mice most resembled those from α5-null mice, in that ACh-induced currents did not fall below levels observed in nicotine-naïve controls and were instead elevated in the first few days of withdrawal, the time of highest risk for relapse (Hughes et al., 2004). Zhang and colleagues (Zhang et al., 2012) showed that, during withdrawal, phasic DA release is increased relative to tonic DA release in WT animals, thereby increasing DA's "signal-to-noise" ratio. Although the effect of α5 disruption on this phenomenon is unknown, it is possible that increased α4ß2 nAChR function in DA neurons from α5-null and α5-SNP mice results in a higher propensity for fast, "phasic" firing than WT mice. Therefore, the DA signal produced by an acute nicotine re-exposure would be especially elevated in the α5-SNP mice, producing a DA response that may alter the vulnerability to some forms of relapse. To that end, a recent preclinical study demonstrated an enhanced propensity to nicotine-primed reinstatement in α5-SNP rats (Forget et al., 2018), whereas another study suggests that smokers expressing rs16969968 are less responsive to cigarette cues than those with fully functional α5-containing nAChRs (Janes et al., 2012). These individuals have a three-fold increased likelihood of responding to pharmacologic cessation treatments that include nicotine replacement therapy or DA and norepinephrine reuptake inhibition (bupropion), compared to smokers with low-risk genetic variants in the same gene cluster (Chen et al., 2012). Our findings might provide a mechanism by which drugs that enhance/maintain DA activity are more effective during nicotine cessation in individuals carrying the CHRNA5 polymorphism.

### Summary

Our data demonstrate the significant role of α5-nAChRs in the effects of chronic nicotine exposure on VTA DA neurons. Lack of α5 subunits or the expression of the rs16969968 SNP leads to long-lived alterations of VTA nAChR receptors. Further research is needed to investigate how these DA neuron adaptations to nicotine influence the broader mesolimbic DA system to impact nicotine use and abuse.

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## Highlights

1. CHRNA5 mutation significantly reduces VTA nAChR function at baseline.
2. Chronic nicotine exposure enhances nAChR function when CHRNA5 is mutated.
3. nAChR currents decrease during extended withdrawal in control mice.
4. nAChR currents remain enhanced during nicotine withdrawal when CHRNA5 is mutated.

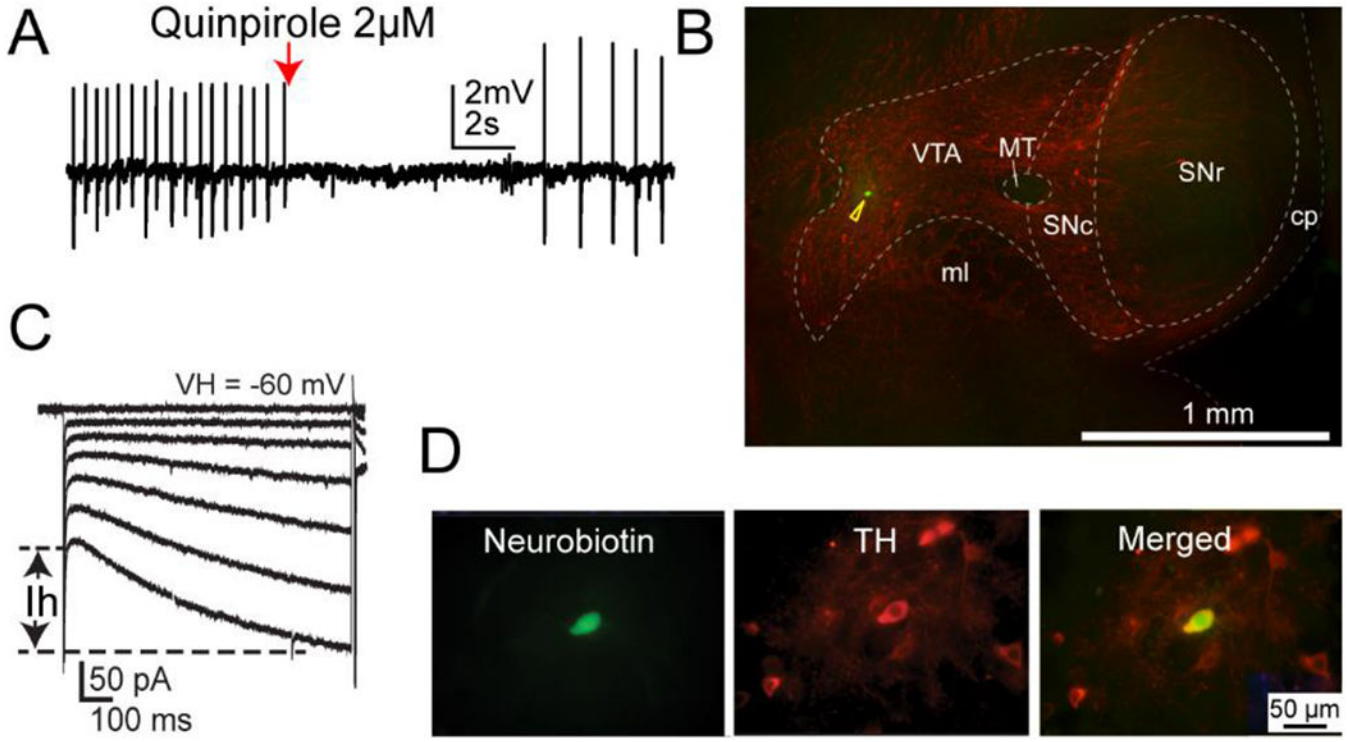


**Figure 1.**

Identification of VTA DAergic neurons. Cell DAergic identity was confirmed by several approaches. DAergic cells displayed inhibited response to quinpirole (A), were anatomically localized to the VTA region (B), and were defined by the presence of hyperpolarization-activated cationic currents (H-currents, C). In addition, recorded cells were back-filled with neurobiotin and confirmed as DAergic neurons using TH immunohistochemical staining (D).

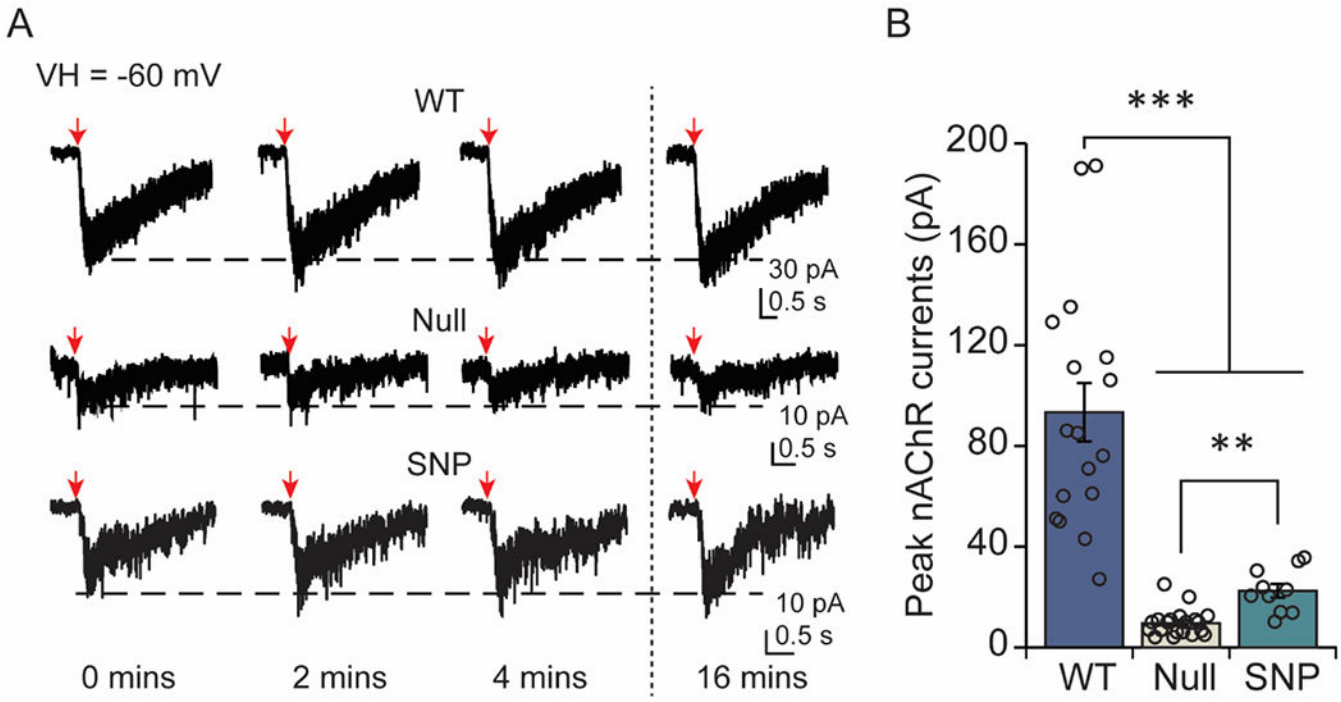


**Figure 2.**

The α5 subunit regulates baseline response to ACh in DA neurons. (A) Example traces are shown from WT, α5-null, and α5-SNP neurons. Downward arrows indicate ACh puffs, which were applied once every 2 mins. ACh induced stable inward currents in DA neurons that were stable and persistent. Recordings were taken at the indicated times (in mins). (B) Currents (mean ± SEM) in response to the ACh puff. The amplitude of the response to ACh was significantly higher in WT neurons than neurons from animals without α5 or with the α5 SNP. **p<0.01, ***p<0.001

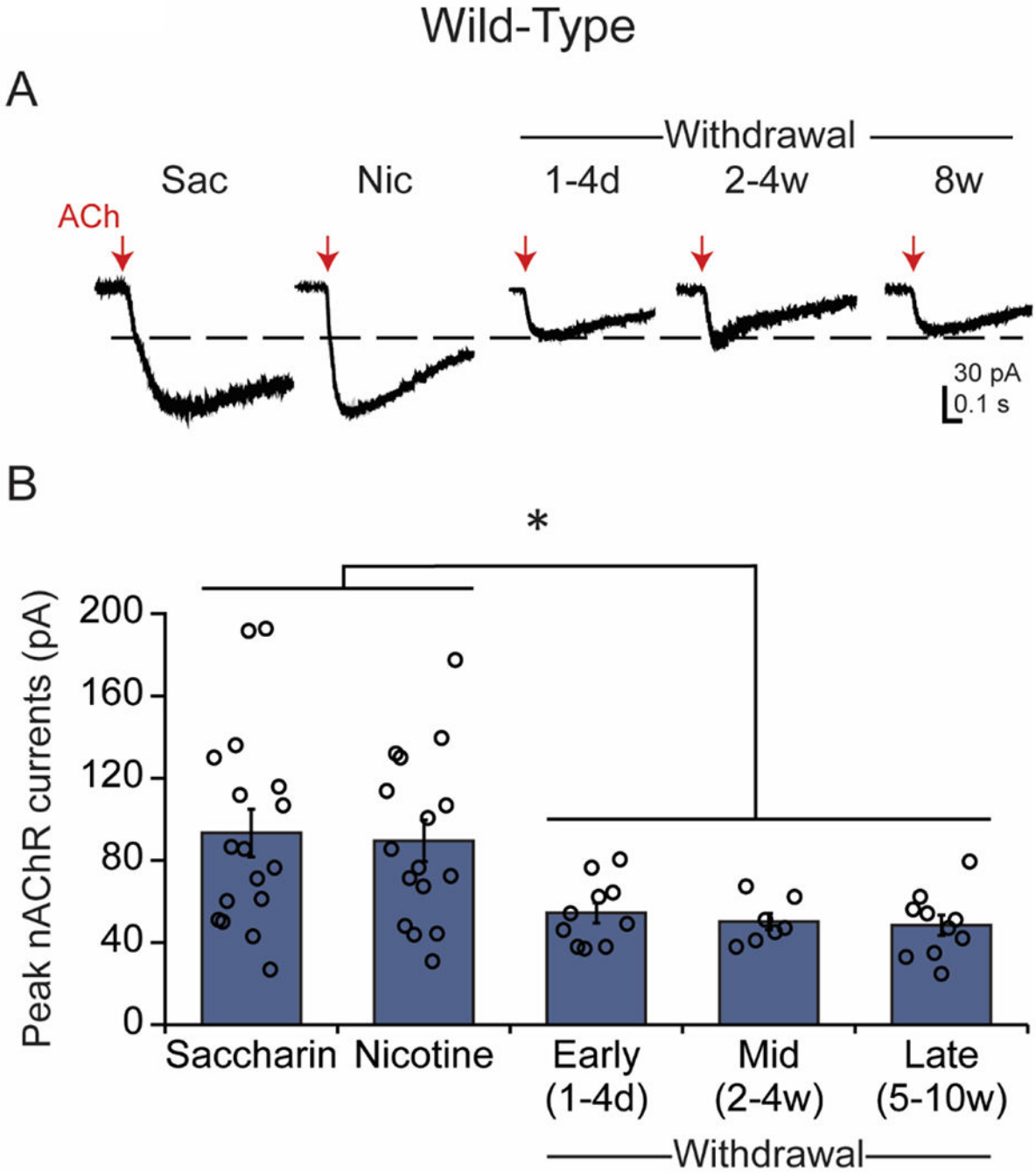


**Figure 3.**

Long-term withdrawal depresses the response to ACh in WT DA neurons. (A) Example traces are shown from WT DA neurons under saccharin/drug-naïve and chronic nicotine conditions, and in different stages of withdrawal. Red arrows indicate the ACh puffs. (B) Current (mean ± SEM) responses to the ACh puff. The amplitude of the response to ACh was significantly reduced during withdrawal compared to saccharin/drug-naïve and nicotine-treated conditions. *p<0.05

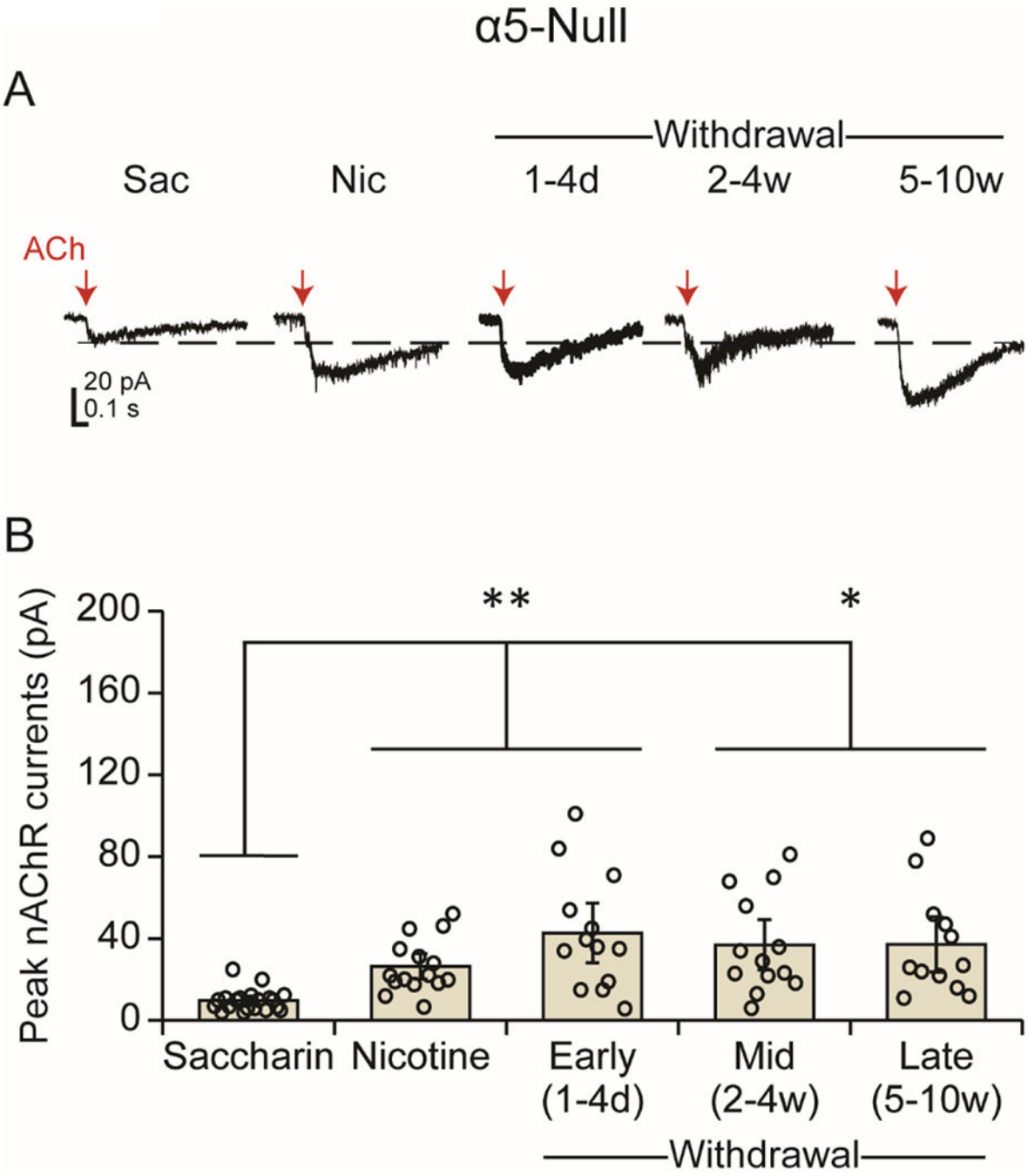


**Figure 4.**

In the absence of the α5 subunit, the ACh-induced currents are smaller than WT (Fig 2), but chronic nicotine leads to an increase in the ACh response that continues into withdrawal in VTA DA neurons. (A) Example traces are shown from α5-null DA neurons under saccharin/drug-naïve and chronic nicotine conditions and in different stages of withdrawal. Red arrows indicate the ACh puffs. (B) Current (mean ± SEM) responses to the ACh puff. The amplitude of the response to ACh was significantly enhanced under chronic nicotine

treatment and throughout withdrawal relative to saccharin/drug-naïve conditions. *p<0.05, **p<0.01

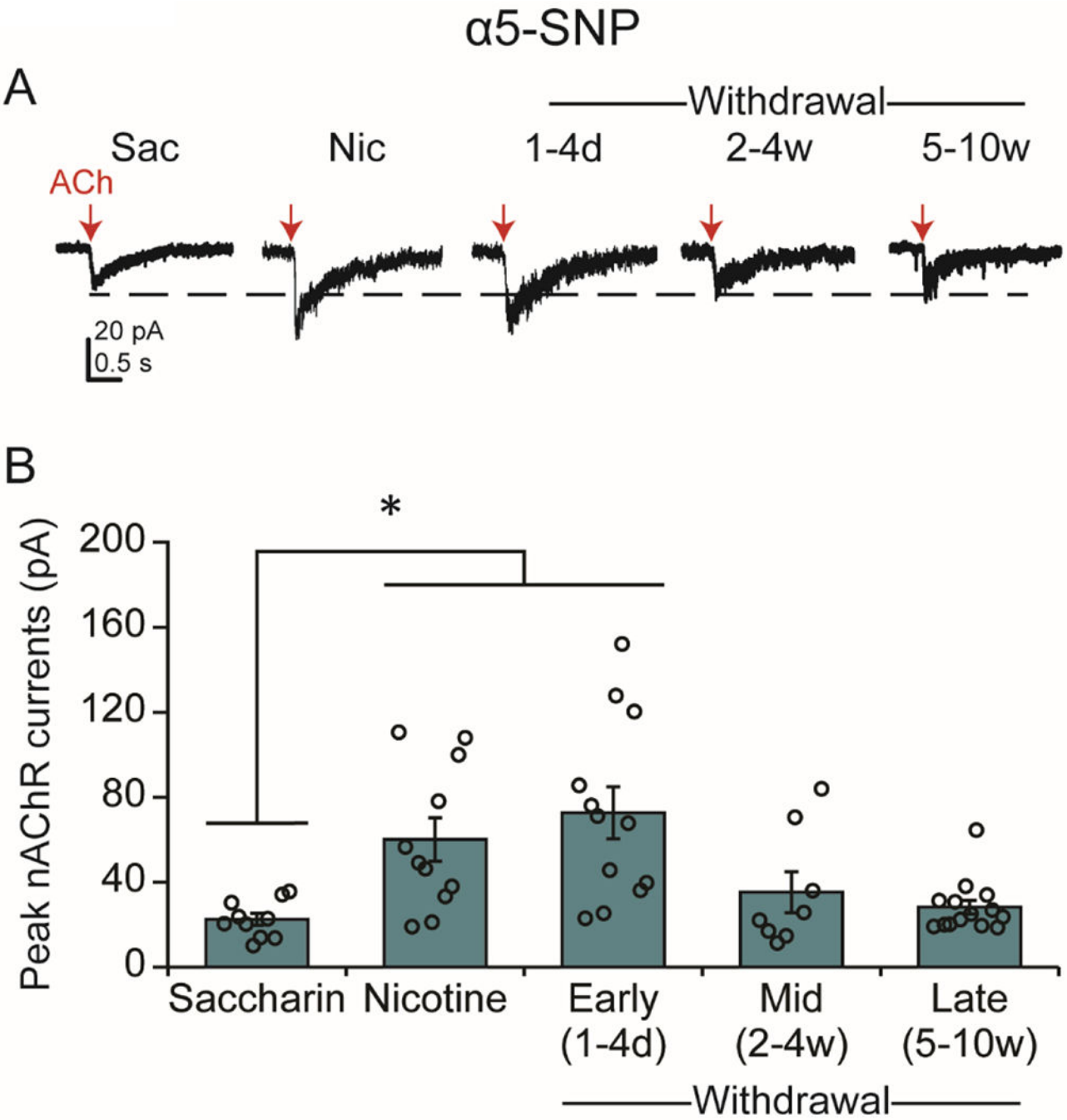


**Figure 5.**

Re-expression of the α5-SNP in VTA DA neurons of α5-null mice produces a complex response to nicotine and withdrawal. (A) Example traces from α5-SNP DA neurons under saccharin/drug-naïve and chronic nicotine conditions and in different stages of withdrawal. Red arrows indicate the ACh puffs. (B) Current (mean ± SEM) responses to the ACh puffs. The amplitude of the response to ACh was significantly enhanced under chronic nicotine

treatment and the initial stage of withdrawal, but currents returned to saccharin/drug-naïve levels at later stages of withdrawal. *$p<0.05$