Behavioral/Cognitive

Nicotine Enhances Excitability of Medial Habenular Neurons via Facilitation of Neurokinin Signaling

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The medial habenula (MHb) densely expresses nicotinic acetylcholine receptors (nAChRs) and participates in nicotine-related behaviors such as nicotine withdrawal and regulating nicotine intake. Although specific nAChR subunits are identified as being involved in withdrawal behavior, the cellular mechanisms through which nicotine acts to cause this aversive experience is unclear. Here, we demonstrate an interaction between the nicotinic and neurokinin signaling systems that may form the basis for some symptoms experienced during nicotine withdrawal. Using patch-clamp electrophysiology in mouse brain slices, we show that nicotine (1 μM) increases intrinsic excitability in MHb neurons. This nicotine-induced phenomenon requires α5-containing nAChRs and depends on intact neurokinin signaling. The effect is blocked by preincubation with neurokinin 1 (NK1; L-732138, 10 μM) and NK3 (SB222200, 2 μM) antagonists and mimicked by NK1 (substance P, 100 nM) and NK3 (neurokinin B [NKB], 100 nM) agonists. Microinjections (1 μl) of L-732138 (50 nM) and SB222200 (100 nM) into the MHb induces withdrawal behavior in chronic nicotine-treated (8.4 mg/kg/d, 2 weeks) mice. Conversely, withdrawal behavior is absent with analogous microinjections into the lateral habenula of nicotine-treated mice or in mice chronically treated with a vehicle solution. Further, chronic nicotine reduces nicotine's acute modulation of intrinsic excitability while sparing modulation by NKB. Our work elucidates the interplay between two neuromodulatory signaling systems in the brain through which nicotine acts to influence intrinsic excitability. More importantly, we document a neuroadaptation of this mechanism to chronic nicotine exposure and implicate these mechanisms collectively in the emergence of nicotine withdrawal behavior.

Key words: **alpha 5 nicotinic subunit; medial habenula; neurokinins; nicotine; electrophysiology; withdrawal**

Introduction

Nicotinic acetylcholine receptors (nAChRs) are found throughout the nervous system, consisting of numerous combinations of α (α2–9) and β (β2–4) subunits in the form of homomeric and heteromeric ion channels (Dani and Bertrand, 2007). In the periphery, nAChRs primarily mediate fast synaptic transmission (Zhang et al., 1996; Ullian et al., 1997), whereas in the CNS, they mostly modulate the function of other neurotransmitter systems (McGehee et al., 1995; Gray et al., 1996; Léna and Changeux, 1997). Nicotine is arguably the primary addictive component in tobacco and its initial actions are via nAChRs (Benowitz, 2009; De Biasi and Dani, 2011). Through genome-wide association studies (GWAS), we now know that increased risk of nicotine dependence in humans is associated with specific single-nucleotide polymorphisms located within a particular gene cluster on chromosome 15 that encodes the α5, α3, and β4 nAChR subunits (Boulter et al., 1990; Saccone et al., 2007; Berrettini et al., 2008; Bierut et al., 2008; Thorgeirsson et al., 2008). Of these three subunits, α5 has recently received special attention due to its association with various aspects of nicotine dependence, including the number of cigarettes smoked per day (Berrettini et al., 2008) and early onset of nicotine dependence (Weiss et al., 2008), as well as with tobacco-related diseases such as lung cancer (Amos et al., 2008; Thorgeirsson et al., 2008; Wang et al., 2010) and chronic obstructive pulmonary disease (Wang et al., 2010).

The habenulo-interpeduncular (Hb-IPN) pathway is one of the few areas of the brain where the α5, α3, and β4 subunits are coexpressed (Salas et al., 2004a; Salas et al., 2004b; Salas et al., 2009; Fowler et al., 2011). The Hb-IPN pathway comprises the epithalamic medial (MHb) and lateral habenula (LHb), the interpeduncular nucleus of the midbrain, and the fasciculus retroflexus, a white matter tract composed predominantly of efferents from the habenula to the IPN and other midbrain nuclei (Sutherland, 1982). The habenula mediates negative reward and motivation (Matsumoto and Hikosaka, 2007, 2009; Frahm et al., 2011; Lammel et al., 2012) and the cholinoceptive MHb in particular plays an important function in nicotine-related behaviors, especially those that are aversive (Salas et al., 2009; Fowler et al., 2011; Frahm et al., 2011). We determined that α5- and β4-containing habenular nAChRs are important for the emergence of nicotine

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withdrawal behavior (Salas et al., 2004a; Salas et al., 2009). Others have shown that habenular nAChRs containing the α5 subunit regulate the consumption of nicotine at normally aversive high doses (Fowler et al., 2011). Regarding the underlying physiology, it is known that habenular cholinergic afferents to the IPN corelease glutamate and acetylcholine to excite IPN neurons via fast and slow transmission modes, respectively (Ren et al., 2011). However, the relationship within the MHb between cholinergic signaling and other neuromodulatory influences that regulate cellular physiology and behavior is unknown (Ebner and Singewald, 2006; Merighi et al., 2011). In this study, we investigated the signaling processes that underlie nicotine-induced neuroadaptations that are the basis for nicotine withdrawal behavior.

Materials and Methods

Animals. We studied both male and female C57BL/6J mice or nAChR subunit mutant mice (α5, β2, β4) backcrossed for a minimum of 11 generations into the C57BL/6J background. WT controls and mutant mice were obtained from homozygous breeders that were offspring of heterozygous parents so that both WT and mutant mice had common ancestors. For electrophysiology experiments, mice were used between the ages of postnatal day 17 (P17) and P30. For behavioral experiments, mice were 2–4 months old at the start of nicotine treatment. Mice were weaned at P21 and same-sex littermates were housed in cages with a maximum of five mice per cage. All mice were housed under a 12 h light/dark cycle with *ad libitum* access to food and water. All procedures received preapproval from the Baylor College of Medicine Institutional Animal Care and Use Committee and were performed in accordance with the guidelines for animal intramural research from the National Institutes of Health.

Brain slice preparation. Mice were anesthetized by intraperitoneal injection of a drug mixture containing the following (in mg/kg): 100 ketamine, 20 xylazine, and 3 acepromazine. After decapitation, brains were dissected and immediately placed into ice-cold cutting solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 D-glucose, 4 $MgCl_2$, and 1 $CaCl_2$ bubbled with 95% O_2/5% CO_2. Coronal brain slices containing the MHb were prepared in the same ice-cold cutting solution using a microtome (VT1000S; Leica Microsystems) at a thickness of 250 μm. After slicing, brain slices were transferred to an incubation chamber containing cutting solution, where they were kept at 32°C for 20 min and then at room temperature for at least 40 min until transfer to the recording chamber.

Electrophysiology. Slices were transferred to a recording chamber in an upright light microscope (Axioskop 2 FS; Carl Zeiss) and were continually bathed (1–2 ml/min) in recording solution (same as cutting solution with the following modifications: 1 mM $MgCl_2$, 2 mM $CaCl_2$) maintained at 32–34°C using a temperature controller (TC-324B; Warner Instruments). Whole-cell patch-clamp recordings were obtained from neurons in the MHb under visual guidance using borosilicate glass pipette electrodes. Electrodes were filled with a K-methanesulfonate-based internal solution containing the following (in mM): 130 CH_3KO_3S, 0.1 EGTA, 10 HEPES, 2 MgCl2, 4 Mg-ATP, 0.3 Tris-GTP, and 7 phosphocreatine*di(Tris), pH: 7.3, 280–285 mOsm.

Electrophysiological signals were acquired using a patch-clamp amplifer (Axoclamp 200B; Molecular Devices) and digitizer (Digidata 1322A; Molecular Devices) and recorded using pCLAMP (Molecular Devices). For current-clamp recordings, signals were filtered at 10 kHz and sampled at 25 kHz. In voltage-clamp recording mode, signals were filtered at 5 kHz and sampled at 20 kHz. Series resistance was monitored in voltage clamp using a series of three hyperpolarizing voltage steps (−80 to −100 mV, $\Delta V = 10$ mV) from a −70 mV holding potential. For current-clamp experiments, a holding current was injected through the pipette to maintain a resting potential of −70 mV. After drug application, the holding current was adjusted to preserve the −70 mV resting potential. Input resistance (R_{in}) was measured using a series of hyperpolarizing current steps (−10 to −40 pA, $\Delta I = 10$ pA) and calculating R_{in} from Ohm's law ($V = IR$). Intrinsic excitability was measured in current-clamp mode by injecting a series of 5 2-s-long depolarizing current steps (+10 to +50 pA, $\Delta I = 10$ pA) at a frequency of 0.1 Hz and counting the number of resultant action potentials during each step. After baseline recordings, further recordings were performed after 10 min wash-in periods in which nicotine, substance P (SP), or neurokinin B (NKB) were added to the recording solution. In some experiments, slices were preincubated for at least 10 min with antagonists for nAChRs (mecamylamine or methyllycaconitine) or neurokinin receptors (L-732138, L-733060, or SB222200) before obtaining baseline recordings. Control recordings performed alongside experiments using agonists and antagonists resembled data from Figure 1 and were pooled together during the analysis. All drugs were purchased from either Sigma-Aldrich or Tocris Bioscience/R&D Systems.

Chronic nicotine treatment for electrophysiology. For experiments involving chronic nicotine treatment in animals destined for use in electrophysiology experiments, nicotine was delivered to experimental animals via the mothers' milk. Between P1 and P3, the male in a breeding cage was removed and the nursing dam was implanted with a 28 d osmotic minipump (Alzet) to deliver vehicle or nicotine (free base) at a constant rate of 8.4 mg/kg/d. To verify receipt of nicotine in the pups, blood serum was collected and analyzed for cotinine, a metabolite of nicotine, using an ELISA kit (Calbiotech). After brain slice preparation, slices were incubated in nicotine-free ACSF and used as usual for electrophysiology experiments.

Chronic nicotine treatment, habenula cannulation, and somatic nicotine withdrawal behaviors. Adult C57BL/6J mice were implanted on day 0 with osmotic minipumps (Alzet) to deliver saline alone or nicotine in saline at a constant rate of 8.4 mg/kg/d (concentration expressed as free base). On days 9–10 of nicotine treatment, animals were implanted with bilateral cannulae into the MHb or LHb. These cannulae would be used during behavioral testing to deliver antagonists for neurokinin receptors. Behavioral testing began on day 12 and took place over the course of 3 d. On day 1 of testing, 0.5 μl of either L-732138 (50 nM) in saline or saline alone was microinjected through the cannulae at a rate of 0.25 μl/min. Mice were returned to their home cages and 10 min later were examined for the emergence of somatic withdrawal signs. The total number of cage scratches, wet dog shakes, grooming events, chewing motions, head noddings, and jumps during a 20 min examination period was noted and the cumulative sum of all these events became the score for the nicotine withdrawal assay (Salas et al., 2004a). On day 2 of testing, 0.5 μl of SB222200 (100 nM) in saline was microinjected through the cannulae in animals that had previously received saline microinjections on testing day 1 and 0.5 μl of saline was microinjected through the cannulae in the L-732138-treated animals. Upon return to the home cage, behavioral testing occurred as on day 12. Finally, on testing day 3, animals either received 0.5 μl microinjections of both L-732138 (50 nM) and SB222200 (100 nM) in saline or saline alone and somatic withdrawal signs were assayed for a third time. After the completion of behavioral assays, histology was performed to confirm cannula placement.

Data analysis. All data were analyzed using Microsoft Excel and Statistica (Statsoft) software. For analyses of firing rate in response to depolarizing current steps, two-way ANOVA was used; asterisks in the figures indicate significance levels derived from the main effect of treatment (nicotine or neurokinins). Paired *t* tests were used to compare R_{in}. One-way ANOVA was used to compare nicotine-induced currents. In behavioral experiments, two-way ANOVA was used. For the comparison between nicotine-induced currents (see Fig. 3*B*) and for the withdrawal behavior studies (see Fig. 6), the Newman–Keuls *post hoc* test was used to further compare the groups. *F*-statistics and degrees of freedom displayed are of the main effect of nicotine.

Results

Nicotine enhances the intrinsic excitability of MHb neurons

We conducted patch-clamp experiments on neurons within the ventral two-thirds of the MHb because these comprise the cholinergic and cholinoceptive subdivisions of the nucleus (Fig. 1*A*; Arvidsson et al., 1997; Quick et al., 1999; Schütz et al., 2003; Lein et al., 2007). We first tested whether acutely applied nicotine

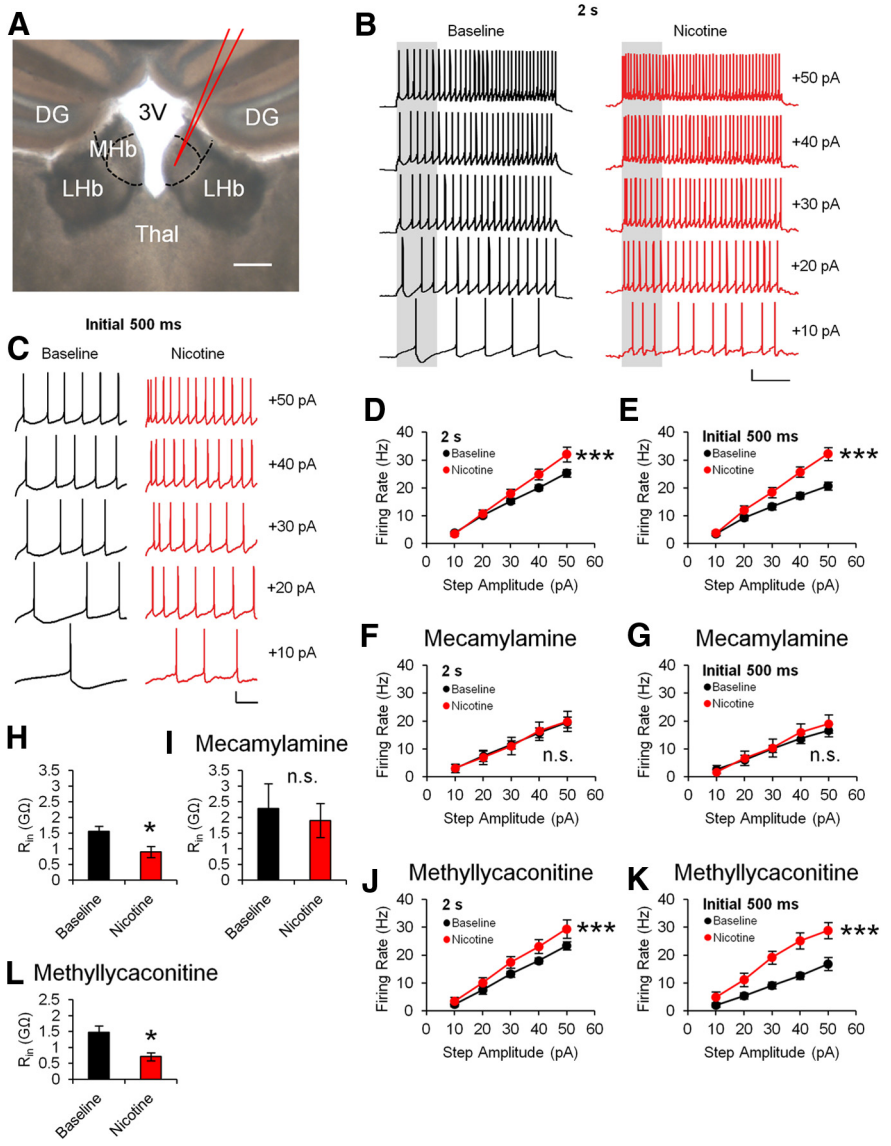

Figure 1. Nicotine enhances the intrinsic excitability of MHb neurons. ***A***, For all electrophysiological experiments, neurons were used from the cholinoceptive and cholinergic ventral two-thirds (red pipette) of the medial habenula (dashed lines) in coronal brain slices. MHb neurons were held at −70 mV in current-clamp configuration and subjected to 2 s depolarizing current steps of increasing amplitudes (10–50 pA, $\Delta I = 10$ pA) to elicit action potentials. After nicotine (1 μM) bath application, the holding current was readjusted to maintain the −70 mV resting potential and the protocol was repeated. ***B***, Example traces show that, after nicotine bath application, the number of action potentials elicited by each current step increased over the corresponding response during baseline, indicating that an increase in intrinsic excitability had occurred. ***C***, Facilitation of firing by nicotine was especially evident during the initial 500 ms of the current steps. Displayed traces in ***C*** are enlarged from the portion of the traces in ***B*** within the gray boxes. ***D***, ***E***, Quantified firing responses to the current steps for the entire 2 s step (***D***) or the initial 500 ms (***E***) reflect the nicotine-induced enhancement of excitability ($n = 20$ cells/14 mice). ***F***, ***G***, Blockade of heteromeric nAChRs by mecamylamine (10 μM) preincubation blocked the enhancement of excitability by subsequent application of nicotine (1 μM; $n = 6$ cells/3 mice). ***H***, ***I***, Nicotine bath application caused a reduction in R_{in} ($n = 20$ cells/12 mice), which was blocked by preincubation with mecamylamine ($n = 6$ cells/3 mice). ***J***, ***K***, Preincubation with methyllycaconitine (5 nM) did not alter the facilitation of intrinsic excitability by subsequent nicotine application ($n = 7$ cells/5 mice). ***L***, Likewise, it did not prevent the decrease in R_{in} by nicotine ($n = 7$ cells/5 mice). Scale bars: ***A***, 200 μm; ***B***, 20 mV/500 ms; ***C***, 20 mV/100 ms. All values are displayed as mean ± SEM. *$p < 0.05$; ***$p < 0.001$. Thal, thalamus; 3V, third ventricle; DG, dentate gyrus.

modulates the intrinsic excitability of MHb neurons by subjecting MHb neurons in current-clamp configuration to current steps of increasing amplitude (10–50 pA) starting from a holding potential of −70 mV. Because MHb neurons fire action potentials spontaneously (McCormick and Prince, 1987), negative current was generally required to maintain the desired resting membrane potential. We compared the action potential firing of MHb neurons to depolarizing current steps (2 s) before and after bath application of nicotine (1 μM; Fig. 1*B*,*D*). In the presence of nicotine, the holding current was increased to maintain the cell at the same resting potential of −70 mV. After nicotine application, despite maintaining the resting potential, a greater number of action potentials was elicited during each depolarizing current step ($n = 20$ cells/14 mice, $F_{(1,95)} = 34.60$, $p < 0.001$). The increase in firing was particularly prominent during the initial portion of the current steps ($n = 20$ cells/14 mice, $F_{(1,95)} = 94.46$, $p < 0.001$). Although the average firing rate across the 2 s step increased by up to ~25% over baseline, the average firing rate during the initial 500 ms of each step increased by up to ~55% over baseline (Fig. 1*C*,*E*). Similar results were obtained using nicotine at 100 nM (data not shown). Preincubation with the nAChR antagonist mecamylamine (10 μM; Papke et al., 2008) blocked the facilitation of intrinsic excitability during nicotine (1 μM) application ($n = 6$ cells/3 mice, $p > 0.05$; Fig. 1*F*,*G*). In contrast, when slices were preincubated with the nAChR antagonist methyllycaconitine (5 nM), which preferentially inhibits α7 homomeric nAChRs (Alkondon et al., 1992; Palma et al., 1996; Yu and Role, 1998), the facilitation of intrinsic excitability by nicotine remained across both the entire 2 s step ($n = 7$ cells/5 mice, $F_{(1,30)} = 16.92$, $p < 0.001$) and within the initial 500 ms of each step ($n = 7$ cells/5 mice, $F_{(1,30)} = 59.95$, $p < 0.001$; Fig. 1*J*,*K*). Therefore, heteromeric nAChRs, which are sensitive to blockade by mecamylamine, likely underlie the facilitative effect of nicotine on intrinsic excitability.

Nicotine decreases the input resistance of neurons in the MHb

An increase in R_{in} could explain the augmented intrinsic excitability upon nicotine application: with increased R_{in}, neurons would respond more to the same depolarizing current steps with greater voltage deflections and a consequent increase in action potential firing. However, when we measured R_{in} before and after nicotine (1 μM) application, we found that it dramatically decreased in the presence of nicotine (baseline: 1.76 ± 0.24 GΩ vs nicotine: 0.92 ± 0.27 GΩ, $n = 20$ cells/12 mice, $p < 0.05$; Fig. 1*H*), which is consistent with the opening of nAChRs. This decrease was abolished by preincubation with mecamylamine (baseline: 2.28 ± 0.79 GΩ vs nicotine: 1.90 ± 0.54 GΩ, $n = 6$ cells/3 mice, $p > 0.05$), but not by preincubation with methyllycaconitine (baseline: 1.47 ± 0.20 GΩ vs nicotine: 0.72 ± 0.13 GΩ, $n = 7$ cells/5 mice, $p < 0.05$;

Fig. 1*I*,*L*). Therefore, an increase in R_{in} is not the mechanism through which nicotine acts to promote intrinsic excitability.

α5 nAChR subunit is required for nicotine-induced increase in excitability

The MHb expresses a large complement (α3–7, β2–4) of nAChR subunits (Sheffield et al., 2000). To determine the nAChR subunits necessary for nicotine's facilitation of excitability, we used mice with null mutations in targeted nAChR subunits. Genetic variants in the *CHRNA5/CHRNA3/CHRNB4* gene cluster, which encodes the α5, α3, and β4 nAChR subunits (Duga et al., 2001), were found in genomic studies to influence various aspects of nicotine addiction (Saccone et al., 2007; Bierut et al., 2008; Thorgeirsson et al., 2008). Expressed in habenular nAChRs, these subunits are important for the aversive aspects of nicotine dependence (Salas et al., 2004a; Salas et al., 2009; Fowler et al., 2011; Frahm et al., 2011). Motivated by these genomic and behavioral studies, we focused our investigation on these nAChR subunits, particularly the α5 and β4 subunits, because of their demonstrated roles in aversive nicotine-related behaviors. Mice null for the α3 subunit were not examined because they have impaired growth and predominantly die before postnatal week 2 (Xu et al., 1999). In mice null for the α5 subunit, the nicotine-induced (1 μM) facilitation of excitability observed in wild-type mice was abolished ($n = 10$ cells/8 mice, $p > 0.05$; Fig. 2*A*,*D*,*G*). Nevertheless, in the initial 500 ms of the step response, an attenuated nonsignificant trend toward facilitation remained at the highest amplitude tested (Fig. 2*D*,*G*). Conversely, in mice null for the β4 subunit, the facilitation of excitability by nicotine remained for both the entire 2 s step ($n = 8$ cells/7 mice, $F_{(1,35)} = 64.15$, $p < 0.001$; Fig. 2*B*) and the initial 500 ms ($n = 8$ cells/7 mice, $F_{(1,35)} = 108.47$, $p < 0.001$; Fig. 2*E*,*H*). Interestingly, although the R_{in} decreased after nicotine application in α5-null mice (baseline: 2.22 ± 0.40 GΩ vs nicotine: 0.59 ± 0.14 GΩ, $n = 12$ cells/7 mice, $p < 0.01$; Fig. 2*J*), the corresponding effect was abolished in β4-null mice (baseline: 1.84 ± 0.41 GΩ vs nicotine: 1.04 ± 0.42 GΩ, $n = 9$ cells/5 mice, $p > 0.05$; Fig. 2*K*).

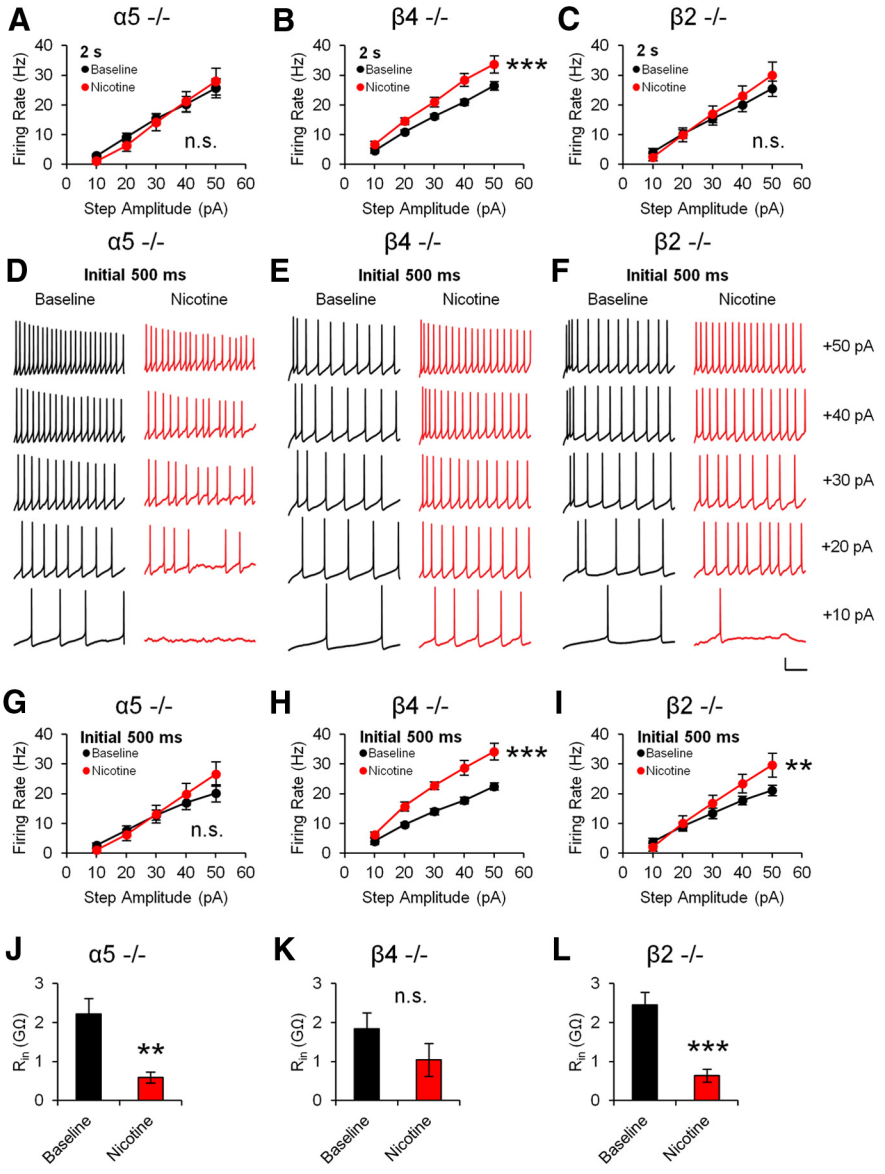

Figure 2. Null mutation of the α5 nAChR subunit blocks facilitation of excitability. Mice mutant for various nAChR subunits were used to address the subunit composition of the nAChRs that underlie nicotine's enhancement of excitability. We initially focused on two subunits encoded for in the *CHRNA5/CHRNA3/CHRNB4* gene cluster, α5 and β4. ***A***, ***D***, ***G***, Null mutation of the α5 subunit abolished the increase in intrinsic excitability after nicotine (1 μM) bath application ($n = 10$ cells/8 mice). This blockade of nicotine's effect was observed both across the entire 2 s step (***A***), as well as during the initial 500 ms (***D***, ***G***). ***B***, ***E***, ***H***, Conversely, the facilitative effect of nicotine persisted in mice bearing a β4 subunit null mutation ($n = 8$ cells/7 mice). ***C***, ***F***, ***I***, We also analyzed the β2 subunit and observed an intermediate effect in mice bearing a β2-null mutation. The facilitative effect of nicotine on intrinsic excitability was blocked when averaging across the entire 2 s step duration, but persisted during of the initial 500 ms ($n = 7$ cells/5 mice). ***J***–***L***, Null mutation of the β4 subunit (***K***) blocked the nicotine-induced decrease in R_{in} ($n = 9$ cells/5 mice), whereas null mutations in the α5 (***J***) or β2 (***L***) subunits did not ($n = 12$ cells/7 mice, 12 cells/5 mice). Scale bar indicates 20 mV/100 ms. All values are displayed as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

Since α5-containing (α5*) nAChRs are heteropentamers containing at least one β subunit (Couturier et al., 1990) and the β4-null mutation did not affect nicotine's enhancement of excitability, we investigated whether the β2 subunit, also expressed in the MHb (Sheffield et al., 2000), is necessary for nicotine's effect on excitability. In β2-null mice, the effect of nicotine (1 μM) on the step-evoked firing response was abolished when averaged across the entire 2 s step ($n = 7$ cells/5 mice, $p > 0.05$; Fig. 2*C*), whereas the increase in the response during the initial 500 ms of the step remained ($n = 7$ cells/5 mice, $F_{(1,30)} = 8.24$, $p < 0.01$; Fig. 2*F*,*I*). Furthermore, the R_{in} decrease after nicotine application was preserved in β2-null mice (baseline: 2.46 ± 0.32 GΩ vs nicotine: 0.64 ± 0.16 GΩ, $n = 12$ cells/5 mice, $p < 0.001$; Fig. 2*L*). Together, these results suggest that nicotine enhances intrinsic excitability by acting at nAChRs that contain the α5 and β2 subunits.

To determine whether the abolishment of nicotine's effect on intrinsic excitability by the α5-null mutation was due to a reduc-

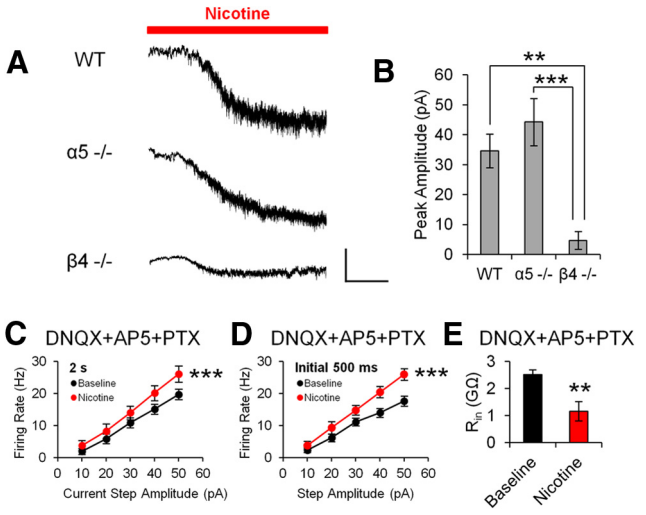

Figure 3. Enhancement of excitability by nicotine does not require postsynaptic nAChR function, ionotropic glutamatergic transmission, or GABAergic transmission. Nicotinic currents were measured in MHb neurons from WT, α5-null, and β4-null mice by bath application of nicotine (1 μM) at a holding voltage of −70 mV. ***A***, Representative traces illustrate that, although the α5-null mutation had no effect on the peak current (middle), the β4-null mutation greatly reduced it (bottom). Nicotine bath application (red bar) began at the start of the trace and perfused steadily into the experimental chamber. ***B***, Quantification of peak currents elicited by nicotine (1 μM) indicated that only 13.6% of the WT nicotinic current remained in the β4-null mouse ($n = 9$ cells/5 mice, 8 cells/6 mice, and 8 cells/3 mice, for WT, $\alpha5^{-/-}$, and $\beta4^{-/-}$, respectively). ***C–E***, Blockade of ionotropic glutamate and GABA receptors by preincubation with DNQX (10 μM)/AP5 (50 μM)/picrotoxin (100 μM) did not block the enhancement of excitability produced by nicotine (1 μM; $n = 12$ cells/9 mice), nor did it affect the nicotine-induced decrease in R_{in} ($n = 7$ cells/2 mice; ***E***). Scale bar indicates 20 pA/4 min. All values are displayed as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

tion in overall nAChR function, we measured current responses evoked by 1 μM nicotine in MHb neurons under voltage clamp (Fig. 3*A*,*B*). Despite the abolishment of nicotine's enhancement of intrinsic excitability in α5-null mice, we found that evoked currents in α5-null mice were similar to those of WT mice (WT: 34.7 ± 5.6 pA vs $\alpha5$ −/−: 44.3 ± 7.8 pA; $n = 9$ cells/5 mice, $n = 8$ cells/6 mice). However, when we analyzed the β4-null mice, nicotine-evoked currents were severely diminished (4.7 ± 3.0 pA, $n = 8$ cells/3 mice; ANOVA: $F_{(2,22)} = 12.15$, $p < 0.001$; Newman–Keuls: $p < 0.01$ vs WT, $p < 0.001$ vs α5) and often there were failures to produce any current at all. This finding suggests that the β4 nAChR subunit is essential to the proper function of postsynaptic nAChRs. Moreover, because the decrease in R_{in} after nicotine application was abolished in β4-null mice, the direct activation of postsynaptic β4* nAChRs is likely responsible for the reduction of R_{in} observed after nicotine application (Fig. 1*H*).

The observation that postsynaptic nicotinic currents were not appropriately correlated with the nicotine-induced enhancement of excitability suggested the involvement of presynaptic mechanisms. The MHb receives substantial glutamatergic and GABAergic innervation from the medial septum/diagonal band and nucleus triangularis, respectively (Qin and Luo, 2009). To test the possibility that glutamatergic or GABAergic mechanisms were involved in nicotine's enhancement of excitability, we preincubated brain slices in a mixture of ionotropic glutamate and GABA receptor antagonists (10 μM DNQX, 50 μM AP5, 100 μM picrotoxin) to block neurotransmission through those receptors. Using current steps to test excitability, we found that the enhancement of excitability by nicotine (1 μM) persisted in this configuration (2 s step: $n = 12$ cells/9 mice, $F_{(1,55)} = 57.00$, $p < 0.001$; initial 500 ms: $n = 12$ cells/9 mice, $F_{(1,55)} = 102.52$, $p < 0.001$; Fig. 3*C*,*D*), leading to the hypothesis that the α5* nAChRs that mediated this effect are not positioned presynaptically on glutamategic or GABAergic afferents. This antagonist combination also did not block the decrease in R_{in} due to nicotine (baseline: 2.52 ± 0.17 GΩ vs nicotine: 1.16 ± 0.36 GΩ, $n = 7$ cells/2 mice, $p < 0.01$; Fig. 3*E*), which is consistent with our hypothesis that the decrease in R_{in} is due to direct activation of postsynaptic β4* nAChRs.

Neurokinins participate in the nicotine-induced enhancement of excitability in the MHb

In addition to glutamate, GABA, and acetylcholine, an array of other neurotransmitters and neuromodulators are expressed in the MHb, including norepinephrine (Gottesfeld, 1983), serotonin (Kinsey et al., 2001), ATP (Edwards et al., 1992), and numerous neuropeptides (Burgunder and Young, 1989; Langlois et al., 2001; Kopp et al., 2002). Because neurokinins can have effects on intrinsic excitability that are similar to those produced by nicotine on MHb neurons (Sculptoreanu and de Groat, 2007; Copel et al., 2009; Xia et al., 2010), we investigated whether an interaction with the neurokinin signaling system mediates nicotine's effects on intrinsic excitability. We initially tested whether blockade of the NK1 or NK3 receptors would reduce the facilitation of excitability by nicotine (1 μM) in MHb neurons. Using L-732138 (10 μM; Cascieri et al., 1994) and SB222200 (2 μM; Goldhill and Angel, 1998; Sarau et al., 2000) to block NK1 and NK3 receptors, respectively, we found that both antagonists separately abolished nicotine's enhancement of excitability when averaged across the entire 2 s test step (L-732138: $n = 8$ cells/6 mice, $p > 0.05$; SB222200: $n = 5$ cells/5 mice, $p > 0.05$; Fig. 4*A*,*B*). L-733060 (3 μM), another antagonist of NK1 receptors, was also tested and had similar effects (data not shown). Interestingly, for any neurokinin receptor antagonist used alone, the nicotine-induced enhancement of excitability during the initial 500 ms of steps was reduced but not completely abolished (L-732138: $n = 8$ cells/6 mice, $F_{(1,35)} = 4.68$, $p < 0.05$; SB222200: $n = 5$ cells/5 mice, $F_{(1,20)} = 8.89$, $p < 0.01$; Fig. 4*D*,*E*,*G*,*H*). However, when both L-732138 (NK1) and SB222200 (NK3) were applied together, nicotine's enhancement of excitability was completely suppressed during the initial 500 ms window ($n = 7$ cells/4 mice, $p > 0.05$; Fig. 4*F*,*I*) and when averaged over the full 2 s step ($n = 7$ cells/4 mice, $p > 0.05$; Fig. 4*C*). The nicotine-induced decrease in R_{in} was unaffected by preincubation with any neurokinin receptor antagonist (L-732138: baseline: 1.65 ± 0.27 GΩ vs nicotine: 0.61 ± 0.29 GΩ, $n = 9$ cells/6 mice, $p < 0.01$; SB222200: baseline: 2.12 ± 0.31 GΩ vs nicotine: 0.66 ± 0.28 GΩ, $n = 8$ cells/5 mice; $p < 0.01$; L-732138 + SB222200: baseline: 1.73 ± 0.31 GΩ vs nicotine: 0.60 ± 0.27 GΩ, $n = 7$ cells/4 mice; $p < 0.05$; Fig. 4*J*–*L*).

SP and NKB are endogenous NK1 and NK3 receptor agonists, respectively, that are present in the MHb (Lein et al., 2007). Given the hypothesis that nicotine's enhancement of intrinsic excitability in the MHb is mediated through neurokinin release, we tested whether application of these endogenous ligands to MHb neurons mimics the observed effects of nicotine. Bath application of SP (100 nM) and NKB (100 nM) mimicked nicotine's facilitation of excitability, both when averaged across the entire 2 s duration of step currents (SP: $n = 7$ cells/ 6 mice, $F_{(1,30)} = 13.39$, $p < 0.001$; NKB: $n = 11$ cells/7 mice, $F_{(1,50)} = 78.72$, $p < 0.001$) and during the initial 500 ms (SP: $n = 7$ cells/6 mice, $F_{(1,40)} = 113.50$, $p < 0.001$; NKB: $n = 11$ cells/7 mice, $F_{(1,50)} = 108.22$, $p < 0.001$; Fig. 5*A*–*F*). Importantly, these agonists produced the observed effects

without lowering R_{in} (SP: baseline: 1.81 ± 0.28 GΩ vs nicotine: 1.65 ± 0.14 GΩ, n = 9 cells/5 mice, $p > 0.05$; NKB: baseline: 2.33 ± 0.22 GΩ vs nicotine: 1.73 ± 0.22 GΩ, n = 15 cells/7 mice, $p > 0.05$; Fig. 5*G*,*H*). Together, these data indicate that the facilitation of MHb neuronal excitability by nicotine occurs via an indirect pathway in which the activation of α5* nAChRs promotes neurokinin signaling onto MHb neurons expressing NK1 and NK3 receptors.

Microinjection of neurokinin receptor antagonists into the MHb precipitates withdrawal behaviors

Because the emergence of somatic withdrawal behavior in mice chronically treated with nicotine critically depends on α5* nAChRs (Salas et al., 2009), we sought to determine whether the enhancement of MHb neuronal excitability through NK1 and NK3 receptor activation is a necessary component of the α5* nAChR-dependent nicotine withdrawal response. Using mice chronically treated with nicotine (8.4 mg/kg/d) via subcutaneous osmotic minipumps, we attempted to elicit nicotine withdrawal behavior by microinjection of NK1 and NK3 receptor antagonists directly into the MHb. Habenular microinjection of L-732138 (NK1) alone, SB222200 (NK3) alone, or both antagonists together each resulted in significantly increased numbers of somatic signs of nicotine withdrawal above vehicle-microinjected controls (Fig. 6*A*, red bars, n = 19 for vehicle, n = 7 for each drug-microinjected group, $F_{(3,56)} = 9.95$, $p < 0.001$). Interestingly, microinjections of SB222200 alone produced an elevated withdrawal score above that of L-732138 microinjections alone or microinjections of both drugs together. These effects were specific to the MHb because similar microinjections into the LHb of mice chronically treated with nicotine did not elicit elevated somatic signs (Fig. 6*A*, pink bars, n = 6 for vehicle, n = 5 for each drug-microinjected group, $p > 0.05$). Furthermore, in another group of animals implanted with minipumps delivering a nicotine-free vehicle solution, analogous microinjections into the MHb also did not elicit elevated somatic signs (Fig. 6*A*, inset, yellow bars, n = 9 for Vehicle, n = 5 for each drug-microinjected group, $p > 0.05$). In sum, these results directly implicate neurokinin signaling within the MHb in the emergence of nicotine withdrawal behavior.

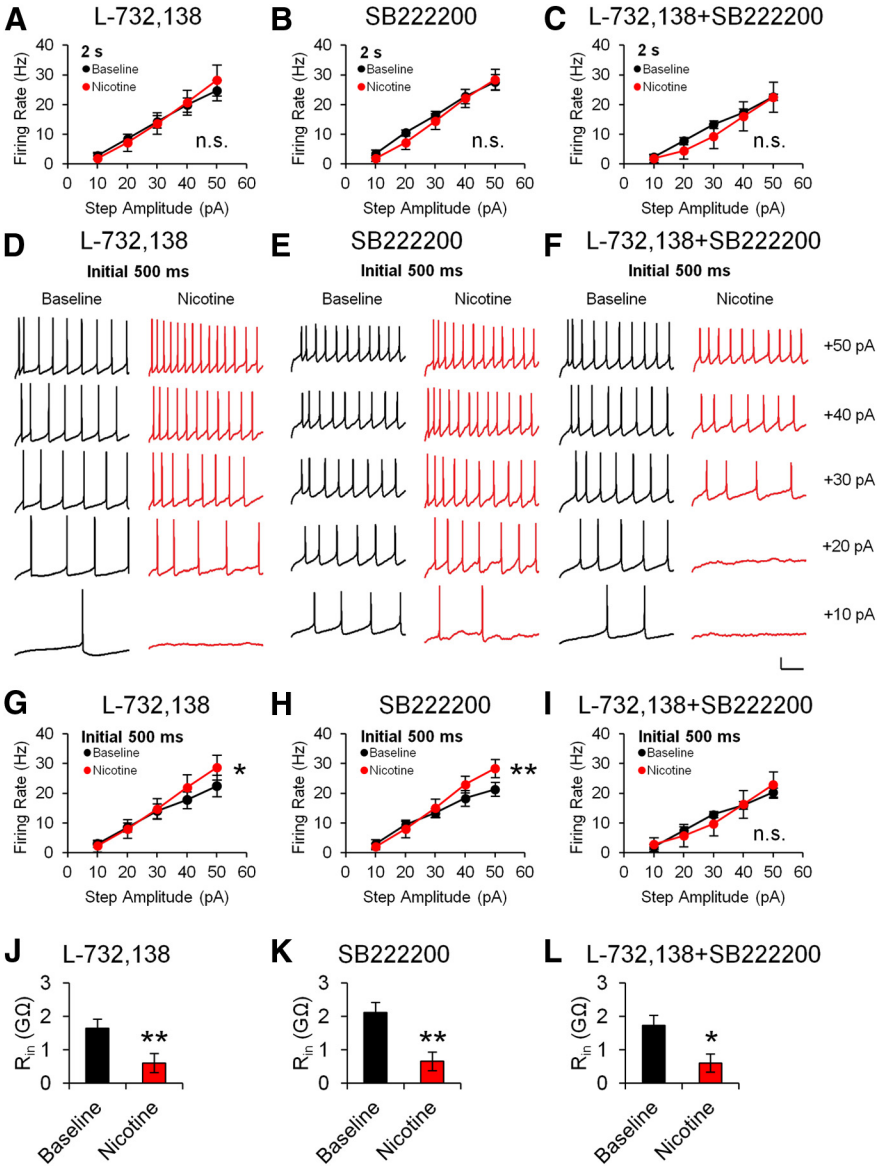

Figure 4. Neurokinin receptor antagonists block nicotine-induced facilitation of excitability. To investigate a potential interplay between the nicotinic and neurokinin signaling systems, we preincubated brain slices with antagonists for NK1 (L-732138) and NK3 (SB222200) receptors both separately and together before assaying for excitability. Preincubation with L-732138 (10 μM; ***A***), SB222200 (2 μM; ***B***), or both antagonists simultaneously (***C***) blocked the nicotine-induced (1 μM) increase in firing rate of action potentials evoked by 2 s current steps (n = 8 cells/6 mice, 5 cells/5 mice, and 7 cells/4 mice, for L-732138, SB222200, and both together, respectively). Conversely, we observed a slightly different outcome upon examination of the initial 500 ms of each step. With either L-732138 (***D***, ***G***) or SB222200 (***E***, ***H***) preincubation alone, the nicotine-induced increase in firing rate of elicited action potentials during the initial 500 ms of each step persisted (n = 8 cells/6 mice, 5 cells/5 mice, respectively). ***F***, ***I***, However, the combined preincubation of both antagonists together completely abolished nicotine's effect on excitability during the initial 500 ms of steps (n = 7 cells/4 mice), as it did for the entire 2 s step. ***J***–***L***, Neurokinin receptor antagonists failed to block the nicotine-induced decrease in R_{in} (n = 9 cells/6 mice, 8 cells/5 mice, and 7 cells/4 mice, for L-732138, SB222200, and both together, respectively). Scale bar indicates 20 mV/100 ms. All values are displayed as mean ± SEM. *$p < 0.05$; **$p < 0.01$.

Chronic nicotine exposure reduces nicotine-induced enhancement of excitability

To gain insight into potential consequences of chronic nicotine over acute nicotinic modulation of intrinsic excitability, we monitored MHb neuronal excitability in mice chronically treated with nicotine or vehicle solution. Because we used adolescent mice for electrophysiology experiments, we chose a chronic nicotine paradigm in which nursing mice obtained nicotine via the milk of their mothers, which had been implanted with osmotic minipumps to deliver nicotine (8.4 mg/kg/d) or vehicle. Nicotine exposure was verified using an ELISA to detect the nicotine metabolite cotinine in the serum of mice. The serum concentration of cotinine was determined to be 16.3 ± 2.6 ng/ml in the offspring of chronic nicotine-treated dams, whereas cotinine was

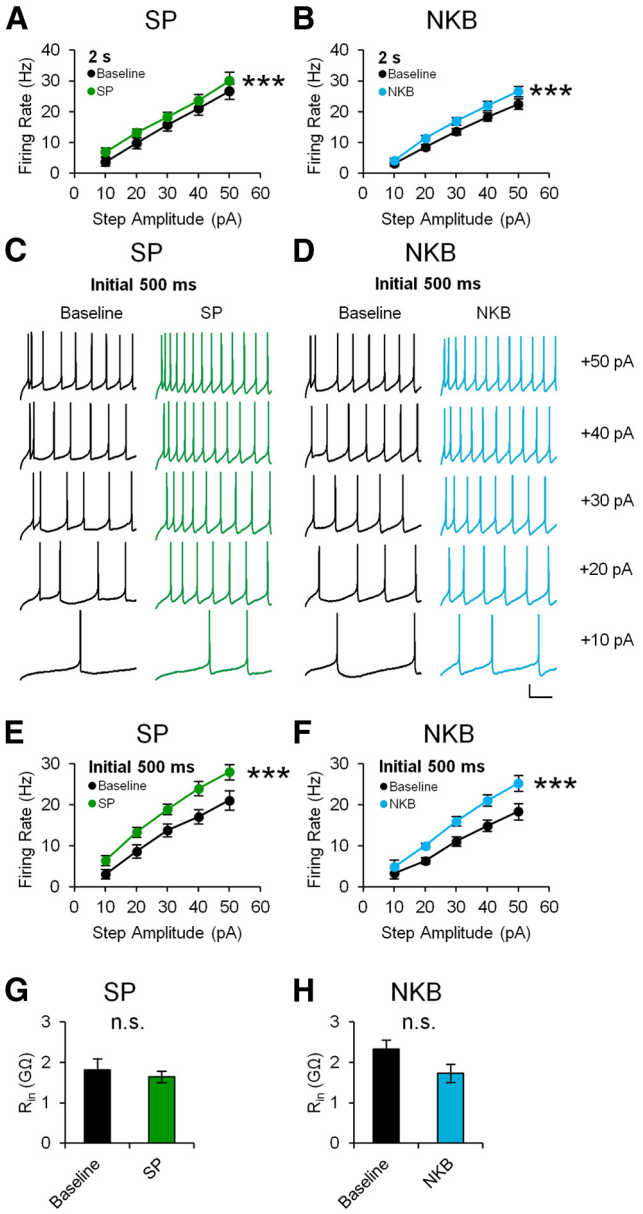

Figure 5. Neurokinins mimic nicotine-induced facilitation of excitability. To test whether nicotine's facilitation of intrinsic excitability could occur through promotion of neurokinin signaling, we used bath application of the neurokinins SP (100 nM) and NKB (100 nM) to mimic nicotine's effect. Bath application of either SP (***A***) or NKB (***B***) increased the firing of action potentials elicited by 2 s depolarizing current steps (10–50 pA, $\Delta I = 10$ pA; $n = 7$ cells/6 mice, 11 cells/7 mice, respectively). ***C–F***, Examination of the initial 500 ms of the step revealed a similar and more pronounced outcome for both SP and NKB, compared with the increase across the entire 2 s step ($n = 7$ cells/6 mice, 11 cells/7 mice). ***G***, ***H***, Bath application of SP or NKB did not affect R_{in} ($n = 9$ cells/5 mice, 15 cells/7 mice, respectively). Scale bar indicates 20 mV/100 ms. All values are displayed as mean ± SEM. ***$p < 0.001$.

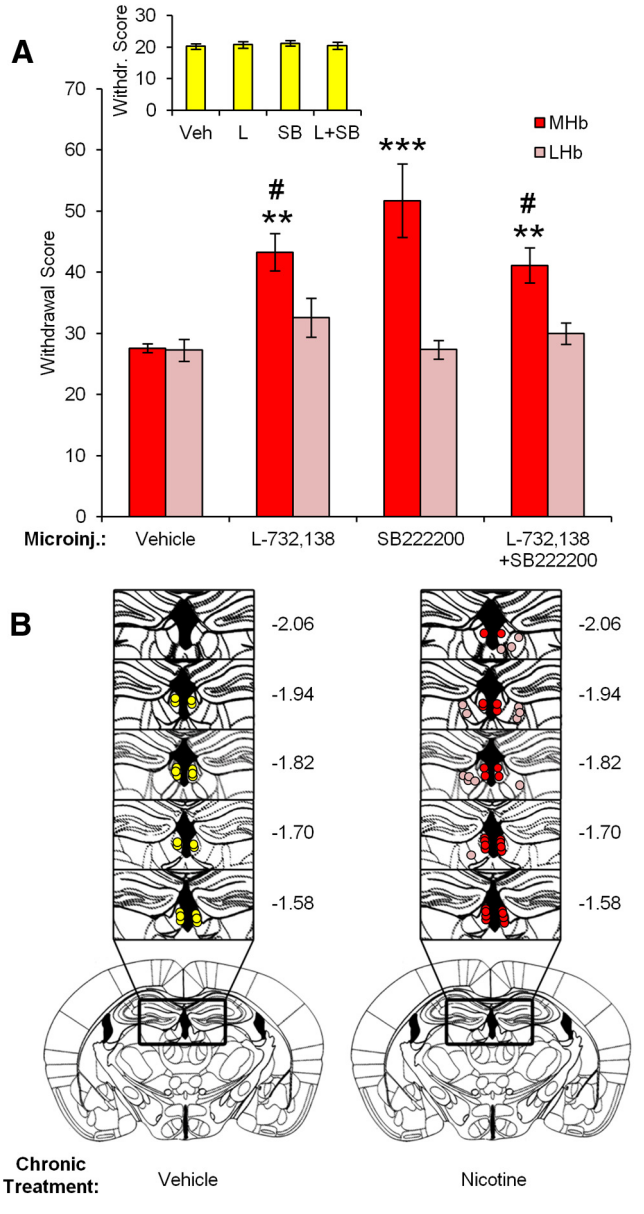

Figure 6. Habenular microinjection of neurokinin receptor antagonists into the MHb precipitates somatic signs of withdrawal in mice chronically treated with nicotine. Mice were chronically treated with nicotine (8.4 mg/kg/d, 2 weeks) or vehicle via osmotic minipumps. ***A***, In mice implanted with nicotine minipumps, the subsequent microinjection of L-732138, SB222200, or both drugs directly into the MHb increased the number of somatic signs observed during a 20 min examination period over that of vehicle-microinjected controls (red bars: $n = 19$ for vehicle, $n = 7$ for each drug-microinjected group). Conversely, analogous microinjections into the LHb of chronic nicotine-treated mice did not elicit elevated somatic signs over vehicle-microinjected controls (pink bars: $n = 6$ for vehicle, $n = 5$ for each drug-microinjected group), nor did analogous microinjections into the MHb of mice implanted with vehicle minipumps (inset, yellow bars: $n = 9$ for vehicle, $n = 5$ for each drug-microinjected group). ***B***, Schematic representation of microinjection sites. All values are displayed as mean ± SEM. **$p < 0.01$, ***$p < 0.001$ vs chronic nicotine/vehicle-microinjected group; #$p < 0.05$ vs chronic nicotine/SB222200-microinjected group.

undetectable in samples from vehicle-treated mice. In comparison, the serum cotinine concentration from the nicotine-treated dams was >100 ng/ml, the concentration of the highest cotinine standard in the assay.

Brain slices from vehicle-treated mice responded similarly to naive mice (Fig. 1*A–E*): acutely applied nicotine (1 μM) increased the excitability of MHb neurons both across the entirety of 2 s depolarizing current steps ($n = 4$ cells/2 mice, $F_{(1,15)} = 10.45$, $p < 0.01$) and during the initial 500 ms ($n = 4$ cells/2 mice, $F_{(1,15)} = 12.59$, $p < 0.01$; Fig. 7*A*,*D*,*G*). However, in brain slices from mice chronically treated with nicotine, the enhancement of excitability by acutely applied nicotine was attenuated (Fig. 7*B*,*E*,*H*). In particular, although a significant enhancement of excitability remained during the initial 500 ms of current steps ($n = 9$ cells/5 mice, $F_{(1,40)} = 5.53$, $p < 0.05$), the enhancement observed when averaged across the entire 2 s of the current steps was abolished ($n = 9$ cells/5 mice,

$p > 0.05$). This was not due to an altered baseline state in response to chronic nicotine, because baseline intrinsic excitability and R_{in} were unchanged after chronic nicotine treatment (data not shown). Furthermore, to determine whether the reduction in nicotine's modulation of intrinsic excitability occurs through an effect on nAChRs or downstream, we tested whether the modulatory effect of NKB on intrinsic excitability is altered by chronic nicotine treatment. In brain slices from mice chronically treated with nicotine, the application of 100 nM NKB significantly enhanced the excitability of MHb neurons (2 s step: $n = 14$ cells/9 mice, $F_{(1,65)} = 30.77$, $p < 0.001$; initial 500 ms: $n = 14$ cells/9 mice, $F_{(1,65)} = 111.8$, $p < 0.001$; Fig. 7*C*,*F*,*I*) in a fashion similar to that observed in naive mice (Fig. 5*B*,*D*,*F*). Last, vehicle or chronic nicotine treatment did not affect the reduction in R_{in} by acute nicotine application (vehicle: baseline: 1.95 ± 0.34 GΩ vs nicotine: 0.47 ± 0.27 GΩ, $n = 4$ cells/2 mice, $p < 0.05$; chronic nicotine: baseline: 2.06 ± 0.39 GΩ vs nicotine: 0.83 ± 0.27 GΩ, $n = 8$ cells/5 mice; $p < 0.05$; Fig. 7*J*,*K*) and NKB (100 nM) application did not alter R_{in} in mice chronically treated with nicotine (baseline: 1.96 ± 0.18 GΩ vs nicotine: 1.74 ± 0.12 GΩ, $n = 13$ cells/7 mice, $p > 0.05$; Fig. 7*L*).

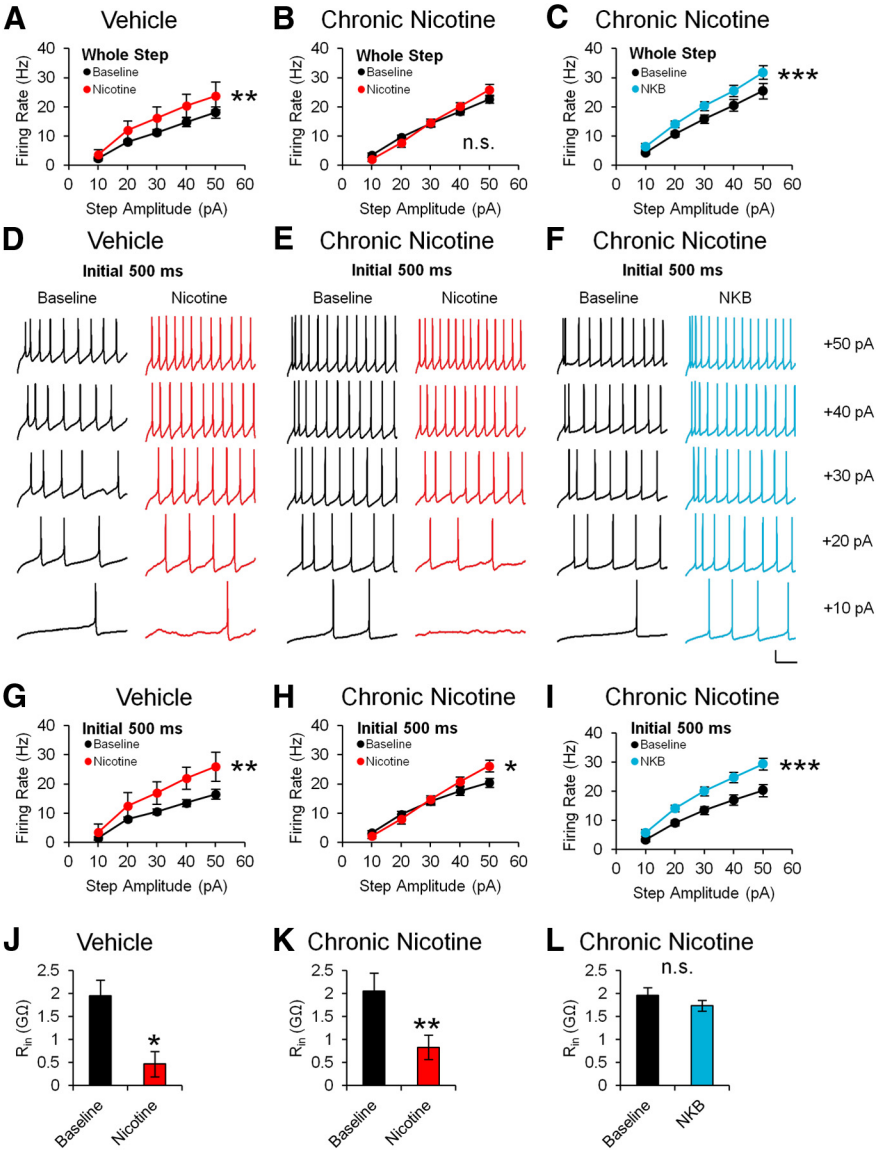

Figure 7. Chronic nicotine treatment reduces the enhancement of excitability by nicotine. We investigated whether chronic nicotine exposure alters the acute ability of nicotine to modulate excitability. ***A***, ***D***, ***G***, In cells from mice chronically treated with vehicle, acute nicotine application (1 μM) increased the number of action potentials elicited by 2 s depolarizing step currents both across the entire 2 s duration and during the initial 500 ms of the steps ($n = 4$ cells/2 mice). ***B***, ***E***, ***H***, However, after chronic nicotine treatment, acute nicotine application only slightly augmented the firing response to 2 s depolarizing current steps during the initial 500 ms of steps, but not across the entire 2 s duration ($n = 9$ cells/5 mice). ***C***, ***F***, ***I***, Ability of NKB (100 nM) to enhance excitability remained after chronic nicotine treatment ($n = 14$ cells/9 mice). ***J***, ***K***, Acute nicotine application (1 μM) reduced R_{in} in neurons from both vehicle- and chronic nicotine-treated mice ($n = 4$ cells/2 mice, 8 cells/5 mice, respectively). ***L***, NKB had no effect on R_{in} after chronic nicotine treatment ($n = 13$ cells/7 mice). Scale bar indicates 20 mV/100 ms. All values are displayed as mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Discussion

α5 nAChR subunit: evidence from mouse behavior and GWAS

Our studies were guided by GWAS of the determinants of risk for nicotine dependence highlighting the involvement of the CHRNA5/CHRNA3/CHRNB4 (α5, α3 and β4) gene cluster (Saccone et al., 2007; Berrettini et al., 2008; Bierut et al., 2008; Thorgeirsson et al., 2008; Berrettini and Doyle, 2012). In particular, we focused on the α5 nAChR subunit, the subunit most prominently featured in those GWAS (Amos et al., 2008; Berrettini et al., 2008; Thorgeirsson et al., 2008; Weiss et al., 2008; Wang et al., 2010). Previously we showed that the nAChRs in the MHb, including those that contain the α5 subunit, are crucial for the emergence of physical signs of withdrawal in mice chronically treated with nicotine (Salas et al., 2004a; Salas et al., 2009). Other evidence indicates that habenular α5* nAChRs regulate the aversive aspects of nicotine during self-administration (Fowler et al., 2011; Frahm et al., 2011). Our present results suggest a mechanism for these behaviors, that α5* nAChRs mediate the enhancement of excitability by acutely applied nicotine through augmentation of neurokinin signaling. This occurs despite an opposing secondary effect of nicotine to reduce R_{in}, which sometimes manifested as decreased excitability at weaker stimuli, particularly in α5-null mice (Fig. 2*D*, +10 pA traces). Furthermore, we show that, under conditions of chronic nicotine exposure, the modulatory effect of nicotine to enhance excitability is attenuated.

Neurokinins become mechanistic candidates for nicotine's effects

Surprisingly, although removal of the β4 subunit caused dramatic decreases in nAChR currents, this had no effect on nicotine-induced increases of intrinsic excitability. Nicotine's excitability enhancement also persisted through blockade of ionotropic glutamate and GABA receptors and these results led us to explore other possible routes for excitability modu-

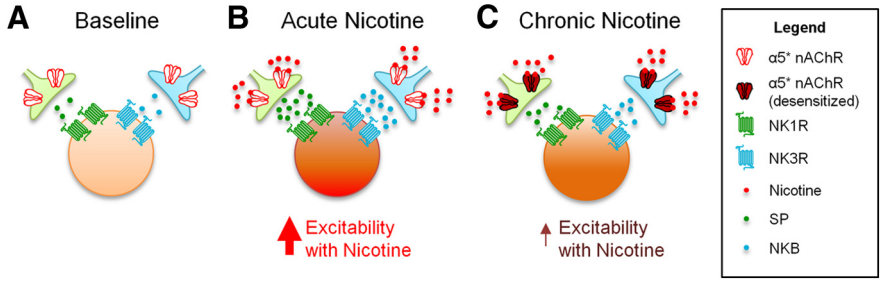

Figure 8. Model of modulation of intrinsic excitability by nicotine in the MHb. ***A***, Under basal conditions, SP and NKB release onto MHb neurons modulates their intrinsic excitability. ***B***, Acutely applied nicotine binds to and activates α5* nAChRs to promote SP and NKB release onto MHb neurons. The greater activation of NK1 and NK3 receptors by increased SP and NKB levels augments intrinsic excitability. ***C***, Chronic nicotine reduces the responsiveness of the α5* nAChRs, likely through desensitization of the nAChRs by chronic nicotine. Therefore, the acute ability of nicotine to enhance excitability is reduced when the drug is chronically present.

lation by nAChRs. Due to the high expression of neurokinins and their receptors in the MHb (Burgunder and Young, 1989; Yip and Chahl, 2001), as well as their analogous roles on excitability in the MHb and other brain areas (Norris et al., 1993; Ogier and Raggenbass, 2003; Budai et al., 2007), we chose to study this neuromodulatory system.

Nicotine's enhancement of excitability was blocked by preincubation with either NK1 or NK3 receptor antagonists. This result suggests a handful of scenarios, but the likeliest is that nicotine acts at different neuronal subpopulations within the MHb, possibly due to the heterogenous mixture of neurons with differing expression of NK1 or NK3 receptors (Norris et al., 1993). Alternatively, nicotine may act at a heterogenous population of neurokinin synapses, for example, modulating the release of both SP and NKB. Regardless, only under blockade of both NK1 and NK3 receptors did we observe a complete abolishment of nicotine's enhancement of excitability, which provides further evidence that nicotine acts via a combination of mechanisms.

Neurokinins participate in nicotine withdrawal behavior

We reasoned that if nicotine's facilitation of neurokinin signaling is an important aspect of the nicotine-induced adaptations that take place in the brains of tobacco smokers, neurokinin receptor blockade during chronic nicotine treatment should mimic the cessation of nicotine intake and consequently lead to the emergence of withdrawal symptoms. We addressed this hypothesis by microinjecting antagonists for NK1 and NK3 receptors into the MHb and found that this triggered somatic withdrawal behavior in mice chronically treated with nicotine. For this reason, we believe that the removal of neurokinin signaling modulation by nicotine, mediated through α5* nAChRs, is a primary cause for the elimination of somatic nicotine withdrawal behavior in α5-null mice (Salas et al., 2009). As with most behavioral pharmacology experiments, it is difficult to control for the diffusion of drugs into nontargeted tissue. However, our attempt at eliciting withdrawal behavior by microinjection of neurokinin antagonists into the LHb, with the lack of an observed effect, further corroborates our model. β4-null mice also lack withdrawal behavior (Salas et al., 2004a), but that behavior likely arises from the absence of nAChR currents (Fig. 3*A*,*B*) and the consequent deficiency of direct excitatory drive onto MHb neurons by nicotine. Therefore, in these two mouse lines, which both lack nicotine withdrawal behavior, different mechanisms may lead to convergent behavioral outcomes.

Neuroadaptation during chronic nicotine exposure

Chronic nicotine treatment reduced the nicotine-induced increase of neuronal excitability in the MHb. Additional details of how reduced responses to acute nicotine can arise under chronic nicotine exposure are suggested from currently known desensitization profiles of β2* and β4* nAChRs, particularly with respect to how densensitization is altered when the α5 subunit is present (Gerzanich et al., 1998). The inclusion of α5 into either β2* or β4* nAChRs increases both the rate and the magnitude of desensitization. However, in α5/β2* nAChRs, the total amount of desensitization (68%) is greater than that of α5/β4* nAChRs (41%). In addition, in β4* nAChRs lacking α5, desensitization is notably low compared with that in β2* nAChRs lacking α5 (21% vs 46%). Therefore, the enhancement of intrinsic excitability by nicotine, which is abolished by the α5-null mutation and reduced by the β2-null mutation, may be especially sensitive to nAChR desensitization in the chronic presence of nicotine. In addition, the incorporation of α5 subunits into nAChRs preferentially stabilizes the assembly of β2* vs β4* nAChRs and greatly reduces the upregulation of the receptors in response to chronic nicotine treatment (Gahring and Rogers, 2010). Together, our results suggest that chronic nicotine attenuates nicotine's acute excitability modulation by desensitizing the nAChRs that modulate neurokinin signaling. Broadly, this might include both short and longer-lasting functional deficits. Further, the disruption of endogenous neurokinin signaling in the MHb of nicotine-withdrawn mice might ultimately trigger somatic withdrawal behavior. Consistent with this hypothesis, we find that NKB-induced excitability facilitation is maintained in mice chronically treated with nicotine.

Neurokinins and neurokinin receptors in the MHb

SP and NKB are prominent endogenous ligands of NK1 and NK3 receptors (Pennefather et al., 2004). Although both receptors are activated by either ligand, SP preferentially activates NK1 receptors and NKB preferentially activates NK3 receptors. In contrast to the expression of NKB, which is extremely dense and ubiquitous throughout the MHb (Lucas et al., 1992; Marksteiner et al., 1992; Lein et al., 2007), strong SP expression is confined to the dorsal one-third of the MHb (Lein et al., 2007; Aizawa et al., 2012). Both NK1 and NK3 receptors are strongly expressed in the cholinoceptive/cholinergic ventral two-thirds of the MHb (Langlois et al., 2001; Lein et al., 2007; Commons and Serock, 2009). For this study, we focused on neurons within the ventral two-thirds of the MHb because this subsection comprises the cholinoceptive and cholinergic MHb (Fig. 1*A*). Based on the expression pattern, the α5* nAChRs modulating excitability might largely facilitate NKB release onto MHb neurons, with additional facilitation of SP release. Because of the anatomical expression, we suspect that the source of NKB onto the cholinoceptive/cholinergic MHb originates from local neurons in addition to neurons throughout the MHb. Conversely, the source of possible SP release onto the cholinoceptive/cholinergic MHb might be

from the dorsal one-third of the MHb, given evidence of dorsal to ventral projections with en passant synaptic boutons within the MHb (Kim and Chang, 2005). Presynaptic nAChRs can enhance neurotransmitter release in part by mediating a calcium influx (Dani and Bertrand, 2007). Heteromeric nAChRs that contain the α5 subunit have high calcium permeability (Gerzanich et al., 1998; Tapia et al., 2007) and thus could mediate a direct influence over neurotransmitter release.

Synthesis and conclusion

The present study supplies a potential mechanism for the emergence of nicotine withdrawal behaviors. We propose a novel interplay in the MHb between the nicotinic and neurokinin neuromodulatory systems. We showed that acutely applied nicotine exhibits bidirectional control over the intrinsic excitability of MHb neurons. In one direction, nicotine reduces R_{in}, likely via direct postsynaptic activation of β4* nAChRs. In the opposite direction, which is the more dominant effect, nicotine enhances excitability via activation of α5* nAChRs that facilitate release of neurokinins (SP and NKB) onto both NK1 and NK3 receptors. Interestingly, blockade of habenular NK1 and NK3 receptors in mice chronically treated with nicotine precipitates physical symptoms of withdrawal. Lastly, chronic nicotine treatment mitigates the enhancement of excitability by acutely applied nicotine while sparing the enhancement of excitability by NKB application. Through these data, we present a model whereby nicotine acutely modulates neurokinin signaling via activation of α5* nAChRs to ultimately promote intrinsic neuronal excitability. Under chronic nicotine treatment, the neuromodulatory influence of nicotine is reduced at the level of nAChR function (Fig. 8). Nicotine's weakened modulation of intrinsic excitability may contribute to the unrewarding and aversive experience during nicotine withdrawal, despite the removal of an excitatory drive (nicotine) on a nucleus that encodes for aversion. Already, mechanisms involving neurokinins are reported to be involved in addiction to ethanol, cocaine, and opiates (Ripley et al., 2002; Davidson et al., 2004; George et al., 2008). Expansion of the present findings and similar investigative avenues may lead to neurokinin-based improved therapies for the treatment of dependence to nicotine and other drugs of abuse.

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