Neuronal Excitability

Acetylcholine Acts through Nicotinic Receptors to Enhance the Firing Rate of a Subset of Hypocretin Neurons in the Mouse Hypothalamus through Distinct Presynaptic and Postsynaptic Mechanisms\textsuperscript{1,2}

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Abstract

Hypocretin/orexin neurons regulate many behavioral functions, including addiction. Nicotine acts through nicotinic acetylcholine receptors (nAChRs) to alter firing rate of neurons throughout the brain, leading to addiction-related behaviors. While nAChRs are expressed in the hypothalamus and cholinergic fibers project to this structure, it is unclear how acetylcholine modulates the activity of hypocretin neurons. In this study, we stimulated hypocretin neurons in mouse brain slices with ACh in the presence of atropine to dissect presynaptic and postsynaptic modulation of these neurons through nAChRs. Approximately one-third of tested hypocretin neurons responded to pressure application of ACh (1 mM) with an increase in firing frequency. Stimulation of postsynaptic nAChRs with ACh or nicotine resulted in a highly variable inward current in approximately one-third of hypocretin neurons. In contrast, ACh or nicotine (1 \mu M) reliably decreased the frequency of miniature EPSCs (mEPSCs). Antagonism of nAChRs with mecamylamine also suppressed mEPSC frequency, suggesting that an endogenous, tonic activation of presynaptic nAChRs might be required for maintaining functional mEPSC frequency. Antagonism of heteromeric (\alpha 4/\beta 2) or homomeric (\alpha 7) nAChRs alone suppressed mEPSCs to a lesser extent. Finally, blocking internal calcium release reduced the frequency of mEPSCs, occluding the suppressive effect of presynaptic ACh. Taken together, these data provide a mechanism by which phasic ACh release enhances the firing of a subset of hypocretin neurons through postsynaptic nAChRs, but disrupts tonic, presynaptic nAChR-mediated glutamatergic inputs to the overall population of hypocretin neurons, potentially enhancing the signal-to-noise ratio during the response of the nAChR-positive subset of neurons.

Key words: acetylcholine; hypocretin; nicotine; presynaptic

Introduction

A small group of neurons that express hypocretin (Hcrt, also known as orexin) resides in the perifornical and lateral hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998), and these neurons project throughout the brain and spinal cord (Peyron et al., 1998; van den Pol, 1999; Bayer et al., 2002). Hcrt+ neurons play important roles in modulating...
Neurons expressing the neuropeptide hypocretin regulate many behavioral functions, including sleep, motivation, and behaviors related to addiction. The ability of nicotine to stimulate nicotinic acetylcholine receptors (nAChRs) is essential for its addictive properties, but little is known about whether, and how, nicotine and the endogenous neurotransmitter acetylcholine affect hypocretin neurons. This study suggests that phasic acetylcholine release can enhance the firing of a subset of hypocretin neurons through postsynaptic nAChRs, while disrupting tonic activation of presynaptic nAChRs necessary for maintaining functional glutamatergic inputs to hypocretin neurons. We propose that this mechanism could enhance the signal-to-noise ratio of the electrical response to nicotine or acetylcholine in the nAChR-positive subset of neurons.

Materials and Methods

Animals
Male and female adult Hcrt-GFP mice (Li et al., 2002) (2–5 months old, backcrossed onto the C57BL6/J genetic background for at least 10 generations) were group housed and maintained on a 12–12 h light–dark cycle with food and water available ad libitum. Use of animals was in strict accordance with NIH Care and Use of Laboratory Animals Guidelines.

Brain slice and electrophysiology
Briefly, mouse brains were harvested following acute decapitation. Brains were immediately immersed in ice cold, oxygenated artificial cerebrospinal fluid (ACSF). ACSF contained (in mM): 125 NaCl, 26 NaHCO3, 10 glucose, 2.3 KCl, 1.26 KH2PO4, 2 CaCl2 and 1 MgSO4, pH 7.4. After being trimmed to a small tissue block containing the hypothalamus, brain chunks were cut on a vibratome into coronal slices (300 μm). Acute slices were incubated in a holding chamber with protective NMDG ACSF (containing (in mM): 110 N-methyl-D-glucamine, 110 HCl, 2.5 KCl, 1.2 NaH2PO4, 25 NaHCO3, 25 glucose, 10 MgSO4, 0.5 CaCl2, pH 7.4) at 36°C for 30 min, then transferred to regular ACSF and maintained at room temperature (ac-
according to the method described in http://www.brainslice
methods.com).

All experimental measurements were performed at 32–34°C. Whole-cell recordings were made from visually-
identified, GFP-positive neurons in hypothalamus under
voltage (holding $V = -60$ mV) or current clamp configura-
tion. Electrical signals were amplified with a Multiclamp
700B and digitized with Digidata 1440A (Molecular De-
vices). Micropipettes with a tip resistance of 4–7 MΩ were
made of borosilicate glass (Warner Instruments) using a
Sutter micropipette puller (P-97) and back filled with an
intracellular solution containing (in mM): 135 K-gluconate,
2 MgCl$_2$, 10 Na$_2$-phosphocreatine, 3 Na$_2$-ATP, 0.3 Na$_2$-
GTP, and 10 HEPES (pH 7.3). Only recordings with stable
series resistance (<30 MΩ) were analyzed.

Drug application
All drugs were dissolved in ACSF. For bath perfusion,
D-2-amino-5-phosphonovalerate (AP-5), 6-cyano-7-
nitroquinoxaline-2, 3-dione disodium (CNQX), picotoxin
(PTX), tetrodotoxin citrate (TTX), and atropine (Sigma)
were dissolved in ACSF at their final concentration. For
focal pressure application (puff), acetylcholine (1 mM) or
nicotine (100 μM) was loaded into a glass micropipette
(2 – 4 MΩ), and the pipette tip positioned 40 – 50 μm
from the cell body. Drug solutions were pressure-ejected
via a computer-driven picospritzer (puff duration: 5 s)
during the electrical recording. The picospritzer was used
to control the pressure, timing, and duration of the puff.
For fast bath application, 200 μL of nicotine (5 μM),
mecamylamine (50 μM), dihydro-β-erythroidine hydrobro-
mide (50 μM), methyllycaconitine (50 nM), dantrolene (250
μM), and (−)-xestospongin C (25 μM) were pipetted into
the recording chamber (1 mL of volume), at 5×
final concentration, carefully along the chamber wall slope.

Data analysis
Electrical recording data of nAChR currents were ana-
yzed using Clampfit 10 (Molecular Devices). Traces were
filtered with Gaussian low-pass with 50 Hz cutoff before
current amplitude measurements. Other electrical data
were analyzed using Axograph X 1.5.5 (Axograph Scien-
tific). To analyze miniature EPSCs, we took 150 – 200
events for baseline and washout from each cell, during a
time period ranging from 60 to 120 s. For Ach, we took all
events from within 90 s after application of ACh. Traces
were filtered with Gaussian low-pass with 500 Hz cutoff
before event searching. A template EPSC was defined
with amplitude $-20$ pA, rise 0.5 ms, and decay 3 ms, and
only those EPSCs with amplitude $\geq -10$ pA were
counted. Following programmed event detection, all
events were examined by eye to be counted as EPSCs. All
statistics were done on raw data. Unpaired Student’s $t$
tests and chi square ($\chi^2$) test were used for determining
statistically difference. Significance was set as $p < 0.05$,
and high significance as $p < 0.01$. Values are presented
as mean ± SEM.

Results
Nicotinic stimulation boosts spontaneous action
potential firing in hypocretin neurons
To determine whether phasic nicotinic stimulation mod-
ulates the electrical output of Hcrt+ neurons, we patched
the cells with an ATP-rich (5 mM, vs 3 mM in other ex-
periments) intracellular solution and investigated the
effect of pressure-applied ACh on spontaneous action
potential firing. An adequate level of intracellular ATP is
required for normal Hcrt+ neuron function (Liu et al.,
2011), potentially because Hcrt+ neurons are involved in
the regulation of energy status (Girault et al., 2012; Gao
and Horvath, 2014). In the presence of atropine, Hcrt+
neurons regularly fired action potentials. Mechanical in-
terference as a result of puffing bath solution induced a
temporary reduction in firing frequency (<50% of base-
line) in 7 out of 11 cells (Fig. 1B1,C), despite inducing no,
or minimal, outward current (<10 pA, data not shown).
Firing in 4 out of 11 cells was not altered by puffing bath
solution (50% < change < 200% at 0 – 10 s). In contrast,
in 7 out of 20 cells, a puff of ACh (1 mM) increased the
firing rate (> 200% of baseline) with a significant depo-
larization of the membrane potential (Fig. 1B2,D). We note
that this experimental design is not suitable to detect
decreases in firing due to presynaptic mechanisms. Only
blocking all mEPSCs using CNQX+AP-5 induces even a
small change in firing rate of a hypocretin neuron (~2 Hz
to ~1.6 Hz; Li et al., 2002), which is not distinguishable
using the current criteria (50% < change < 200%). In nine
cells, an ACh puff decreased the firing rate, and in four
cells the ACh puff had no effect. Thus, stimulation of
nAChRs depolarizes approximately one-third of Hcrt+
neurons and boosts the action potential firing of these
cells.

To identify the receptor type(s) that mediate this depolar-
ization and increase in firing rate, we applied an ACh puff
in the presence of the heteromeric (α4β2) nAChR antag-
onist, dihydro-β-erythroidine (DHβE), or the homomeric
(α 7) nAChR antagonist, methyllycaconitine (MLA). When
applied alone, DHβE significantly decreased the firing rate
by 62.9% ($p = 0.00027$) at 10 μM, whereas MLA did not
change the firing rate of Hcrt+ neurons at a concentration
of 10 nM (Fig. 2, top; 20 – 30 s after puff vs 10 – 20 s
before puff; $p = 0.58$). However, after addition of either
reagent, most cells recovered to a new stable firing state
that lasted for several minutes with attachment of the
recording pipette (Fig. 2, bottom). DHβE (10 μM) signifi-
cantly reduced the ability of ACh to increase firing in 11 of
11 Hcrt+ neurons ($\chi^2$ test, $p = 0.0069$) and increased
the likelihood that an ACh puff could decrease the firing rate
($p = 6.8E-6, 10 – 20 s after puff; Fig. 1E$). In contrast,
although MLA (10 nM) antagonized the effects of an ACh
puff in the majority of Hcrt+ neurons, ACh still increased
the firing rate in 2 of 16 cells in the presence of the
homomeric nAChR antagonist ($\chi^2$ test, $p = 0.00039; Fig.$
1F). In this data set, some cells increased their firing rate
during the ACh puff and returned to normal right after puf
cession, whereas 10 of 17 cells responded with a pro-
longed period of firing increase. These prolonged periods
of firing may be due to increased activity of Hcrt neurons triggering action potentials in a recurrent circuit.

Muscarinic acetylcholine receptors (mAcHRs) are expressed by ~30% of hypocretin neurons (Sakurai et al., 2005), and may contribute significantly to cholinergic modulation of these cells. To determine the overall response of hypocretin neurons to cholinergic inputs, we tested the effect of ACh in the absence of atropine to engage both nAcHRs and mAcHRs. ACh induced depolarization and increased firing rate in 57% (8 out of 14) of hypocretin neurons (Fig. 1G), a significant increase compared to the presence of atropine ($\chi^2$ test, $p = 0.0040$). The remaining cells showed no effect (1 out of 14 cells) or responded to ACh with decreased firing (5 out of 14 cells).

**Postsynaptic nAcHR currents can be induced in one third of hypocretin neurons**

Although mRNAs encoding nAcHR subunits are expressed in lateral hypothalamus (Clarke et al., 1985; Wada et al., 1989) where Hcrt+ neurons reside, it is not yet clear on which cell types and in what subcellular structure nAcHRs are functional, and whether Hcrt+ neurons express nAcHRs. To dissect the mechanisms underlying nicotinic stimulation of Hcrt+ neurons, we evaluated postsynaptic nAcHRs on Hcrt+ neurons selectively by using synaptic blockers to rule out contributions from glutamate- and GABA-activated currents. We recorded from Hcrt+ neurons in voltage clamp mode in the presence of TTX (0.5 $\mu$M), PTX (100 $\mu$M), CNQX (10 $\mu$M), AP-5 (30 $\mu$M), and atropine (4 $\mu$M), while puffing 1 mM ACh onto the cell body of the recorded cell. A brief puff of ACh evoked an inward current in about one-third of Hcrt+ neurons (32 out of 92 cells). The peak amplitudes of the inward currents ranged from −10 to −850 pA, with an average size of $−71.1 \pm 30.9$ pA ($n = 32$). We also tested the nAcHR agonist nicotine (100 $\mu$M). A nicotine puff produced a response similar to that induced by ACh in Hcrt+ neurons. Inward currents were recorded in 9 out of 29 cells, ranging from −10 to −1500 pA, with an average size of $−413 \pm 178$ pA ($n = 9$; Fig. 3). We also wished to determine whether the small currents might be sufficient to alter the function of the Hcrt+ neurons. Injection of a small inward current (−10 pA) markedly increased the spontaneous action potential firing rate to 324% ± 101% (Student’s $t$ test, $p = 1.0E-5$, $n = 3$) of the baseline level (Fig. 1B4), suggesting that nicotinic currents are likely to have a significant effect on the output of these cells. nAcHRs are known to be easily desensitized by agonists (Giniatullin et al., 2005). Consistent with these observations, we observed that postsynaptic nAcHR-mediated currents on Hcrt+ neurons were desensitized following 5 s exposure to 1 mM ACh or 100 $\mu$M nicotine. Previous studies have shown that most nAcHR subtypes, including $\alpha_4\beta_2$, $\alpha_3\beta_4$ and $\alpha_7$, recover from the desensitization induced by brief (1−5 s) exposure to 1 mM ACh in 60 s or less (Reitstetter et al., 1999; Meyer et al., 2001; Paradiso and Steinbach, 2003; McCormack et al., 2010). While the majority of currents did not recover rapidly following ACh stimulation, we observed a rare subset of Hcrt+ neurons (3 out of 121 cells) that responded to...
repeated ACh or nicotine puffs every 60 s (Fig. 3A2), which happened only when the current size was greater than 500 pA.

**Acetylcholine and nicotine decrease the frequency of mEPSCs in hypocretin neurons**

Next, we tested whether nAChRs are expressed in presynaptic glutamatergic terminals that impinge onto Hcrt+/H11001 neurons, and what physiological changes might occur to synaptic transmission following presynaptic receptor stimulation. To do this, we applied TTX, PTX, and atropine in the bath and recorded spontaneous mEPSCs from the Hcrt+/H11001 neurons. A brief puff of ACh above the tissue surface next to the Hcrt+/H11001 neuron reliably decreased mEPSC occurrence for ~1 min, followed by full recovery (Fig. 4A, B). The average instantaneous frequencies of mEPSCs at baseline, upon ACh application, and after washout were 3.76 ± 0.19 Hz, 0.64 ± 0.12 Hz, and 3.48 ± 0.31 Hz, respectively. The average peak amplitudes of mEPSCs at baseline, upon ACh application, and washout were 26.4 ± 0.3 pA, 21.8 ± 0.8 pA, and 30.0 ± 0.5 pA, respectively. Analysis shows that phasic stimulation of preterminal nAChRs significantly reduced vesicle release probability by 83.0% (p = 2.06E-39). The average peak size of mEPSCs was also slightly reduced by 17.4% (p = 1.88E-7; Fig. 4C, D).

Nicotine is a strong exogenous agonist of nAChRs, and also a prevalent drug of addiction. During cigarette smoking, nicotine concentration increases rapidly in the brain (Berridge et al., 2010; Rose et al., 2010). To mimic this fast onset of brain nicotine concentration, we pipetted 5× nicotine solutions into the recording chamber within 5 s, to a final concentration of 1 μM (Henningfield et al., 1993). In 4 out of 11 tested cells, the mEPSC frequency was immediately depressed from 5.37 ± 0.59 Hz to 2.02 ± 0.23 Hz (Fig. 5A-C). In an additional four cells, the immediate effect of nicotine was not significant, with frequency changed from 2.97 ± 0.40 Hz to 3.39 ± 0.52 Hz (p = 0.527; Fig. 5D-F, T-1), but following this initial 1–2 min period, suppression of mEPSC frequency (0.96 ± 0.17 Hz, p = 6.8E-6) was observed for 1–2 min (Fig. 5D–F, T-2). Finally, there were three cells with no obvious response to nicotine exposure.

**Antagonists of nAChRs mimic the effect of agonist application on mEPSC frequency**

Under physiological conditions, activation of nAChRs induces excitatory inputs by evoking inward currents. The suppressive effect on mEPSCs might therefore be due to desensitization of nAChRs. To test this possibility, we applied mecamylamine (MEC), a nonselective antagonist of nAChRs. The application of MEC alone caused a substantial suppression of mEPSCs (Fig. 6), with instantaneous frequency decreased to 37.6% (from 6.49 ± 0.32 Hz to 2.44 ± 0.24 Hz, p = 1.82E-23), and amplitude decreased to 80.7% (from 31.6 ± 0.4 pA to 25.5 ± 0.4 pA,
$p = 1.47\text{E-22}$ of baseline values. Furthermore, both the heteromeric (predominantly \(\alpha 4\beta 2\)) nAChR antagonist DH/\(\beta\)E (10 \(\mu\)M) and the homomeric, \(\alpha 7\) nAChR antagonist MLA (10 nM) partially reduced event frequency to 62.5% (from 6.45 \(\pm\) 0.46 Hz to 4.04 \(\pm\) 0.28 Hz, \(p = 1.09\text{E-5}\)) and 76.8% (from 9.88 \(\pm\) 0.57 Hz to 7.58 \(\pm\) 0.47 Hz, \(p = 1.94\text{E-3}\)) of baseline values. The mEPSC peak amplitude was also reduced to 87.4% (from 21.4 \(\pm\) 0.5 pA to 18.7 \(\pm\) 0.4 pA, \(p = 5.74\text{E-5}\)) and 81.5% (from 24.3 \(\pm\) 0.6 pA to 19.8 \(\pm\) 0.4 pA, \(p = 8.18\text{E-10}\)) of their baseline levels, respectively (Fig. 6D–G), consistent with what was observed following ACh or nicotine application (Figs. 4, 5).

**Blockade of Ca\(^{2+}\) internal stores occludes the effect of ACh on mEPSC frequency**

Synaptic vesicle release is a calcium-dependent process (Neher and Sakaba, 2008; Südhof, 2012). Spontaneous vesicle release, unlike action potential-evoked events, is independent of extracellular calcium and voltage-gated calcium channel opening, but is instead driven largely by calcium release from internal stores (Llano et al., 2000; Emptage et al., 2001; Han et al., 2001). To determine whether nAChR stimulation affects calcium release from internal stores, we applied dantrolene (50 \(\mu\)M) and (-)-Xestospongin C (XeC) (5 \(\mu\)M) to block ryanodine and IP\(_3\) receptors in the endoplasmic reticulum membrane (Dickinson et al., 2008). Following 30 min incubation with dantrolene and XeC, the mEPSCs instantaneous frequency was reduced to 50.7% of its baseline value (4.90 \(\pm\) 0.48 Hz to 2.48 \(\pm\) 0.38 Hz, \(p = 7.12\text{E-4};\) Fig. 7), in agreement with previous reports that approximately half of spontaneous vesicle release is due to internal calcium stores (Emptage et al., 2001). The peak amplitude was reduced to 80.6% (21.1 \(\pm\) 0.7 pA to 17.0 \(\pm\) 0.5 pA, \(p = 5.18\text{E-5}\)). In the continued presence of dantrolene and XeC, we pressure applied ACh onto Hcrt+ neurons and found that both amplitude (from 17.0 \(\pm\) 0.5 pA to 18.0 \(\pm\) 0.5 pA, \(p = 0.664\)) and frequency of mEPSCs (from 2.48 \(\pm\) 0.38 Hz to 2.25 \(\pm\) 0.37 Hz, \(p = 0.151\)) remained unchanged (Fig. 7C,D). Thus, blockade of calcium release from internal store occluded the effect of subsequent nAChR stimulation.

**Discussion**

Hypocretin/orexin neurons are involved in multiple behaviors, including those related to arousal and addiction (Mahler et al., 2012; de Lecea and Huerta, 2014). Understanding how the electrical activity of Hcrt+ neurons is modulated by endogenous neurotransmitters and exogenous drugs such as nicotine is critical for understanding the role of these cells in complex behaviors, including nicotine dependence. As a candidate modulator, nAChRs have been shown to be expressed in hypothalamus. Stimulation of these receptors can activate Hcrt+ neurons (Pasumarthi et al., 2006; Pasumarthi and Fadel, 2008; Morgan et al., 2013); however, the underlying mechanisms by which nAChRs modulate the activity of Hcrt+ neurons are still unclear.

In the current study, we used pressure application to mimic phasic ACh neurotransmission (Alexander et al., 2009) and investigated the effects of ACh and nicotine acting through presynaptic and postsynaptic nAChRs on glutamatergic transmission and postsynaptic electrical activity in Hcrt+ neurons. We found that nAChRs are expressed postsynaptically in around one-third of Hcrt+ neurons, as well as presynaptically on glutamatergic axon
terminals to Hcrt+/H11001 neurons. Stimulation of presynaptic or postsynaptic nAChRs produced opposing effects on electrical activity in Hcrt+/H11001 neurons, however. Stimulation of postsynaptic nAChRs evoked an inward current in a fraction (approximately one-third) of Hcrt+/H11001 neurons (Fig. 3). This current, which was as small as 10 pA, ranging to more than 1000 pA, depolarized the membrane potential and increased spontaneous firing of Hcrt+ neurons (Fig. 1). In contrast, stimulation of presynaptic nAChRs reliably decreased the frequency of mEPSCs in Hcrt+ neurons (Fig. 4), indicating the efficacy of glutamatergic transmission was decreased. At a concentration consistent with

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cigarette smoking, nicotine suppressed mEPSCs in a majority (~70%) of Hcrt+ neurons (Fig. 5). The nonselective nAChR antagonist mecamylamine depressed mEPSC frequency to a similar extent (Fig. 6), suggesting that the suppressing effect of ACh on mEPSCs was likely mediated by desensitization of presynaptic nAChRs, and that an endogenous, tonic activation of presynaptic nAChRs might be required for maintaining normal, functional mEPSC frequency. It should be noted that inhibitory inputs impinging on hypocretin neurons are also likely to express nAChRs, and therefore could also be regulated by ACh; however, inhibitory inputs were excluded using picrotoxin in this study.

To explore the mechanism underlying the ACh-mediated decrease in mEPSC frequency, we determined the role of calcium release from internal stores (Llano et al., 2000; Cheng and Lederer, 2008), since intracellular calcium stores are thought to be required for spontaneous vesicle release (Emptage et al., 2001). In the presence of the ryanodine receptor blocker dantrolene and the IP3

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**Figure 5** Effects of nicotine exposure on glutamatergic mEPSCs onto Hcrt+ neurons. **A**, Histograms of mEPSC instantaneous frequency showing the effect of nicotine (Nic) exposure (100 μM) on mEPSCs onto Hcrt+ neurons. The arrow indicates the time of nicotine application. Depending on the response pattern, two periods of time (baseline and Nic) were used for analysis. **B, C**, Mean value of amplitude (B) and instantaneous frequency (C) at baseline and under the influence of nicotine. n = 4 of 11 cells. **D, E, F**, Mean value of amplitude (E) and instantaneous frequency (F) of mEPSCs during baseline recording and at two periods after nicotine exposure. n = 4 of 11 cells.
receptor blocker XeC (Dickinson et al., 2008), mEPSC frequencies in Hcrt+ neurons were significantly decreased. The application of ACh did not further decrease mEPSC frequencies (Fig. 7), indicating that phasic nAChR activation (i.e., endogenous ACh release from cholinergic synapses) could desensitize tonically active nAChRs and thereby decrease calcium release from internal stores, preventing Ca2+–dependent spontaneous vesicle release. Previous studies have shown that nicotine can transiently facilitate neurotransmitter release, and one study in mouse vas deferens indicated that this is mediated by calcium-induced calcium release from a ryanodine-sensitive calcium store in nerve terminals (Brain et al., 2001). Here, we propose that in the mouse central nervous system, at least in glutamatergic synapses impinging onto Hcrt+ neurons, this mechanism is also present. Tonic activation of presynaptic nAChRs contributes to spontaneous vesicle release via transient opening of ryanodine receptors and/or IP3 receptors in local internal calcium stores. Levels of nicotine delivered through cigarette smoking are sufficient to interfere with this function, and this mechanism could therefore contribute to behaviors related to nicotine dependence.

The opening of nAChRs channels leads to the influx of cations, and generally excites the postsynaptic neurons (Léna and Changeux, 1997; Zhou et al., 2001; Mansvelder et al., 2002; Sharma et al., 2008; Huang et al., 2011). In the current study, this direct depolarizing effect of nAChR activation was also demonstrated for mouse Hcrt+ neurons. nAChR antagonist, partially suppressed mEPSC amplitude (F) and instantaneous frequency (G). n = 8 cells.

**Figure 6** Nicotinic AChR antagonists suppressed glutamatergic mEPSCs onto Hcrt+ neurons. **A**, Mecamylamine, a nonselective nAChR antagonist, strongly suppressed mEPSCs onto Hcrt+ neurons. The bar above the graph indicates the duration of MEC (10 μM) exposure. Miniature EPSCs from two periods of time (Baseline and MEC) were used for analysis. **B**, Cumulative probability plot of the mEPSC amplitude at baseline and during MEC exposure was made from the recordings of four cells. Inset shows the mean values of the mEPSC amplitude at baseline and following MEC exposure. **C**, Inter-event intervals of mEPSCs at baseline and upon MEC application from four cells (same cells as in **B**). Inset shows the mean instantaneous frequency of the mEPSCs at baseline and following MEC exposure. **D**, **E**, DHβE, an antagonist of heteromeric nAChRs (particularly α4β2 nAChRs), moderately suppressed the amplitude (**D**) and instantaneous frequency (**E**) of mEPSCs onto Hcrt+ neurons. n = 7 cells. **F**, **G**, MLA, a relatively selective α7 nAChR antagonist, partially suppressed mEPSC amplitude (**F**) and instantaneous frequency (**G**). n = 8 cells.
Hcrt+ nicotinic responses, but are less prominent. Blockade of α4β2* receptors by DHβE not only decreases mEPSCs by disrupting presynaptic nicotinic signaling, but also prevents the activation of postsynaptic nicotinic receptors, and therefore affects spontaneous firing of Hcrt+ neurons. In contrast, the role of presynaptic nAChRs has not been reported previously. In our experimental paradigm, either pressure application of ACh (1 mM) onto the soma and the proximal processes or bath application of nicotine (1 μM) suppressed the spontaneous mEPSC frequency in Hcrt+ neurons, which is consistent with effects reported in the arcuate nucleus of the hypothalamus (Huang et al., 2011). We also observed a consistent, though modest, reduction in mEPSC size that always

Figure 7 Inhibiting calcium release from internal stores occludes the effect of ACh on mEPSCs onto Hcrt+ neurons. A, B, mEPSCs recorded at baseline (A) and after 30 min incubation with the ryanodine receptor antagonist dantrolene (50 μM) and the IP3 receptor antagonist (-)-Xestospongin C (5 μM; B). C, D, Mean values of amplitude (C) and instantaneous frequency (D) of mEPSCs at baseline, after 30 min incubation with dantrolene (Dan) and XeC and upon subsequent ACh puff in the continued presence of dantrolene and XeC. n = 3 cells. Both amplitude and instantaneous frequency were significantly decreased by dantrolene+XeC, but there was no significant difference between mEPSC recordings in the presence of dantrolene and XeC and upon subsequent ACh puffs in the continued presence of these inhibitors. **p < 0.01; NS, not significant (p > 0.05).

Figure 8 ACh increased action potential (AP) firing or had no effect. In the same cells, however, ACh decreased mEPSC occurrence despite of its effect on action potential firing. A, A Hcrt+ cell increased action potential firing rate in response to ACh, in presence of atropine. In the same cell, a puff of ACh decreased mEPSC frequency. Yellow bars indicate the time duration of ACh application. C, Another Hcrt+ cell did not change action potential firing rate upon ACh puff. D, In the same cell, a puff of ACh decreased mEPSC frequency.
accompanied the reduction in mEPSC frequency (Figs. 4 – 7). This could be due to effects of nicotinic signaling on activity of postsynaptic ionotropic glutamate receptors, in particular AMPA receptors. It is also possible that large presynaptic vesicles (Wojcik et al., 2004) or proximal synapses (which produce mEPSCs with larger amplitudes than distal ones; Bekkers and Stevens, 1996) are more significantly recruited by nicotinic signaling. Interestingly, the nicotinic antagonist, MEC, had a similar effect as ACh and nicotine on mEPSC frequency and amplitude (Figs. 4 – 6). Partial blockade of α4β2 or α7 nAChRs by more selective antagonists also reduced mEPSC frequency, indicating that both heteromeric and homomeric nAChRs in brain are present in presynaptic glutamatergic terminals impinging on Hcrt+ neurons. These results suggest that both ACh and nicotine likely desensitize nAChRs and interrupt glutamatergic transmission onto Hcrt+ neurons, further suggesting that tonic activation of presynaptic nAChRs might be necessary for the normal function of neurotransmission in glutamatergic synapses impinging onto these neurons (Brain et al., 2001). Phasic cholinergic release may temporarily interrupt or reduce this tonic glutamatergic transmission. It is also possible that stimulation of postsynaptic nAChRs depolarizes membrane potential and releases calcium from internal stores, facilitating release of retrograde signaling molecules, such as endocannabinoids (Huang et al., 2007) or dynorphin (Li and van den Pol, 2006), that result in negative feedback. These paradoxical presynaptic and postsynaptic effects of ACh may improve the signal-to-noise ratio of selective Hcrt+ firing during phasic release, as might occur during exposure to behaviorally relevant stimuli (Dalley et al., 2001; Parikh et al., 2007), or in response to nicotine during smoking or behaviors relevant to drug reinforcement (Hollander et al., 2008). In particular, phasic ACh release would be expected to stimulate the firing of the approximately one-third of Hcrt+ neurons expressing nAChRs, while also reducing activity in the remaining two-thirds of Hcrt+ neurons by blocking synaptic excitatory events supported by tonic activation of presynaptic nAChRs (Fig. 8).

In conclusion, these results suggest that ACh modulates the Hcrt system through a multifaceted, nAChR-mediated mechanism, which is complementary to the effects of muscarinic modulation of these neurons (Sakurai et al., 2005). More importantly, nAChR-mediated effects of ACh may enhance the output of a selective group of Hcrt+ neurons expressing nAChRs, and distinguish this subset from the overall hypocretin network.

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