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Nicotine regulates activity of lateral habenula neurons via presynaptic and postsynaptic mechanisms

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There is much interest in brain regions that drive nicotine intake in smokers. Interestingly, both the rewarding and aversive effects of nicotine are probably critical for sustaining nicotine addiction. The medial and lateral habenular (LHb) nuclei play important roles in processing aversion, and recent work has focused on the critical involvement of the LHb in encoding and responding to aversive stimuli. Several neurotransmitter systems are implicated in nicotine’s actions, but very little is known about how nicotinic acetylcholine receptors (nAChRs) regulate LHb activity. Here we report in brain slices that activation of nAChRs depolarizes LHb cells and robustly increases firing, and also potentiates glutamate release in LHb. These effects were blocked by selective antagonists of $\alpha_6$-containing ($\alpha_6^*$) nAChRs, and were absent in $\alpha_6^*$-nAChR knockout mice. In addition, nicotine activates GABAergic inputs to LHb via $\alpha_4\beta_2$-nAChRs, at lower concentrations but with more rapid desensitization relative to $\alpha_6^*$-nAChRs. These results demonstrate the existence of diverse functional nAChR subtypes at presynaptic and postsynaptic sites in LHb, through which nicotine could facilitate or inhibit LHb neuronal activity and thus contribute to nicotine aversion or reward.

Nicotine, a major bioactive component in tobacco that drives addiction to smoking, has both strong rewarding and aversive effects\textsuperscript{1}. Although reward has been considered important for addiction, both effects may be critical for promoting smoking, since nicotine aversion can promote craving for nicotine and intake in addicted individuals\textsuperscript{12}. It is well-known that nicotine reinforcement involves midbrain dopaminergic relays to nucleus accumbens\textsuperscript{1}, but the circuitry mediating aversive effects is less clear. Several lines of evidence suggest that nicotine may induce reward and aversion through opposite actions on the mesolimbic system\textsuperscript{13,14}. In addition, recent studies of aversion have focused on the lateral and medial habenula (LHb and MHb)\textsuperscript{1}. The MHb is known to negatively regulate nicotine intake and contribute to nicotine aversion\textsuperscript{1,4–6,8}. There is also much interest in the LHb; LHb neurons are activated by several aversive stimuli\textsuperscript{9,10}, which in turn excites GABAergic neurons in the rostromedial tegmental nucleus (RMTg)\textsuperscript{9} that inhibit midbrain dopaminergic neurons\textsuperscript{11,12}. In this way, LHb could encode aversion to unpleasant stimuli\textsuperscript{13,14} as well as nicotine aversion\textsuperscript{15}. Although the LHb is critical for both nicotine reinstatement\textsuperscript{16} and nicotine related anxiety\textsuperscript{15}, little is known about the mechanisms through which nicotine regulates LHb neuronal activity. Previous work has shown that nicotine robustly excites RMTg neurons\textsuperscript{17}, perhaps indirectly by stimulating the LHb.

Nicotine acts primarily through nicotinic acetylcholine receptors (nAChRs) composed of multiple subunits, which are present throughout the brain including in LHb\textsuperscript{17} and MHb\textsuperscript{18}. Studies from transgenic mice suggest that many nAChR subtypes can contribute to nicotine withdrawal, reinforcement and aversion\textsuperscript{1,19,20}, $\alpha_6^*$-containing nAChRs in particular are highly expressed in mesolimbic pathways and mediate nicotine-related reward\textsuperscript{21–23}, though the relevant brain locations have not been completely identified\textsuperscript{11,24–26}. Given the prominent aversive effects of nicotine\textsuperscript{1} and the LHbs importance for aversion, we used a combination of electrophysiology and pharmacology approach and $\alpha_6^*$-knockout mice to investigate whether different nAChRs contribute to nicotine modulation of LHb neuronal activity.

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Results
Nicotine excites LHb neurons. As reported previously, there is a clear border between the LHb and the adjacent MHB (Figs 1A1 and 2). We thus distinguished the MHB and LHb neurons according to the clear border. In addition, consistent with previous reports, we observed that LHb neurons were loosely dispersed, had heterogeneous morphological but similar membrane properties, and were capable of producing burst firing.

In the current study, we recorded from ~750 LHb neurons. Nicotine increased firing in both cell-attached (Fig. 1B–D) and whole-cell modes (Fig. 1F). Nicotine accelerated spontaneous firing of 77/95 LHb neurons in a dose-dependent manner, with an EC50 of 610 nM (F6.87 = 15.2, p < 0.001), and reached the plateau at 10 μM (Fig. 1B–E). Interestingly, upon bath application of 100 nM nicotine, firing first decreased (to 90.4 ± 3.8% of baseline, p < 0.05; Fig. 1D), then sharply accelerated (p < 0.001), returning to baseline level after washout (Fig. 1B–D). To confirm that the site of nicotine action was within the LHb, recordings in a subset of experiments were made from slices with MHB removed (Fig. 1A3). Nicotine acceleration of LHb neuron firing in these slices (F3.23 = 19.9, p < 0.001; post hoc p < 0.001, Fig. 1E blue triangle) was similar to that with MHB (p > 0.5 with MHB vs without MHB).

In agreement with nicotine enhancement of firing, in the presence of tetrodotoxin (TTX), nicotine also dose-dependently depolarized LHb neurons under current-clamp mode (Fig. 1F–H), with an EC50 of 410 nM (F6.87 = 8.2, p < 0.001; Fig. 1H). Similarly, nicotine induced inward currents (Fig. 1I) in 68/81 neurons under voltage-clamp at −70 mV (in presence of blockers of sodium channels, GABA, glycine, NMDA- and AMPA-type-glutamate receptors) which were dose-dependent (F3.73 = 27.5, p < 0.001; Fig. 1J) with an EC50 of 350 nM. Thus, nicotine depolarized LHb neurons and increased their firing.

To identify nAChR subtypes that mediated nicotinic excitation of LHb neurons, we examined the effects of several nicotine antagonists on nicotine-induced inward currents and on firing. Since 10 μM nicotine produced the maximal effect, the comparative neuronal activity of inward currents induced by 10 μM nicotine in the absence and presence of various nAChR antagonists, which were applied for 5–8 min before co-applying nicotine or ACh. Nicotine currents were greatly reduced (F6.42 = 6.5, p < 0.001, One-way ANOVA; Fig. 2A,C) by mecamylamine (MEC, 1 μM, p = 0.002), a non-selective nAChR antagonist, α-CTx-MII (100 nM, p < 0.001), an selective α6* and α3/32-nAChRs antagonist22,29, MII[H9A; L15A] (100 nM, p = 0.036), an α6*-nAChR blocker, dihydro-beta-erythroidine (DHβE, 100 nM, p = 0.006), relatively selective for α4/32-nAChRs, but not methyllycaconitine (MLA, 10 nM), an α7-nAChR antagonist (p = 0.5). Thus, α-CTx-MII-sensitive (α6* ± α3/32-) and DHβE-sensitive (α4/32) nAChRs contributed to the nicotine-induced LHb depolarization (Fig. 2C). In addition, although 10 μM nicotine is higher than occurs physiologically in human smokers,21 this concentration of nicotine was maximal and thus useful for determining in impact of different nAChR antagonists on nicotine modulation of different aspects of LHb physiology.

We then examined the nAChR subtypes which mediated the nicotine enhancement of LHb firing. Nicotine enhancement of firing was greatly attenuated by the nAChR antagonists tested above (p < 0.049), an selective α6*-nAChR mRNA32 that project to the LHb. To minimize indirect effects through MHB, we locally applied ACh (30 μM) onto the somata of recorded LHb neurons by pressure injection from a second micropipette. In the presence of atropine, ACh induced an inward current (25.6 ± 7.1 pA) in 6/10 neurons tested (p = 0.005) which was significantly depressed by MII [H9A; L15A] to (33.0 ± 4.0% of ACh, p = 0.016; Fig. 2E). These experiments confirmed that there was a direct, MII[H9A; L15A]-sensitive nAChR (likely α6*-nAChR)-mediated depolarization of LHb cells. Indirect effects via MHB were also unlikely since nicotine depolarized LHb cells (Figs 1F–H and 2A–D) in the presence of TTX and glutamate and GABA antagonists, which would block the corresponding synaptic inputs from MHB neurons, and also because nicotine activated LHb neurons in brain slices where MHB was cut off (Fig. 1E).

Nicotine enhances synaptic potentials and currents (IPSCs and EPSCs) in LHb neurons. We next examined whether nicotine would alter inhibitory and excitatory post-synaptic currents (IPSCs and EPSCs, respectively) in LHb neurons. We first examined how nicotine affects paired-pulse transmission when two PSCs were evoked at a brief inter-stimulus interval (50 msec). Nicotine (10 μM) selectively enhanced the first IPSC of each pair (IPSC1; p < 0.05; Fig. 3A,C), thus reducing the paired-pulse ratio (PSC2/PSC1 = PPR) (p < 0.01; Fig. 3D). Similarly, nicotine enhanced only EPSC, of each pair (p < 0.01; Fig. 3B–D) and reduced the EPSC-PPR (p < 0.05). Since decreased PPR can reflect increased transmitter release at the synapse, these results suggest that nicotine might potentiate synaptic transmission in LHb by increasing presynaptic release of both glutamate and GABA.

To better understand the impact of nicotine on GABA and glutamate signaling in the LHb, we then tested the effects of nicotine on spontaneous synaptic currents. Nicotine induced a transient shift to higher frequencies and larger amplitudes of spontaneous IPSCs (sIPSCs) (Fig. 3E–G), with an EC50 of 40 nM for frequency (F6.88 = 9.2, p < 0.001; Fig. 3N, black line) and 10 nM for amplitude (F6.88 = 3.9, p = 0.002; Fig. 3O, black line). By contrast, nicotine induced a slowly developing increase in spontaneous EPSC (sEPSC) frequency (Fig. 3H,I) with an EC50 of 140 nM (F6.105 = 8.9, p < 0.001; Fig. 3N, blue line), and a small but significant increase in sEPSC amplitude (p < 0.001, Kolmogorov-Smirnov test; Fig. 3J) with an EC50 of 210 nM (F6.105 = 4.9, p < 0.001; Fig. 3O, blue line). At a holding potential of −40 mV, sEPSCs and sIPSCs appeared as distinct inward and outward currents, and nicotine (1 μM) induced a transient outward current followed by a sustained inward current and increased frequency of sIPSCs and sEPSCs (Fig. 3K,M). Similar changes were seen under current clamp, with an initial hyperpolarization followed by depolarization and increased frequency of sIPSPs and sEPSPs (Fig. 3L,M). Thus, nicotine has...
**Figure 1. Nicotine regulates LHb activity.** (A) Brain atlas diagram for approximate location of MHb and LHb (A1). Representative images of coronal slices containing the LHb with (A2) and without MHb (A3). (B–D) Nicotine first slowed and then accelerated LHb firing. Red arrow: initial firing reduction; (E) Concentration-response of nicotine-induced % increase of firing rate, recorded in cell-attached mode, in brain slices containing both LHb and MHb (●). Nicotine (1 μM and 10 μM) caused similar increases in firing rate of LHb neurons in brain slices with removed MHb (▲). ns = no significance, with MHb vs without MHb. Number of neurons is indicated. Nicotine depolarization of LHb neurons (F), even in presence of TTX (G,H). (I,J) Nicotine-induced inward currents at a holding potential of −70 mV, and in the presence of Inhibitor Cocktail. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.
a biphasic action in LHb, causing a transient enhancement of ongoing GABA release and a slower but sustained increase in glutamate release.

We also examined the impact of nicotine on miniature synaptic currents, where spontaneous release was determined in the presence of TTX, Ca\(^{2+}\)-free ACSF (where CaCl\(_2\) was replaced by MgCl\(_2\)) or in presence of 100 \(\mu\)M

Figure 2. nAChR subtypes mediating nicotine effects in LHb. (A) Nicotine-induced inward currents were blocked by MEC, \(\alpha\)-CTx-MII, MII[H9A;L15A] and DH\(\beta\)E, but not MLA. (B) Nicotine-induced increases in firing rate was prominently reduced by MEC, and MII [H9A; L15A], and weakly but significantly reduced by MLA. (C,D) Summary of above data. (E) Local ACh application induced an MII [H9A; L15A]-sensitive inward current. * \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\).
Figure 3. Nicotine enhances IPSCs and EPSCs in LHb neurons. (A,B) Nicotine action on paired evoked IPSCs (eIPSCs) (A) and evoked EPSCs (eEPSCs) (B). eIPSCs (recorded at $V_{H} = +40$ mV) were abolished by gabazine (10μM), a GABA<sub>A</sub> receptor antagonist; eEPSCs (at $V_{H} = −70$ mV) were abolished by DNQX (20μM), an AMPA receptor blocker. (C) Nicotine (10μM) enhanced the first of PSC pairs. (D) % change in amplitude (AMP) of first PSCs and paired pulse ratio (PPR = PSC<sub>2</sub>/PSC<sub>1</sub>). (E–J) Nicotine increased spontaneous sIPSC frequency and amplitude (E–G) as well as sEPSC frequency (H–J). (K,L) Simultaneous recording of sIPSC/sIPSPs and sEPSC/sEPSPs at −40 mV under voltage- (K) and current-clamp (L). (M) Different time course of nicotine action on the frequency of sIPSC/Ps and sEPSC/Ps ($n = 9$). (N,O) Concentration-dependence of increased sEPSC and sIPSC frequency (N) and amplitude (O). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 
cadmium chloride (CdCl₂, a non-selective blocker of voltage-gated calcium channels). All of these treatments allow determination of the effects of nicotine on synaptic release in the absence of possible effects on presynaptic action potential generation (TTX) or activity-dependent effects on release (calcium-free or cadmium-containing media). Nicotine caused a smaller but still significant increase in the frequency of spontaneous miniature EPSCs (F_3,77 = 5.3, p = 0.004) and IPSCs (F_3,66 = 7.6, p = 0.001) in the presence of TTX, or calcium free ACSF, or CdCl₂, **p < 0.01, ***p < 0.001, Student’s paired t-test for nicotine application vs pre-nicotine control. *p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA followed by Tukey’s multiple comparison test for nicotine application vs nicotine plus TTX/calcium free ACSF/CdCl₂.

We next examined the nAChR subtypes that mediate the nicotinic enhancement of spontaneous synaptic currents. Increased sIPSC frequency was greatly attenuated (F_5,123 = 5.5, p = 0.001; Fig. 4A1,C) in the presence of TTX, or calcium free ACSF, or CdCl₂, **p < 0.01, ***p < 0.001, Student’s paired t-test for nicotine application vs pre-nicotine control. *p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA followed by Tukey’s multiple comparison test for nicotine application vs nicotine plus TTX/calcium free ACSF/CdCl₂.
were important for mediating the nicotinic enhancement of sIPSC frequency. In contrast, the enhancement of sEPSC frequency was reduced (\(F_{5,132} = 7.4, p < 0.001\); Fig. 5B,C) by MEC (\(p < 0.001\)), \(\alpha\)-CTx-MII (\(p < 0.001\), MII[H9A; L15A] (\(p = 0.001\)), less by MLA (\(p = 0.009\)), but not significantly by DH\(\beta\)E (\(p = 0.125\)). Thus, MII-sensitive (\(\alpha_6*\)±\(\alpha_3\beta_2\)) and MLA-sensitive (\(\alpha_7\))-nAChRs mediated nicotine’s effects on glutamatergic inputs to LHb neurons, similar to what was observed for the nicotine enhancement of LHb firing (Fig. 2H).

Figure 5. nAChR subtypes mediating nicotine effects on sIPSCs and sEPSCs. (A,B) Effects of various nAChR blockers on nicotine-induced acceleration of sIPSCs (A) and sEPSCs (B). (C) Summary of above data. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). (D) Proposed schematic of nAChR subtype distribution in the LHb. (E,F) Nicotine enhanced firing (E) and mEPSC frequency (F) recorded in brain slices from wild-type (WT) but not \(\alpha_6\)-nAChR knockout (\(\alpha_6\) KO) mice. *\(p < 0.05\), **\(p < 0.05\), **\(p < 0.01\). (G) Summary of above data.
Increased firing and mEPSC frequency by nicotine are absent in α6-αChR knock-out. Since pharmacology experiments suggested that α6*-αChRs were critical for nicotine actions in the LHb (Fig. 2), we directly tested the importance of α6-αChRs by comparing nicotine actions on LHb neurons from wild-type (WT) and α6-αChR knock-out (α6-KO) mice. Compared to the WT mice, LHb neurons in the α6-KO mice exhibit higher basal firing rate (WT: 6.6 ± 1.4 Hz; α6-KO: 11.8 ± 2.1 Hz; p = 0.043 WT vs KO) and basal mEPSC frequency (WT: 1.0 ± 0.1 Hz; α6-KO: 2.0 ± 0.5 Hz; p = 0.045) but not basal mEPSC amplitude (WT: 12.5 ± 0.7 pA; α6-KO: 12.3 ± 0.7 pA; p = 0.84). Importantly, nicotine (10 μM) significantly increased LHb firing in WT but not α6-KO mutants (p = 0.03 WT vs KO; Fig. 3E,G), and increased LHb mEPSC frequency in WT but not α6-KO mutants (p = 0.004; Fig. 3E,G). These results directly demonstrate the importance of α6*-αChRs for the nicotine-induced enhancement of LHb activity. Alternatively, these results could reflect an occlusion of the ability of nicotine to increase firing in α6-KO mutants which have a higher basal firing rate.

Mechanism of nicotine’s biphasic action on LHb neurons. As described in Fig. 1, upon the application of 100 nM nicotine, LHb firing was first decreased; and then sharply accelerated, returning to baseline after washout (Fig. 1A–C). We proposed that the decrease of firing was mediated by nicotinic potentiation of GABAergic transmission, and the enhancement of firing was mediated in part by nicotinic potentiation of glutamatergic transmission. To test this hypothesis, we compared the effects of 100 nM nicotine in the absence and presence of a GABA_A receptor antagonist (20 μM gabazine), glutamate receptor antagonists (20 μM DNQX plus 50 μM AP5), or a cocktail of both GABA_A and glutamate receptor blockers (gabazine + DNQX + AP5).

As shown in Table 1, nicotine-induced inhibition of firing was completely abolished (F3,37 = 7.7, p < 0.001) by gabazine (p = 0.042) or by the cocktail (p = 0.011), but not by DNQX + AP5 (p > 0.5). In contrast, nicotine-induced enhancement of firing was partly decreased (p = 0.013) by the cocktail (p = 0.04), but not by gabazine alone (p > 0.5). Thus, the ability of nicotine to increase and decrease firing was mediated by nicotine enhancement of IPSCs and EPSCs, respectively, although the excitatory effect of nicotine also involved mechanisms other than glutamate receptors.

Discussion

Converging evidence indicates that the LHb is activated by aversive stimuli38. Nicotine can have strong aversive effects37, which can play an important role in promoting nicotine addiction1,2. In keeping with this, we found that nicotine strongly activated LHb neurons in vitro, as has been observed in vivo39; in vivo, LHb activation of RMTg neurons would in turn inhibit midbrain dopamine (DA) neurons and thus contribute to aversion23,39. In addition, we found that the actions of nicotine in LHb brain slice were mediated by distinct αChR subtypes. Pharmacology and α6*-knockout experiments suggested that α6*-αChRs played a prominent role in the sustained enhancement of LHb firing and glutamate release, while DH/βE-sensitive αChRs, likely reflecting α4β2-αChRs, were important for the transient increase in GABAergic transmission. Our results show that different αChR subtypes played a significant role in the regulation of LHb activity, which may contribute to both nicotine aversion and reward.

At different concentrations found in the blood of human smokers (25–444 nM)31, nicotine acted at diverse αChR subtypes to modulate LHb activity in brain slices from rats. Low concentrations (EC50 ~20–50 nM) potentiated IPSCs via DH/βE sensitive (α4β2) αChRs, leading to rapid but only brief hyperpolarization, consistent with α4β2-αChRs propensity to desensitization41–42. Higher nicotine concentrations (EC50 ~200 nM) elicited a more sustained increase in glutamate release via MII-sensitive (α6 ± α3/32)- and MLA-sensitive (α7)-containing αChRs; pharmacology and knockout experiments combined suggest a particular role for α6*-αChRs in nicotine excitation of LHb neurons. Nicotine’s actions on synaptic transmissions depended in part on calcium and TTX-sensitive sodium channels. At higher concentrations (EC50 ~400 nM), nicotine directly depolarized LHb neurons via MII- and DH/βE-sensitive αChRs but not by MLA-sensitive αChRs. Similarly, the nicotine-induced increase in firing was sensitive to MII, MLA and DH/βE. The EC50 of nicotine for enhancing firing (~600 nM) was about 1.5 fold greater than the EC50 of the nicotine-induced current responses. The difference between the EC50 for nicotine enhancement of firing and depolarization may reflect the presence of different αChRs at presynaptic terminals versus postsynaptic neurons in LHb.

Genetic variations in the CHRNA6–CHRNA3 gene cluster increase vulnerability to tobacco smoking43–45, α-CTx-MII infusion into α6*-αChRs-expressing mesolimbic regions (midbrain and nucleus accumbens)36–48 decreases nicotine self-administration by rats1,2. Our findings show that the LHb is another critical brain region for α6*-αChR-mediated actions for nicotine. First, global disruption of α6-αChRs increase basal mEPSC frequency as well as firing rate, indicating α6-αChRs is a critical regulator of LHb neuronal activity. Second, although the MIIb also expresses many αChRs32 and projects to LHB46, the effects we observed probably originated mainly in the LHb, since α6*-mediated LHB currents were elicited by local applications of ACh or by bath applications of nicotine in the presence of TTX, and persisted when the MIIb was cut away from the LHB. Moreover, nicotine excitation of MIIb neurons in vitro46 does not require α6*-αChRs. Previous immunoreactivity studies39 suggest that α6-αChRs are mainly expressed in the ventral inferior MIIb, with little expression in the LHb. In the current study, using the patch-clamp electrophysiology technique, one of the most sensitive ways to detect functional activity of receptors, we were able to detect that nicotine modulated physiological activity of LHb neurons through action of αChRs containing the α6 subunit.

Nicotine has both negative and positive motivational effects which contribute to its abuse potential1. Here we demonstrate that nicotine, acting through α6*-αChRs, depolarizes LHb neurons and enhances glutamatergic signaling. Manipulating LHb activity, especially through α6*-αChRs, may therefore be of great value in treating nicotine addiction. In addition, we have shown that, over a wide range of concentrations (10 nM–100 μM), nicotine robustly activates LHb neurons. In contrast, nicotine at <100 nM has no significant effects on DA neurons in the ventral tegmental area (VTA); only at ≥250 nM does nicotine increase DA neuronal firing and burst
Nicotine-induced change of firing rate

|                      | ACSF            | +Gabazine | +DNQX + AP5 | +Gabazine + DNQX + AP5 |
|----------------------|-----------------|-----------|-------------|-------------------------|
| Inhibition (% of baseline) | 90.4 ± 3.8 (n = 8) | 101.2 ± 1.0* (n = 8) | 86.9 ± 3.5 (n = 8) | 103.4 ± 2.5* (n = 8) |
| Excitation (% of baseline)  | 149.8 ± 7.3 (n = 14) | 155.4 ± 6.8 (n = 8) | 122.4 ± 3.5* (n = 8) | 126.5 ± 3.9* (n = 8) |

Table 1. GABA or and glutamate transmissions mediate nicotine–induced changes in firing of LHb neurons. Nicotine (100 nM)-induced inhibition of spontaneous firing was abolished by the GABA A receptor antagonist gabazine, while nicotine-induced acceleration of firing was partly attenuated by glutamate receptor antagonists DNQX + AP5. *p < 0.05, One-way ANOVA followed by Tukey’s multiple comparison test for nicotine application in ACSF vs nicotine plus these blockers.

Nicotine application in ACSF vs nicotine plus these blockers.
Spontaneous firing was recorded by the loose-patch cell-attached technique, allowing long-lasting recordings without perturbing the cytoplasmic contents, and in whole-cell mode to measure membrane potential and input resistance. Currents induced by nicotine or acetylcholine (ACh) were recorded in TTX, AP5, DNQX, gabazine, and strychnine. Compounds were applied by bath perfusion, except acetylcholine (30 μM, with 0.5 μM atropine to block muscarinic receptors) which was ejected by pressurized air from a micropipette placed near the recorded cell33.

**Drugs.** We purchased common salts and [L-1-methyl-2-(3-pyridyl) pyrrolidine] freebase (nicotine), 6,7-dinitroquinoxaline-2,3-dione (DNQX), DL-2-amino-5-phosphono-valeric acid (AP5), strychnine, mecamylamine hydrochloride, methyllycaconitine, gabazine, atropine, acetylcholine, CdCl2, TTX (from Sigma (St Louis, MO), and dihydro-3-erythroxydine hydrobromide from Santa Cruz Biotechnology (Santa Cruz, CA). α-conotoxin MII (α-CTx-MII) and MII [H9A; L15A] were synthesized30.

**Data Analysis and Statistics.** Baseline electrophysiological data were recorded for 5–10 min, before drug superfusion and during the washout. To calculate the percent change in EPSCs/IPSCs/firing frequency or amplitude for a given cell, recordings during the initial control period were averaged and normalized to 100%. eIPSC and eEPSC amplitudes were calculated by averaging the peak current from six sweeps during baseline and during each drug application. For the measurement of inward currents induced by bath application of nicotine, traces were filtered at 10 Hz, and the means of 30-s baseline before nicotine application and 30 s during the maximal effects of nicotine were calculated and subtracted to give the magnitude of the nicotine current. The peak of nicotinic currents induced by puffing ACh onto neuronal somata was measured after the traces were filtered at 500 Hz. All values given in the text and figures indicate mean ± S.E.M. Statistical significance was assessed by a two-tailed Student’s t test, a one-way ANOVA with a Tukey’s post hoc test for multiple group comparisons, or a Kolmogorov-Smirnov (K-S) test. Dose-response data were fitted to the logistic equation: \( y = \frac{y_{max}}{1 + \frac{100x}{x_{0}}}, \) where \( y \) is the percentage change, \( x \) is the concentration of nicotine, \( \alpha \) is the slope parameter, and \( x_{0} \) is the nicotine concentration which induces a half-maximal change.

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Author Contributions
W.Z., C.X., M.G. and R.F. collected data, W.Z., J.-H.Y., C.X., F.W.H., K.K., X.F., W.H., J.W., A.B. and J.M.M. elaborated the study design and drafted the article. All authors critically reviewed content and approved final version for publication.

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