Cellular/Molecular

Cholinergic Modulation of Appetite-Related Synapses in Mouse Lateral Hypothalamic Slice

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Nicotine administration reduces appetite and alters feeding patterns; a major deterrent to smoking cessation is hyperphagia and resultant weight gain. We demonstrate here that lateral hypothalamic (LH) circuits involving melanin-concentrating hormone (MCH) neurons are subject to cholinergic modulation that may be related to the effects of nicotine on appetite control. Cholinergic input to the perifornical LH area of the mouse is confirmed by examination of immunostaining for vesicular acetylcholine (ACh) transporter (VAT) in conjunction with antibodies to MCH and the vesicular GABA transporter (vGABAT). vAChT-positive neurons border the LH, and VAT-positive projections are detected throughout the perifornical area. MCH-positive dendrites appear studded with vGABAT-positive contacts, consistent with recordings of GABAergic inputs to LH/MCH neurons identified by their location, morphology, electrophysiological profile, and MCH expression. Activation of presynaptic nicotinic ACh receptors (nAChRs) enhances GABAergic transmission. GABAergic transmission is potentiated by (1) direct nicotine application, (2) increasing local ACh concentration, and (3) stimulation of cholinergic projections. Based on pharmacological studies and comparisons of wild-type versus α7 nAChR subunit mutant mice, we propose that α7*-nAChRs are required for the modulation of GABAergic inputs in LH. Prenatal exposure to nicotine elicits a persistent elevation of GABAergic transmission in the LH of postnatal pups. Furthermore, GABAergic inputs to LH of prenatal nicotine-exposed pups are insensitive to subsequent nicotine challenge. Our studies support the hypothesis that nicotine administration or elevated cholinergic tone enhance inhibition of perifonical LH/MCH neurons via activation of presynaptic α7*-nAChRs.

Key words: **MCH; orexin; nicotine; motivation; reward; obesity**

Introduction

Nicotine, the major addictive component in tobacco, reduces appetite and alters feeding patterns typically resulting in reduced body weight (Grunberg, 1986; Grunberg et al., 1986; Miyata et al., 1999; Jo et al., 2002). Particularly striking is the hyperphagia and resultant weight gain that typically accompanies smoking cessation (Grunberg et al., 1986; Klesges et al., 1989; Pomerleau et al., 2000). Direct application of nicotine in the region of the LH (this paper) or in synaptic preparations of LH neurons *in vitro* enhances GABAergic transmission (Jo and Role, 2002). The effects of ACh per se appear to be more complex, because activation of muscarinic ACh receptors (AChRs) oppositely modulated GABAergic transmission. Previous *in vitro* studies underscored an important potential role of endogenous cholinoceptive systems in the lateral hypothalamus (LH) and further implicated ACh in the regulation of feeding-related circuits and synapses.

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The LH is the recipient of several sources of cholinergic projections with inputs from the brainstem (pedunculopontine and laterodorsal tegmental areas) as well as from local cholinergic cell groups (substantia innominata and zona incerta) (Grove, 1988; Woolf, 1991; Bayer et al., 1999). There are numerous reports demonstrating that chronic nicotine exposure can regulate the expression and/or function of nicotinic AChRs (nAChRs) (Yates et al., 1995; Olale et al., 1997; Peng et al., 1997; Ke et al., 1998; Molinari et al., 1998; Wang et al., 1998; Fenster et al., 1999; Buisson and Bertrand, 2001; Gentry and Lukas, 2002; Slotkin et al., 2002; Pakkanen et al., 2005). In contrast, there is relatively little information about effects of prenatal nicotine exposure on postnatal alterations in synaptic transmission or on persistent changes in the profile of nAChR-mediated modulation of CNS transmission in general and in hypothalamic circuits in particular. The current study establishes a potential role of nAChR activation in the short- and long-tern regulation of excitability in feeding-related synapses.

Mice lacking melanin-concentrating hormone (MCH) have lowered body weight, are lean, and display marked hypophagia compared with control animals (Shimada et al., 1998). Recent studies showing that MCH receptor-deficient mice, like those lacking MCH expression, are lean, further supporting the importance of MCH in appetite regulation (Marsh et al., 2002). Projections of the MCH neurons include the nucleus accumbens and the ventral tegmental area, prominent reward-related areas. As

such, these relays may play a unique role in enhancing the hedonic value of food intake.

We focused the current analysis on testing whether the activation of nAChRs might modulate GABAergic inputs involved in the regulation of feeding circuits within the LH. We also initiated studies testing the hypothesis that prenatal exposure to nicotine might exert long-lasting effects on the GABAergic transmission at LH synapses and alter the responsiveness of these circuits to subsequent cholinergic modulation.

Materials and Methods

Slice preparation. Transverse brain slices were prepared from wild-type C57BL/6 or α7 knock-out (KO) mice (Orr-Urtreger et al., 1997) at postnatal day 10 (P10) to P17. Animals were anesthetized with a mixture of ketamine and xylazine. After decapitation, the brain was transferred into a sucrose-based solution bubbled with 95%O_2/5% CO_2 and maintained at ~3°C. This solution contained the following (in mM): 248 sucrose, 2 KCl, 1 $MgCl_2$, 1.25 KH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Transverse coronal brain slices (350 μM) were prepared using a Vibratome (VT1000S; Leica, Nussloch, Germany). Slices were equilibrated with an oxygenated artificial CSF (aCSF) for >1 h before transfer to the recording chamber. The slices were continuously superfused with aCSF at a rate of 2 ml/min (unless noted in the text) containing the following (in mM): 113 NaCl, 3 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, and 10 glucose [in 95% O_2/5% CO_2 at room temperature (24–26°C)].

Electrophysiological recordings. Brain slices were placed on the stage of an upright infrared–differential interference contrast microscope (BX50WI; Olympus Optical, Tokyo, Japan) mounted on a Gibraltar X-Y table (Zeiss, Oberkochen, Germany) and visualized with a 40× water immersion objective by infrared microscopy (camera by Dage-MTI, Michigan City, IN). Membrane currents were recorded at room temperature (24–26°C) with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA) in the whole-cell configuration (unless otherwise indicated). The external solution contained the following (in mM): 113 NaCl, 3 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, and 10 glucose (except as noted) (in 95% O_2/5% CO_2). CNQX (10 μM), DL-AP-5 (50 μM), and atropine (500 nM) were continuously present in the external solution. The internal solution contained the following (mM): 130 KCl or CsCl, 2 $MgCl_2$, 10 EGTA, 5 $CaCl_2$, 10 HEPES, 1 Na_2ATP, and 0.5 Na_2GTP. Pipettes resistance ranged from 2.5 to 4 MΩ.

For perforated patch-clamp recording using gramicidin (Sigma, St. Louis, MO), the pipette tip was filled with an internal solution (150 ml KCl and 10 ml HEPES, pH 7.3) and backfilled with the same solution containing gramicidin (100 μg/ml). Access resistance was stable between 20 and 30 MΩ after ~1 h.

Eliciting monosynaptic postsynaptic currents and detection of miniature postsynaptic currents. For extracellular stimulation, an electrode prepared from "theta (θ)" glass (World Precision Instruments, Sarasota, FL) filled with extracellular solution was placed close to the recorded neuron. Pairs of stimuli were delivered at 0.1 Hz (interval of 150 ms; 1 ms; from −20 to −100 μA); the paired-pulse modulation ratio is defined as $[(I_2 - I_1) \times 100]/I_1$. To induce the release of endogenous ACh, burst stimulation (50 Hz for 1 s, five times; interval between stimuli, 5 s) was applied in the area in which we detected cholinergic cell bodies (~2–3 mm from recording site).

Spontaneous miniature postsynaptic currents (mPSCs) were recorded in the presence of tetrodotoxin (TTX) (1 μM; Sigma). Autodetected events with amplitude of more than −5 pA and rise time of <3 ms were also visually examined to correct for noise fluctuation. Analysis of mPSC decay phase was based on the following criteria: (1) single events only (i.e., no multiple events), (2) events having stable baselines 15 ms before the rise, and (3) smooth transition from 0 current to peak amplitude [<20% deviation in $d(pA)/dt$ during rise]. All data were stored for off-line analysis on a personal computer with a Pentium III or IV processor after being filtered at 5 kHz by the Axopatch 200B amplifier. Analysis used pClamp8 (Axon Instruments) and Mini analysis 5.0 (Synaptosoft, Decatur, GA).

Immunocytochemical staining. The recorded neurons that were relocated and processed for subsequent immunohistochemical analyses were injected with Alexa-Fluor 488 (Invitrogen, Carlsbad, CA). The brain slices were then fixed and remounted, and cryostat sections were prepared as delineated below.

For routine immunocytochemical studies, P15 mice under deep anesthesia were perfused with 4% formaldehyde in PBS. The entire brain was dissected and postfixed intact for 3 h in 4% formaldehyde/4%sucrose (PBS) and then equilibrated in 30% sucrose (PBS). Tissue was embedded in OCT, frozen, and sliced in 14 μm sections. Brain sections were incubated with 0.3% Triton X-100 (PBS) for 30 min, with 8% donkey serum (in 0.3% Triton X-100, PBS) for 30 min to block nonspecific binding and again with 0.3% Triton X-100 (PBS) for another 30 min at room temperature. Primary antibodies against MCH (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and vesicular ACh transporter (VAT) (1:500, Sigma; or 1:500, Chemicon, Temecula, CA) and/or α-bungarotoxin αBgTx conjugated to fluroprobe (Alexa-Fluor 488) were incubated overnight at 4°C. The brain sections were incubated with 0.3% Triton X-100 (PBS) for 10 min, rinsed twice with PBS, and reincubated for 2 h at room temperature in secondary antibodies FITC-conjugated anti-rabbit IgG for VAT and a rhodamine-conjugated anti-goat IgG for MCH (diluted 1:200; both from Jackson ImmunoResearch, West Grove, PA). Brain slices were rinsed twice in PBS and mounted onto slides.

Prenatal and perinatal exposure to nicotine. Pregnant females at 14 d after mating were given nicotine through the drinking water with sucrose (2%) and nicotine (200 μg/ml) or with sucrose alone (control group) throughout the period of pregnancy and lactation. Based on other reports using this technique in mice, steady-state levels of nicotine achieved under these conditions are ~175 ng/ml in pup blood (Pauly et al., 2004).

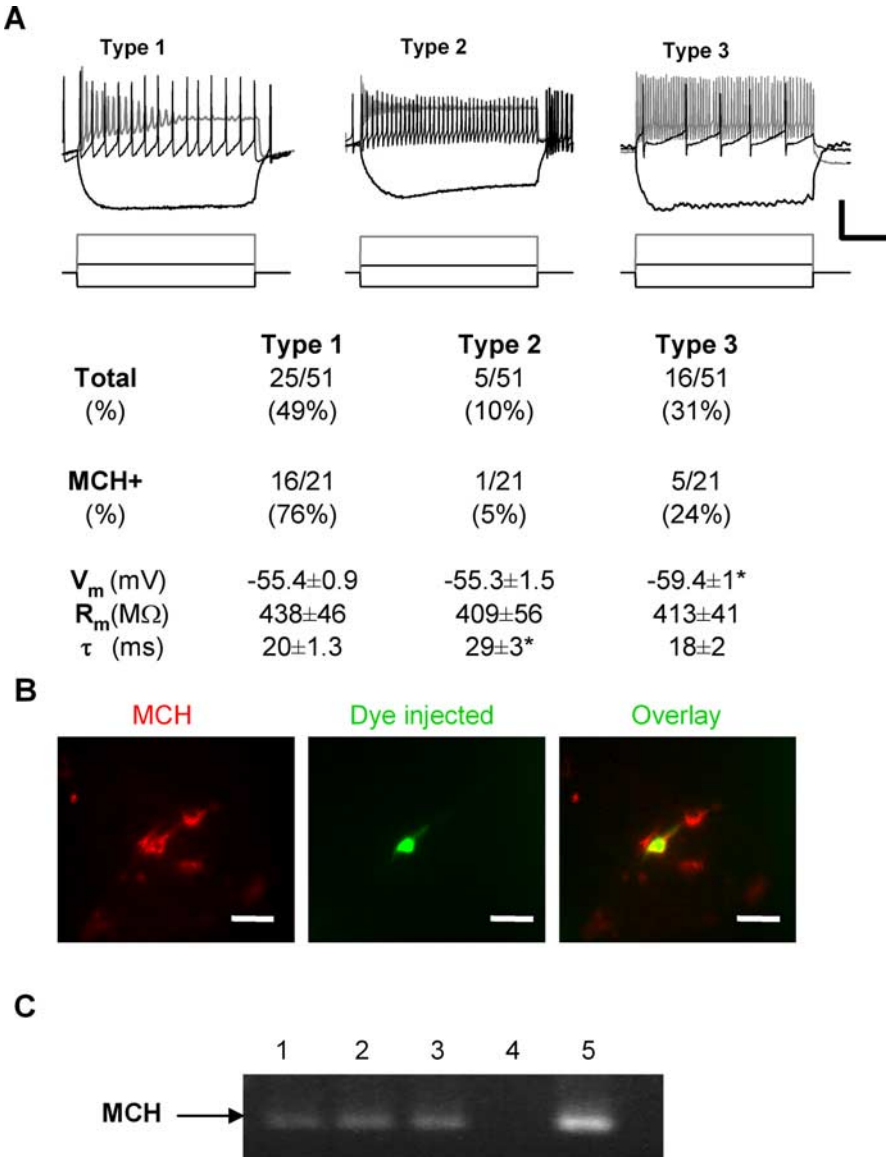
Single cell reverse transcription-PCR. Single-cell samples were collected from brain slice preparations via aspiration into the patch pipette. The initial reverse transcription (RT) reaction was conducted after pressure ejection of the single-cell samples into freshly prepared RT mix A solution (20 U of RNase OUT, 300 ng of random primers, 0.5% NP-40, and RNase free water). Samples were sonicated in a total volume of 10 μl at 4°C for 5 min and then incubated for 3 min at 65°C before addition of 10 μl RT mix B (500 μM dNTP, 1× RT buffer, 5 mM $MgCl_2$, 10 mM DTT, and 200 U of Superscript II l). The tubes were incubated at 25°C for 5 min, at 42°C for 1 h, and at 65°C for 10 min.

Each cDNA sample obtained was split into two aliquots, one for analysis of MCH transcripts and the other for analysis of neuronal enolase. Two rounds of amplification were done for the detection of MCH transcripts and one round of amplification for the analysis of neuronal enolase. In the first amplification (final 50 μl), the reaction mixture contained 10 μl of cDNA, 1× PCR buffer with Mg^{2+} (Roche Products, Indianapolis, IN), 0.2 mM dNTP, 0.1 μM of each primer, and 2 U of *Taq* polymerase (Roche Products). For the second amplification, the reaction mixtures contained 3 μl of the first-round PCR product, 1× PCR buffer with Mg^{2+} (Roche Products), 0.5 mM dNTP, 1 μM of each primer, 1 M of Betaine (Sigma), and 2 U of *Taq* polymerase in a final volume of 30 μl. After denaturation by 3 min at 94°C, the target cDNAs were amplified by 35 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 30 s, followed by 10 min at 72°C). As positive control, 1 ng of brain RNA was subjected to RT-PCR in parallel with the single-cell samples. After amplification, the PCR products (532 bp for enolase and 220 bp for MCH) were analyzed on 2% agarose gels. The primers used were as followed: MCH sense, 5′-CAGCTTCCAAGTCCATAAGG-3′; MCH antisense, 5′- AGTGGCAGCCCGTGAGTTAC-3′; enolase sense, 5′-GCTTTGCCCCCAATATCCTG-3′; enolase antisense, 5′-CACAGTCCGACGACAAGATC-3′. The primers were purchased from Invitrogen.

Results

Basic characteristics of LH neurons studied

This report is based on recordings from >300 neurons within a 200 μm radius, lateral to the fornix in acute slices of hypothalamus from WT and α7 nAChR (−/−) mice. We first examined basic electrophysiological features of perifornical (PF) LH neurons to assess the properties of immunopositive MCH neurons within this area. Brief pulses (400 ms) of hyperpolarizing and



	Type 1	Type 2	Type 3
Total	25/51	5/51	16/51
(%)	(49%)	(10%)	(31%)
MCH+	16/21	1/21	5/21
(%)	(76%)	(5%)	(24%)
V_m (mV)	-55.4±0.9	-55.3±1.5	-59.4±1*
R_m(MΩ)	438±46	409±56	413±41
τ (ms)	20±1.3	29±3*	18±2

Figure 1. Characteristics of PF LH neurons. ***A***, Electrical properties of PF LH neurons. PF LH neurons were classified into three groups based on the active and passive membrane properties. Top, Responses of LH neurons to three different depolarizing and hyperpolarizing current steps from the resting membrane potential level in current-clamp mode. Type 1 neurons typically displayed moderate spike accommodation in response to sustained depolarization and were multipolar. Type 2 neurons were distinguished by the presence of a hyperpolarization-activated current and a more prolonged membrane time constant. Type 3 neurons showed tonic firing in response to direct depolarization and had a more hyperpolarized resting membrane potential than type 1 or 2 neurons. Bottom, Summary of the basic characteristics of PF LH neurons. The majority of MCH-positive neurons in PF LH are type 1. Asterisks represent a significant difference between groups ($p < 0.05$). V_m, Resting membrane potential; R_m, input resistance; τ, membrane time constant. Calibration: 50 mV, 0.65 nA, 100 ms. ***B***, After electrophysiological characterization, neurons were injected with dye for relocalization. Example of a type 1 neuron (green) that was immunostained with an antibody to MCH (red). Scale bar, 20 μm. ***C***, Single-cell RT-PCR was used to assess the expression of MCH and neuronal enolase in 25 cells as typically examined in the PF LH. Representative results are shown. Lane 5 is a positive control (total brain mRNA). Lanes 1–3 are positive for both neuronal enolase and MCH mRNA. The cell in lane 4 was neuronal enolase positive but MCH negative.

depolarizing currents (from −200 to 400 pA; step, 50 pA; 400 ms) were applied to the patch pipette with the neuron in current-clamp configuration. The majority of PF LH neurons studied in this manner could be grouped into three classes based on the apparent shape of the cell body and the neuritic arbor confirmed from *post hoc* analysis of dye (Alexa-Fluor 488)-filled cells and on the passive and active membrane properties (Fig. 1*A*). Among the population of recorded neurons, direct injection of depolarizing current elicited firing with moderate accommodation in ~50% of neurons (Fig. 1*A*). This first group, referred to as type 1 neurons, is typically multipolar. Type 2 neurons, which comprised ~10% of the population studied, were distinguished by a transient hyperpolarization-activated current with a more prolonged time constant than type 1 or 3 neurons. Type 3 neurons (31%) displayed continuous firing without spike accommodation during a direct depolarizing pulse and were significantly more hyperpolarized at rest membrane potential than either type 1 or type 2 (Fig. 1*A*).

A subset of the electrophysiologically characterized neurons ($n = 51$) were successfully relocated after the recording session and examined for immunofluorescent staining with an antibody to MCH. Comparison of the electrophysiological profiles, location, shape, and immunohistochemical data revealed that the majority of neurons with a type 1 profile were positively stained for MCH (Fig. 1*B*). Overall, 43% of the neurons that were electrophysiologically characterized and relocalized after immunocytochemical analysis were MCH positive, with the majority (76%) of these MCH-positive neurons conforming to type 1 characteristics. We find a similar fraction of PF LH neurons to be MCH positive by single-cell PCR analysis (Pakkanen et al., 2005). Previous reports from other laboratories have associated the bipolar morphology and tonic firing rate profile as seen in our type 3 neurons with orexin-expressing cells in LH slices (Li et al., 2002; Eggermann et al., 2003; Gao et al., 2003). Also, van den Pol and colleagues demonstrated that MCH-positive neurons in culture, as well as in brain slice preparations, show spike-frequency adaptation with sustained depolarization and responses to hyperpolarizing current that are similar to that of our type 1 neurons (Gao et al., 2003; van den Pol et al., 2004). In addition, we collected samples from 25 individual cells that were within the PF LH and selected based on the criteria delineated above. Single-cell RT-PCR was used to assess the expression of MCH and neuronal enolase (Fig. 1*C*). Together, it appears that the MCH-expressing neurons are readily distinguished from at least two other major classes of neurons within the PF LH, by electrophysiological and morphological criteria.

Glucose and nicotine sensitivity of GABAergic inputs to PF LH neurons

Previous *in vitro* studies demonstrated that GABAergic transmission among LH neurons was modulated by nAChR activation (Jo and Role, 2002). To pursue these observations in the more intact brain slice preparations, we examined nAChR-mediated effects

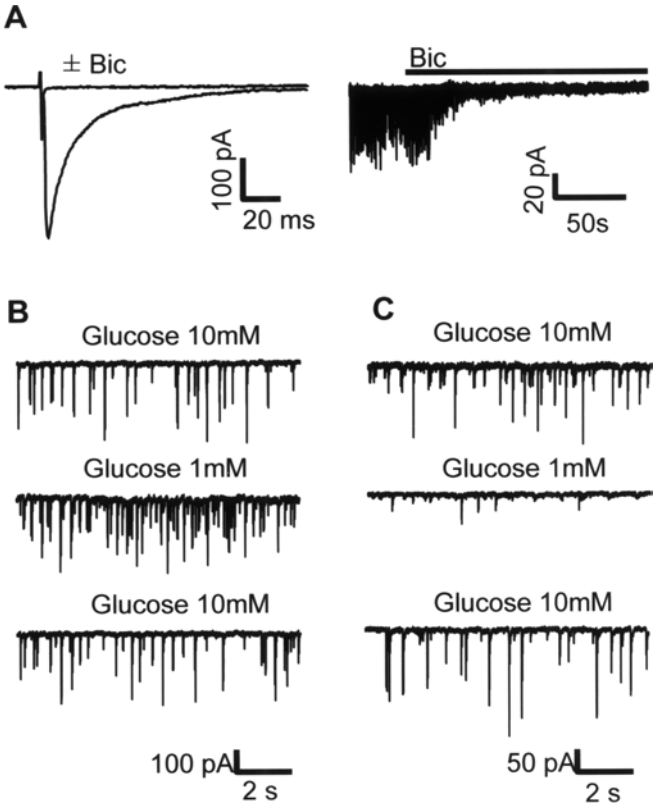


Figure 2. Properties of presynaptic GABAergic inputs to PF LH neurons. ***A***, All recordings shown are in the presence of CNQX and DL-APV to block glutamatergic transmission. The addition of the $GABA_A$ receptor antagonist bicuculline (Bic; 10 μM) completely blocks both the evoked and spontaneous synaptic currents recorded, consistent with their being mediated by $GABA_A$ receptors. ***B***, ***C***, GABAergic inputs are sensitive to the concentration of external glucose. Lowering external [glucose] increased the frequency of GABAergic sIPSCs in approximately half of the neurons tested (***B***) and depressed GABAergic transmission in ~30% of neurons (***C***).

on GABAergic transmission in slice recordings with a mixture of NMDA and AMPA receptor blockers. Under these conditions, all stimulus evoked, spontaneous, and TTX-resistant (miniature) postsynaptic currents detected are sensitive to bicuculline (10 μM), consistent with our monitoring GABAergic transmission in isolation ($n = 16$ neurons) (Fig. 2*A*).

We next assessed how the GABAergic inputs to PF LH neurons respond to peripheral and/or exogenous signals, including glucose and nicotine. If the GABAergic input to PF LH neurons participates in appetite-related neural circuitry, then, based on previous literature, we expect certain profiles of glucose response characteristics of the synaptic transmission in PF LH (Liu et al., 2001; Song et al., 2001; Wang et al., 2004). In these experiments, the external concentration of glucose was lowered from 10 to 1 mM. Lowering $[glucose]_{ext}$ resulted in modulation of GABAergic transmission within <1 min of switching the perfusion media in ~70% of the neurons tested [Fig. 2*B* ($n = 7$ of 15 positively modulated), *C* ($n = 4$ of 15 neurons negatively modulated)]. Thus, the majority of the PF LH neurons studied appear to receive GABAergic inputs that are effected by changes in $[glucose]_{ext}$, consistent with their potential involvement in feeding-related circuits (Song et al., 2001; Wang et al., 2004).

Cholinergic projections within the LH

Having identified a population of PF neurons that receive both glucose- and nicotine-sensitive GABAergic inputs, we next examined whether locally released ACh might modulate synaptic excitability via nAChRs. If endogenous ACh normally participates in the regulation of PF LH synapses, then cholinergic inputs should be detectable in mouse LH (Rao et al., 1987; Tago et al., 1987; Woolf, 1991; Oh et al., 1992). Visualization of immunoreactivity for VAT demonstrated positive staining in fiber-like structures throughout the LH (Fig. 3*B*). In addition to these cholinergic nerve processes, we also detected numerous VAT-positive cell bodies in the area adjacent to the optic tract, along the LH/zona incerta border and near the substantia innominata, as observed previously in other rodent studies (Tago et al., 1987; Grove, 1988; Woolf, 1991).

Examination of the LH area with antibody probes for MCH (Fig. 3*A*,*C*) revealed MCH-positive neurons in a broad swath across the lateral and more medial regions of the lateral hypothalamic area. MCH-positive neurons were particularly plentiful in the PF area of the LH, in which both our recording studies and immunohistochemical identifications of cholinergic fibers were conducted. Figure 3*C* shows higher-magnification views of double immunostaining of a typical PF LH area studied with antibodies against both VAT and MCH. VAT-positive fibers appear in close proximity with MCH-positive as well as MCH-negative neurons.

Having confirmed the existence of endogenous sources of ACh to PF LH neurons, we then tested whether the effect of increasing the local concentration of ACh could alter the overall suprathreshold activity of PF LH neurons. These initial recordings in current-clamp configuration used perforated patch with gramicidin to maintain intracellular Cl^-. Based on results of previous studies of nicotine application in *in vitro* preparations of LH (Jo and Role, 2002), we sought to assess the potential role of nAChRs in the modulation of GABAergic inputs *in vivo* and therefore included blockers of both glutamate receptors and muscarinic AChRs. Under these recording conditions in acute LH slice, we observed that increasing the external concentration of ACh ($\uparrow [ACh]_{ext}$) decreased the firing rate of PF LH neurons at resting membrane potential (Fig. 3*D*). The observed decrease in firing rate of PF LH neurons with elevated $[ACh]_{ext}$ could be attributable to an ACh-induced increase in inhibitory tone and/or the activation of outward currents in the postsynaptic neuron. Subsequent studies attempted to discern the mechanisms by which endogenous ACh might affect the synaptic excitability of PF LH neurons in more detail.

We next tested whether the PF LH synapses in these brain slice preparations, like those we had studied previously in dispersed cell culture, were modulated by direct activation of nAChRs (Jo and Role, 2002). Nicotine (0.5–1 μM) increased the amplitude of evoked IPSCs (eIPSCs) (eIPSCs plus nicotine, 217 ± 10% of control; $n = 5$ of 10) (Fig. 3*E*) and enhanced the frequency of spontaneous IPSCs (sIPSCs) (sIPCS plus nicotine, 178 ± 14% of control; $n = 5$ of 10) (Fig. 3*E*). Thus, GABAergic inputs to PF LH neurons within the intact LH slice are sensitive to nicotine.

Facilitation of spontaneous and evoked GABAergic transmission by endogenous ACh

We next pursued the mechanisms of potential physiological contributions of endogenous activation of nAChR on GABAergic inputs to PF LH neurons using two different approaches. Synaptic transmission was monitored in voltage clamp, and we elevated the $[ACh]_{ext}$ by either inhibition of acetylcholinesterase (AChEI) (Figs. 4, 5) or electrical stimulation in areas of vesicular ACh transporter-positive cell bodies (Fig. 6).

AChE inhibitors vary in their relative affinity, off rate, and selectivity for the AChE (Hodge et al., 1992). In preliminary

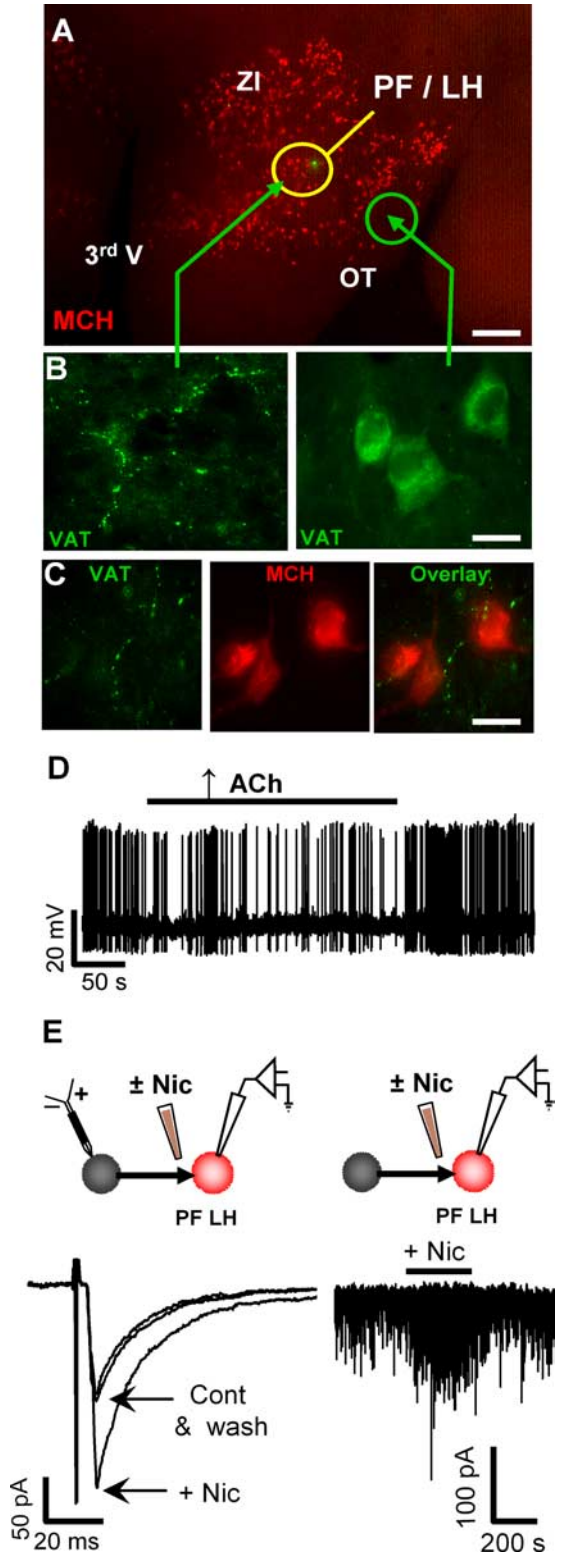


Figure 3. Presence of cholinergic neurons and fibers within the mouse LH. ***A***, MCH-positive neurons (red) are distributed throughout the lateral hypothalamus. The yellow circle indicates the approximate recording area within the PF LH area in all studies (example of a recorded neuron injected with FITC). The green circle indicates the region of cholinergic neuronal cell bodies along the lateralmost border of the LH at which electrodes were placed to stimulate endogenous cholinergic pathways (see Fig. 6). Scale bar, 300 μm. ***B***, VAT-positive cell bodies (green; right) are located within the LH, along the LH border close to the optic tract (OT) and in the neighboring zona incerta (ZI). Cholinergic fibers are evident in the PF LH (green; left). Scale bar, 15 μm. ***C***, VAT-positive immunostained processes appear to contact MCH-positive cell bodies in the PF LH area. Scale bar, 15 μm. ***D***, In the perforated patch-clamp recording using gramicidin to keep the Cl^- concentration intact, an increase of ACh concentration by inhibiting AChE decreases firing rate at the resting membrane potential. V_m of −56 mV. ***E***, Direct application of nicotine facilitates GABAergic transmission at most PF LH synapses. Top, Schematic diagram of recording configuration. Bottom, Nicotine (Nic; 1 μM) increased the amplitude of stimulus-evoked IPSCs (left) as well as increasing the frequency of spontaneous IPSCs (right).

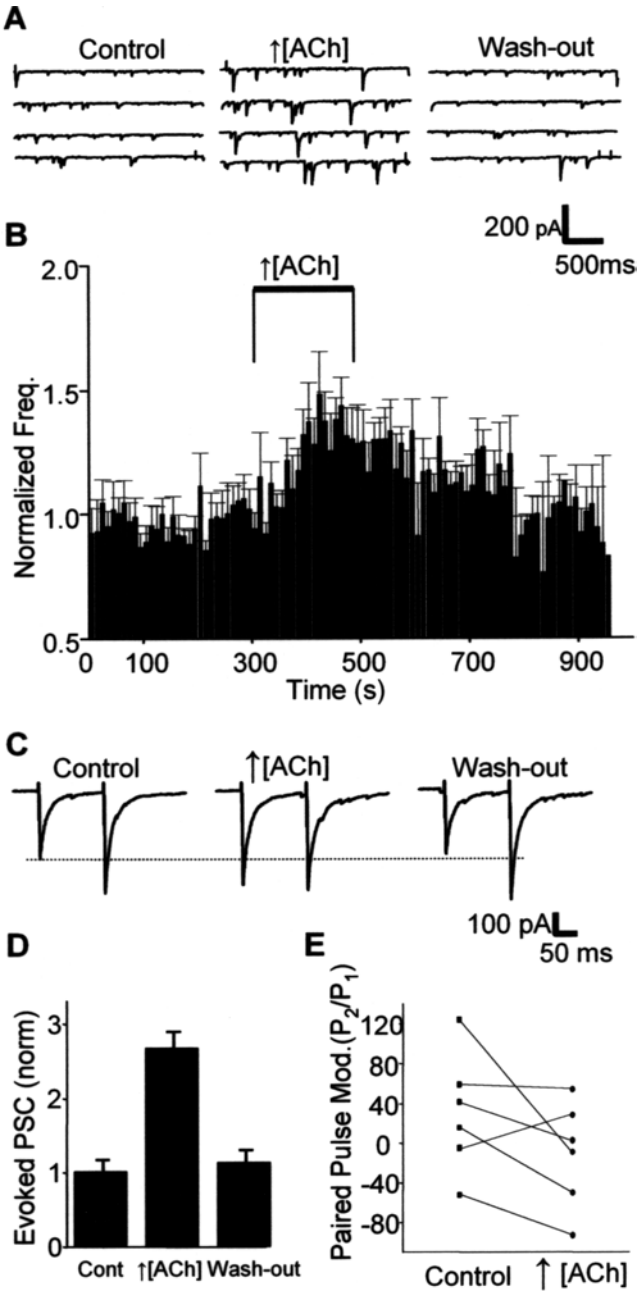


Figure 4. Increasing extracellular acetylcholine levels by AChE inhibition modulates both the spontaneous and evoked GABAergic transmission to PF LH neurons. ***A***, ***B***, Facilitation of spontaneous synaptic transmission by elevated $[ACh]_{ext}$. Sample current traces showing spontaneous GABAergic IPSCs recorded at a holding potential of −70 mV before, during, and after treatment of the slice with the AChE-inhibitor ambenonium at 500 nM (***A***). Pooled event histogram from six separate experiments showing the time course of synaptic current frequency recorded in PF LH neurons before, during, and after treatment of the slice with ambenonium (***B***). Control sIPSC frequency averaged 1.3 ± 0.4 Hz in this series. ***C***, ***D***, Facilitation of evoked synaptic transmission by elevated $[ACh]_{ext}$. Representative recordings of evoked GABAergic IPSCs at a holding potential of −70 mV before, during, and after treatment of the slice with ambenonium (500 nM; ***C***). Each set of traces is the average of five consecutive stimulus pairs under each of the indicated conditions. ***E***, The extent of paired-pulse modulation was also affected by elevated $[ACh]_{ext}$, consistent with involvement of a presynaptic mechanism.

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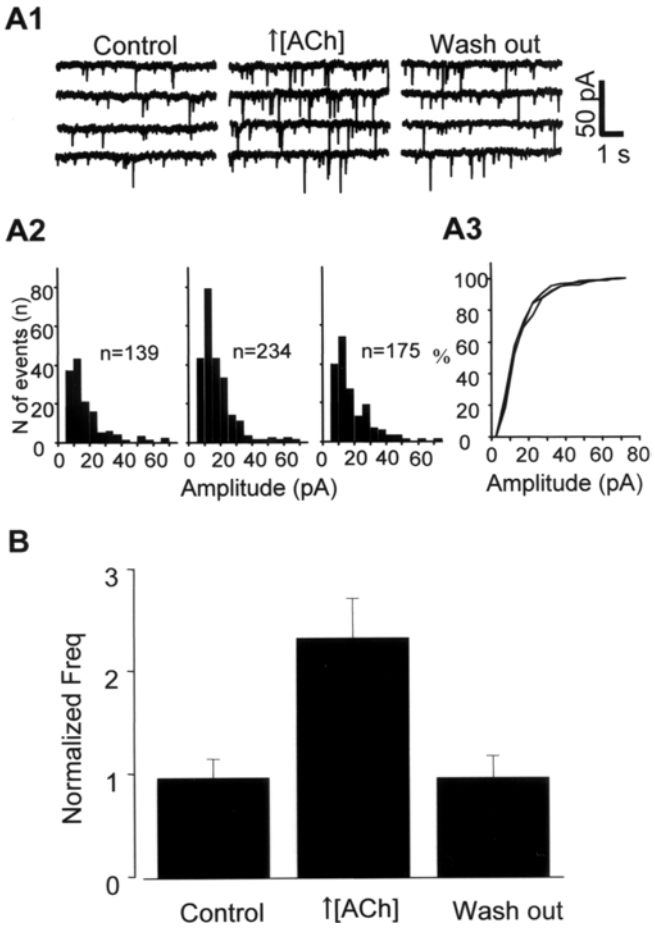

Figure 5. Increasing the ambient concentration of ACh increases the frequency of miniature IPSCs at LH synapses. ***A1–A3***, Enhancement of the frequency without change in amplitude of TTX-resistant miniature GABAergic IPSCs by elevated $[ACh]_{ext}$. Sample current traces showing spontaneous miniature GABAergic IPSCs recorded at a holding potential of −70 mV before, during, and after treatment of the slice with the AChE inhibitor ambenonium at 500 nM (***A1***). Neither the amplitude distribution nor the mean amplitude of mIPSCs was significantly affected (***A2***, ***A3***). ***B***, Increased $[ACh]_{ext}$ enhances the frequency of GABAergic mIPSCs. Summary of spontaneous miniature synaptic current frequency data from all experiments ($n = 14$). Results obtained in the presence or absence of atropine (1 μM) were indistinguishable, consistent with modulation via nicotinic rather than muscarinic AChRs. Control mIPSC frequency averaged 0.6 ± 0.1 Hz in this series.

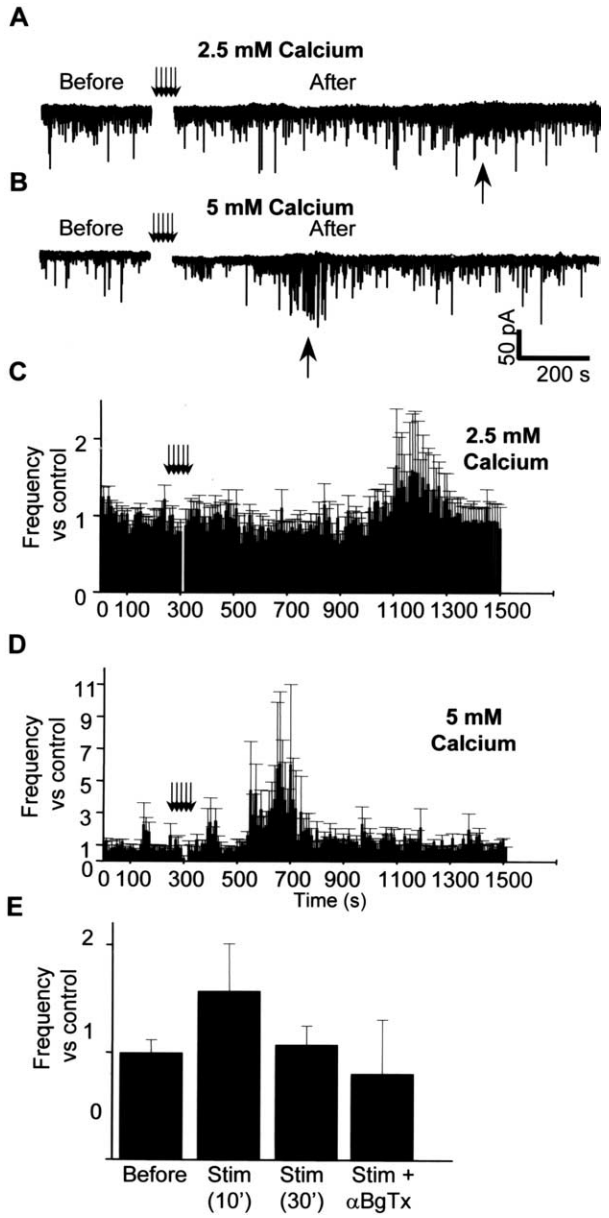

Figure 6. Extracellular stimulation of local cholinergic neurons triggers nAChR-dependent facilitation of GABAergic transmission. ***A***, Extracellular stimulation of nearby cholinergic neurons facilitates spontaneous GABAergic synaptic transmission. Sample current traces showing spontaneous GABAergic IPSCs recorded at a holding potential of −70 mV, before and after extracellular stimulation within the area of cholinergic cell bodies (as indicated in Fig. 3*A*; 50 Hz, 1 s for 5 times; 5 s interstimulus interval; 2.5 mM calcium). ***B***, Modulation of spontaneous synaptic transmission by stimulation of cholinergic neurons in the presence of 5 mM calcium. Sample current traces showing spontaneous GABAergic IPSCs recorded at a holding potential of −70 mV, before and after stimulus burst. ***C***, Pooled event histogram from 15 separate experiments showing the time course of synaptic current frequency recorded in PF LH neurons before, during, and after stimulation within the area of cholinergic cell bodies in LH in the presence of 2.5 mM calcium. The nAChR-mediated increase in GABAergic synaptic transmission occurs at ~10–15 min after the stimulus burst. ***D***, Extracellular stimulation of cholinergic cell bodies is more efficacious in the modulation of spontaneous GABAergic synaptic transmission in the presence of 5 mM calcium. Pooled event histogram from five separate experiments showing the time course of synaptic current frequency in the presence of 5 mM calcium. The nAChR-mediated increase in GABAergic synaptic transmission reaches 10 times the baseline frequency and occurs at ~3–5 min after the stimulus burst. ***E***, Stimulation of cholinergic neurons induces modulation of GABAergic transmission that is mediated by the activation of nAChRs. Summary of synaptic current frequency data from all experiments examining the pharmacology of burst stimulus-evoked modulation. Mecamylamine (data not shown) as well as αBgTx completely block stimulus burst-induced facilitation of GABAergic transmission. Control sIPSC frequency averaged 1.8 ± 0.8 Hz in this series.

work, we examined galanthamine as well as ambenonium effects on synaptic transmission. We chose ambenonium for subsequent studies because of its higher selectivity for AChE per se. In another series of experiments, we used high-frequency extracellular stimulation (50 Hz) of the LH area that includes cholinergic cell groups (Fig. 3) (Rao et al., 1987; Tago et al., 1987; Woolf, 1991; Oh et al., 1992). In all cases, GABAergic transmission was isolated by inclusion of glutamate receptor blockers (see Materials and Methods).

Application of the AChEI ambenonium at 100–500 nM to LH slices increased the frequency of sIPSCs (Fig. 4*A*,*B*). Initial experiments revealed that increased $[ACh]_{ext}$ resulted in an enhancement of GABAergic sIPSCs via activation of nAChRs in both the presence and absence of muscarinic antagonists. In all subsequent experiments, the contribution of nAChRs was isolated from that of nAChRs by inclusion of atropine (0.5–1 μM) (Zwart et al., 1999). Increased $[ACh]_{ext}$ facilitated spontaneous GABAergic IPSCs by 158 ± 1% compared with control; there were no significant changes in the amplitude of these synaptic

currents (control, −53 ± 8 pA; plus AChEI, −56 ± 7 pA; $n = 6$). Approximately half of the GABAergic inputs to type 1 neurons were modulated by increased $[ACh]_{ext}$ as measured by assays of spontaneous IPSCs ($n = 10$). Treatment with AChEI resulted in facilitation of spontaneous GABAergic transmission in all neurons in which 1 mM glucose decreased the frequency of sIPSCs ($n = 4$ of 4 neurons). In neurons in which lowering external glucose concentration caused a facilitation of GABAergic transmission, increasing $[ACh]_{ext}$ was without effect ($n = 0$ of 7 neurons). These data are consistent with the interpretation that ACh modulates a specific subset of glucose-sensitive GABAergic synapses in the PF LH.

We next examined the effects of increased $[ACh]_{ext}$ on stimulus-evoked GABAergic synaptic transmission: evoked IPSCs are also potently modulated by manipulation of $[ACh]_{ext}$ (Fig. 4*C–E*). Paired-pulse stimuli (interval, 150 ms) in the area of inputs to PF LH neurons elicited robust eIPSCs. When paired-pulse stimulation of GABAergic input to PF LH was coupled with increased $[ACh]_{ext}$, the amplitude of the evoked IPSCs was significantly increased in ~30% of neurons tested (6 of 21 neurons; ambenonium, 500 nM) (Fig. 4*C–E*). Treatment with AChEI not only enhanced the average amplitude of eIPSCs (243 ± 42% of control; $n = 6$) but also changed the ratio of paired-pulse modulation (Fig. 4*E*). In contrast, the decay time constant of eIPSCs was not affected (control, 16 ± 2 ms; ambenonium, 17 ± 2ms; $n = 6$ cells; $p > 0.05$). These data are consistent with the interpretation that the enhancement of GABAergic eIPSCs is attributable to nAChR-induced changes in presynaptic function.

Although ACh can facilitate GABAergic transmission, the modulation is independent of superthreshold activity (Fig. 5). In the presence of TTX (1 μM), augmentation of $[ACh]_{ext}$ elicited a reproducible increase in the frequency of miniature IPSCs (mIPSCs) within ~1 min of treatment with AChEI. Neither the amplitude distribution nor the mean amplitude or decay time constants of the mIPSCs were affected by $[ACh]_{ext}$ ($n = 8$; mean amplitude: control, 25 ± 3 pA; plus ambenonium, 23 ± 4 pA; decay time constant: control, 13 ± 2 ms; plus ambenonium, 13.5 ± 2ms; data not shown) (Fig. 5*A*). The mean increase of the mIPSC frequency is 247 ± 58% of control ($n = 8$ of 14) (Fig. 5*B*). Together, our data indicate that an increase of ACh in the synaptic area elicited by inhibition of AChEI is sufficient to produce significant facilitation of ongoing GABAergic synaptic transmission. In view of results obtained in both paired-pulse stimulation experiments and in assays of TTX-resistant spontaneous IPSCs, as well as in the presence and absence of muscarinic receptor antagonists (data not shown), we suggest that the AChR-mediated modulation of GABAergic inputs to type 1, LH/MCH neurons involves nAChR-activated changes in presynaptic aspects of GABA transmission.

A diffuse or nondirected release of ACh may be the principal manner through which cholinergic terminals influence neuronal activity in the CNS (Descarries et al., 1997). Previous studies propose that ACh can diffuse from cholinergic en passant-type terminals, thereby exerting its effects at some distance from the release sites by "volume transmission." To explore potential physiological influences of endogenous cholinergic neurons on the PF LH circuit, we examined the effects of high-frequency stimulation of local cholinergic cell bodies. The stimulation electrode was typically placed ~3 mm from the PF LH neurons selected for recording (as indicated by green circle in Fig. 3*A*). With the high-frequency extracellular stimulation paradigm of 50 Hz, 1 s for five times, with an interstimulus interval of 5 s (see Materials and Methods), we detected an increase in the frequency of sIPSCs in 38% of PF LH neurons tested in slices bathed in 2.5 mM Ca^{2+} containing aCSF (15 of 39 neurons; average enhancement, 156 ± 44% of control; baseline frequency, 2.2 ± 0.7 Hz; mean amplitude: control, 38 ± 7 pA; after, 40 ± 7 pA; $p > 0.05$) (Fig. 6*A*, *C*). A second period of stimulation was also routinely effective in reproducing the observed facilitation of GABAergic transmission (data not shown). Nicotinic ACh receptor antagonists mecamylamine and αBgTx (1 μM; $n = 5$ and 50 nM; $n = 4$, respectively) inhibited the facilitation of GABAergic transmission induced by the stimulus burst (Fig. 6*E*), consistent with the notion that the electrical stimulation induced the release of endogenous ACh, which then enhanced GABA release by activation of nAChRs.

We next examined the effects of applying the same extracellular stimulation paradigm in the presence of 5 mM $[Ca^{2+}]_{ext}$ aCSF to test whether conditions that would increase the release of endogenous ACh might alter the extent and/or time course of nAChR-mediated modulation. In this experimental setting, the same stimulus paradigm induced up to a 10-fold increase in the frequency of sIPSCs without significant alteration of sIPSC amplitude (average enhancement, 554 ± 263% of control; baseline frequency, 2.9 ± 1 Hz; mean amplitude: control, 27 ± 4 pA; after, 31 ± 3 pA; $p > 0.05$; $n = 5$) (Fig. 6*B*, *D*). Likewise, in the presence of 5 mM external Ca^{2+}, the delay between burst stimulation and elevated transmission was considerably decreased, although still substantial (from ~13 to ~5 min delay).

Endogenous ACh-induced facilitation of GABAergic transmission involves activation of α7* nicotinic receptor

To further assess the participation of α7* versus non-α7* nAChRs in the modulation of GABAergic transmission, we assayed AChR-mediated synaptic facilitation in the presence of selective nAChR antagonists and in slices from mice that lack α7 nAChR expression (α7−/−) (Orr-Urtreger et al., 1997). We first confirmed the involvement of nicotinic AChRs per se using broad-spectrum nAChR antagonists that do not discriminate nAChRs subtypes. Mecamylamine (1 μM) completely blocked the facilitation of GABAergic transmission induced by either burst stimulation of cholinergic regions or increasing extracellular ACh by AChE inhibition ($n = 8$; data not shown). To assess the possible contribution of (αβ)* nAChRs, we tested for effects of dihydro-β-erythroidine (DH-β-E) (500 nM) on facilitated GABAergic transmission. Unlike mecamylamine, DH-β-E was without effect on nAChR-mediated modulation ($n = 6$) (Fig. 7*A*). In contrast, the α7-selective antagonist αBgTx (50 nM) completely blocked the facilitation of GABAergic transmission by increased $[ACh]_{ext}$ attributable to treatment with AChE inhibitors ($n = 6$) (Fig. 7*A*) or by extracellular stimulation of cholinergic neurons ($n = 4$) (Fig. 6*E*). In addition, a brief previous exposure of LH slice to low concentrations of nicotine is sufficient to block subsequent effects of AChEI, consistent with nAChR cross-desensitization (nicotine, 100 nM, 1 min) (Fig. 7*A*). The cross-desensitization effect reversed within 15 min of washout (data not shown; $n = 6$).

The involvement of α7* nAChRs in the modulation of GABAergic transmission was further supported by experiments in which we used genetically modified mice that lack α7 nAChR subunit gene expression (α7 KO). In 12 separate experiments on LH slices from α7 KO mice in which baseline GABAergic transmission was similar in amplitude and frequency to +/+ siblings, we never observed AChEI-induced synaptic facilitation (500 nM; $n = 12$) (Fig. 7*A*).

In studies of triple immunostaining in WT slice with αBgTx

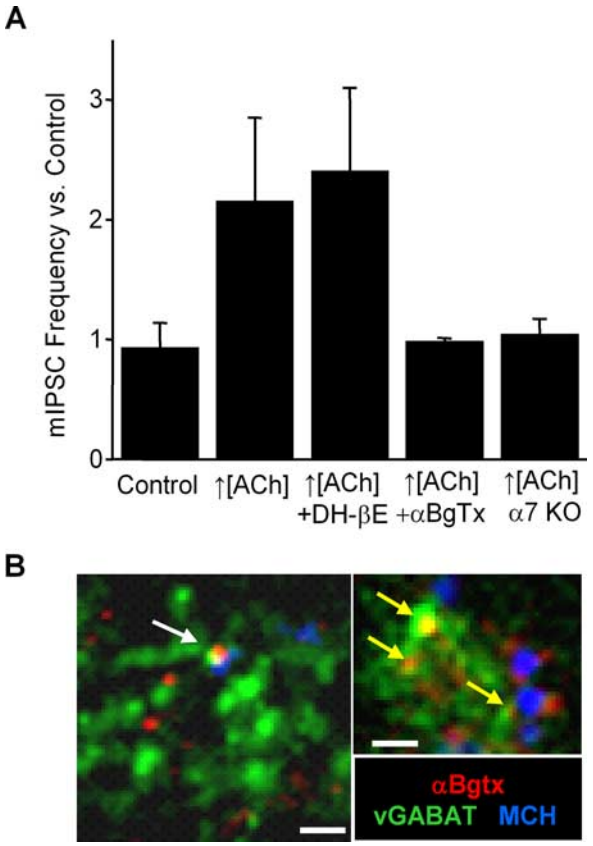

Figure 7. nAChR-induced facilitation of GABAergic transmission involves activation of α7-containing nicotinic receptors. ***A***, Summary of experiments ($n = 39$) examining the pharmacology of nAChR-induced facilitation of GABAergic transmission. The α7-selective antagonist, αBgTx (50 nM), blocked the facilitation of GABAergic transmission elicited by nicotine (500 nM to 1 μM) or by inhibition of AChE (ambenomium; 500 nM), whereas DH-β-E was without effect. Application of AChE inhibitors elicited no detectable change in GABA release in 12 separate experiments on LH slices from α7 KO mice. ***B***, Triple immunostaining was performed with primary antibodies directed against MCH (visualized with secondary antibody coupled to 7-amino-4-methylcoumarin-3-acetic acid; blue), the vesicular transporter for GABA (green), and with a directly conjugated α7 nAChR-selective toxin, αBgTx (red). Structures positive for vGABAT and αBgTx staining (yellow arrows) can be detected in close proximity to MCH-positive dendrites (white arrow). Scale bar, 6 μm

and antibodies for MCH and vesicular GABA transporter (vGABAT), we could detect sites in which both vGABAT-immunopositive and αBgTx-positive structures appeared adjacent to MCH-positive dendrites (Fig. 7*B*). Together, these electrophysiological and immunocytochemical data implicate α7* nAChRs in the ACh-induced facilitation of GABAergic transmission at synapses to the LH/MCH neurons.

Exposure to nicotine during mid-prenatal and early perinatal period exerts long-lasting effects on inhibitory inputs to LH and attenuates nAChR-mediated facilitation of GABAergic transmission

In view of our findings implicating presynaptic nAChR activation in the modulation of GABAergic inputs to LH/MCH neurons, we initiated tests of whether exposure to nicotine during prenatal and early perinatal development would influence the profile of inhibitory transmission in postnatal pups. In studies described above and in previous published work on GABAergic transmission in LH neurons *in vitro*, brief application of nicotine at concentrations of 0.5–1 μM has been shown to elicit a brisk but transient increase in GABAergic transmission (Figs. 3*E*, 7*A*) (Jo and Role, 2002).

Pregnant WT mice were exposed to nicotine from postcoital day 14 through weaning and inhibitory transmission to PF LH neurons was monitored in offspring at P14–P21 to assess the effects of perinatal nicotine exposure. Perinatal nicotine exposure resulted in dramatic differences in baseline GABAergic transmission and abolished the effects of subsequent applications of acute nicotine in the modulation of GABAergic transmission (Fig. 8).

GABAergic transmission in hypothalamic slices of pups from WT control versus WT nicotine-exposed moms was examined by monitoring the frequency and amplitude of sIPSCs. The average baseline frequency of sIPSCs was increased approximately sevenfold in nicotine-exposed groups compared with the control group (control, 2 ± 0.8 Hz, $n = 7$; nicotine-exposed, 13.9 ± 3.5 Hz, $n = 8$) (Fig. 8, compare *A* with *B*, *D*). The mean amplitude of sIPSCs in perinatal nicotine-exposed pups was also increased compared with control (from 33.5 ± 3.4 pA, $n = 7$ cells to 72.1 ± 20.3 pA, $n = 8$ cells) (Fig. 8*D*).

Finally, we compared the effect of brief applications of nicotine on GABA release in prenatal nicotine-exposed versus control pups (Fig. 8, compare *A* with *B*, *E*). Whereas application of nicotine (500 nM to 1 μM) increased GABAergic transmission in control pups, it was without effect in nicotine-exposed animals [Fig. 8*E* (mean change in the frequency, $12 \pm 6\%$; $n = 8$), *C*, *G* (mean increase, $98 \pm 36\%$; $n = 6$)]. These electrophysiological findings complement recent behavioral studies on the effects of prenatal nicotine exposure and nAChR mutant mice (Cohen et al., 2005)

Discussion

There are three principle findings of this study. The first is that MCH-expressing neurons appear to be distinguishable from other perifornical LH neurons by combined analysis of electrophysiology, morphology, immunostaining, and mRNA expression profile. The second major finding of this work is that released ACh modulates the synaptic excitability of the LH/MCH neurons via activation of presynaptic nicotinic AChRs. Finally, initial studies indicate that perinatal exposure to nicotine enhances tonic GABAergic input to LH and attenuates nicotinic modulation of inhibitory transmission in postnatal pups.

Previous studies have demonstrated that activation of presynaptic nAChRs by exogenous application of nicotinic agonists can alter the release of neurotransmitters, including glutamate, GABA, glycine, and dopamine (for review, see Berg and Conroy, 2002; Alkondon and Albuquerque, 2004; Dajas-Bailador and Wonnacott, 2004; Mansvelder et al., 2005) (McMahon et al., 1994; McGehee et al., 1995; Alkondon et al., 1997; Zhu and Chiappinelli, 1999; Wonnacott et al., 2000; Kiyosawa et al., 2001; Mansvelder et al., 2002). In addition, direct nAChR-mediated, fast synaptic transmission occurs in several brain areas (Jones and Yakel, 1997; Alkondon et al., 1998; Frazier et al., 1998; Hefft et al., 1999; Nong et al., 1999). The broad distribution of nAChR-expressing neurons and the diffuse pattern of cholinergic projections throughout the brain contrast with the relatively low levels of total nAChR expression. These characteristics of the nicotinic cholinergic system in the CNS, along with the paucity of classical presynaptic profiles at sites of cholinergic projection, has led to the proposal that ACh released as a "volume transmitter" may be a primary mechanism for nAChR activation in the CNS (Descarries et al., 1997). In this view, tonic released ACh may play an important role in the fine-tuned modulation of synaptic transmission.

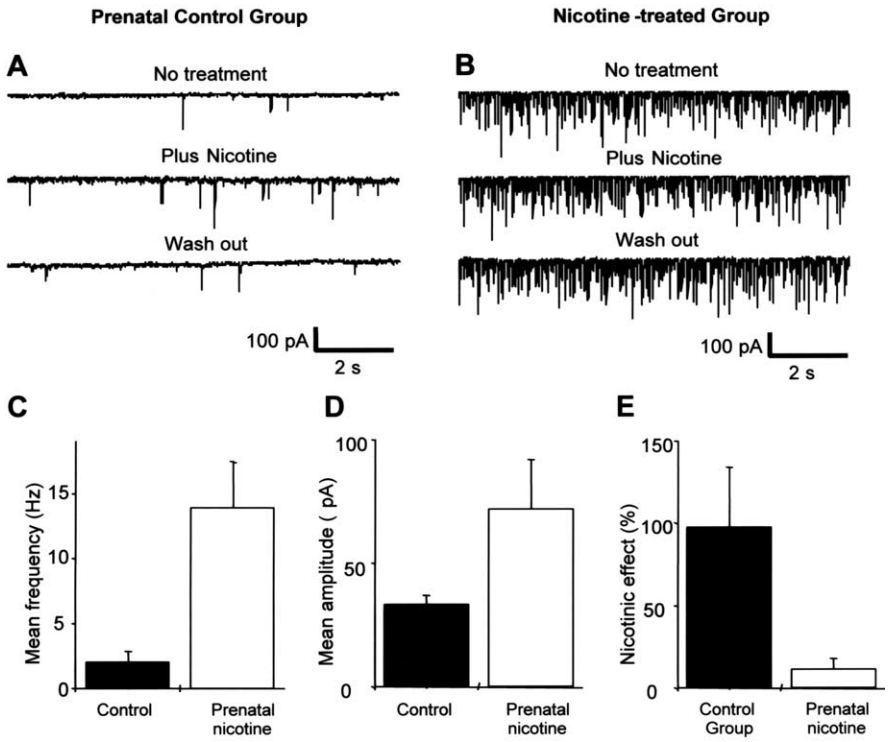

Figure 8. Perinatal exposure to nicotine causes long-lasting increases in tonic GABAergic transmission and ablates short-term nAChR modulation in LH. The effects of perinatal exposure (from postcoital day 14 to weaning) to nicotine were evaluated by recording in slices from 2- to 4-week-old pups. ***A***, Representative recordings from PF LH neurons in acute slice from control group ($n = 7$) showing baseline spontaneous GABAergic transmission (top trace), increased sIPSC frequency elicited by brief application of nicotine (Plus Nicotine; 3 min, 500 nM), and the return to baseline activity 15 min after washout of nicotine (bottom trace). ***B***, Representative recordings from PF LH neuron in acute slice from perinatal nicotine-treated group ($n = 8$) showing baseline spontaneous GABAergic transmission before (top trace), during (middle trace), and 15 min after application of nicotine (Plus Nicotine). Prenatal exposure to nicotine elevates GABAergic transmission and blocks nicotine-induced facilitation. ***C–E***, Summary of the effects of perinatal exposure to nicotine on the baseline frequency (***C***) and amplitude (***D***) of GABAergic sIPSCs. ***E*** summarizes the effects of acute nicotine application in slices from pups exposed to nicotine versus those maintained under control conditions in the prenatal and perinatal periods as delineated in Materials and Methods.

ACh-induced facilitation of GABAergic transmission via α7* nAChRs

A few previous studies have provided evidence for significant effects of endogenously released ACh in the modulation of CNS synapses via nicotinic AChRs (Maggi et al., 2001; Zhou et al., 2001; Mansvelder et al., 2002). In a previous study (Jo and Role, 2002), we studied the effects of direct ACh receptor activation in the lateral hypothalamic synapses *in vitro*. Treatment with nicotine strongly facilitated GABAergic transmission at LH synapses, whereas the activation of muscarinic AChRs depressed GABAergic transmission.

The current study examines the role of endogenous cholinergic systems in nAChR modulation of LH neurons in more intact brain slice preparations. Inhibition of AChE results in an elevation of ACh that, like the application of nicotine, elicits a significant enhancement of GABAergic transmission to LH/MCH neurons. Inhibition of AChE may mimic physiological conditions in which tonic levels of released ACh can facilitate GABAergic transmission. Pharmacological and immunocytochemical studies, as well as tests in genetically modified mice that lack α7 nAChR subunit gene expression indicate that endogenous ACh-induced facilitation of GABAergic transmission involves activation of α7* nicotinic receptors.

High-frequency burst stimulation of regions within and around the LH that include cholinergic cell groups also triggered an nAChR-dependent (αBgTx blockable) facilitation of GABAergic transmission in PF LH. A simple interpretation of the stimulus-evoked, nAChR-mediated facilitation of GABAergic transmission would be that activation of local cholinergic neurons results in increased ACh release, which then activates presynaptic nAChRs on GABAergic projections (Mulle et al., 1992; McMahon et al., 1994; Mansvelder et al., 2002). Stimulus-evoked facilitation was reproducibly blocked by either mecamylamine or αBgTx, indicating that the modulation required nAChR activation. More complex scenarios, however, are indicated by the observation of extensive delays (10 min) between the stimulus burst and nAChR-dependent facilitation of transmission. The prolonged delay was unaffected by poststimulus exposure to TTX, suggesting that the mechanism(s) underlying the delay are action potential independent (data not shown). Blockers of muscarinic AChRs and of both AMPA- and NMDA-type receptors did not effect the delay. In contrast, increasing external Ca (from 2.5 to 5 mM) or perfusion temperature (from ~26 to ~32°C) or lowering perfusion rate decreased the delay, although several minutes still elapsed between stimulation and increased GABAergic transmission. The mechanism of the delay remains a puzzle.

Prolonged delays from stimulus to release of transmitter have been noted in growing neurons and at newly developed synapses (Hume et al., 1983; Young and Poo, 1983; Poo et al., 1985). Long delays after tetanic stimulation is also reminiscent of "silent synapses," a phenomenon attributed to both presynaptic and postsynaptic plasticity (Liao et al., 1995; Malenka and Nicoll, 1997; Gasparini et al., 2000). Our slices were routinely from P14–P17 mice, and the cholinergic projections, although present, may be immature. The burst stimulus used may be of limited efficacy because of immature and/or low probability of release or rapid synaptic fatigue (Zengel and Magleby, 1981; Grinnell et al., 1989). Alternatively, if relatively high levels of ACh release are required for action as a volume transmitter, diffusion and local AChE activity may be rate limiting. A recent study demonstrates that presynaptic α7 nAChRs are often located at extrasynaptic loci in the rat ventral tegmental area and that α7 nAChRs are not directly apposed to cholinergic fibers consistent with their activation by a paracrine mode of acetylcholine release (Jones and Wonnacott, 2004).

Prenatal exposure to nicotine

In vivo exposure to drugs of abuse has been shown to elicit changes in synaptic strength within neuronal circuits associated with reward (Ungless et al., 2001; Melis et al., 2002; Saal et al., 2003). Administration of drugs of abuse, such as cocaine, nicotine, and morphine induces a long-term change of glutamatergic synaptic transmission in midbrain dopamine neurons (Ungless et al., 2001; Saal et al., 2003). *In vivo* ethanol exposure also in-

duces long-lasting potentiation of GABAergic synapses in dopamine neurons (Melis et al., 2002). These previous reports indicate that a single exposure to drugs of abuse could induce long-term changes in synaptic activity.

Despite numerous studies of regulation of the expression and/or function of nAChRs by chronic nicotine exposure *in vivo* and *in vitro* (Yates et al., 1995; Olale et al., 1997; Peng et al., 1997; Ke et al., 1998; Molinari et al., 1998; Wang et al., 1998; Fenster et al., 1999; Buisson and Bertrand, 2001; Gentry and Lukas, 2002; Slotkin et al., 2002; Pakkanen et al., 2005), little information is available about the effect of prenatal nicotine exposure on synaptic activity in the CNS in general and in the hypothalamus in particular. Hence, we sought to define whether prenatal exposure to nicotine alters the inhibitory input to LH or alters cholinergic modulation in the postnatal animal. Our initial studies suggest that offspring of nicotine-exposed pregnant mice may have persistent increases in inhibitory tone in lateral hypothalamic circuits.

Based on current findings and on previous results, we propose that the anorexigenic effects of nicotine exposure may be associated with a decrease in the excitability of LH/MCH neurons via increases in GABAergic inhibitory tone. Consistent with this idea, continuous exposure to nicotine in the prenatal animal enhanced the baseline activity of GABAergic transmission to LH/MCH neurons. Such increased inhibitory tone in the LH could decrease the net excitability of LH neurons associated with motivational aspects of feeding. This is consistent with previous studies showing that *in vivo* exposure to drugs of abuse, including cocaine, nicotine, morphine, and ethanol, enhances glutamatergic as well as GABAergic transmission in reward neural circuit (Ungless et al., 2001; Melis et al., 2002; Saal et al., 2003). The mechanism underlying this upregulation of inhibitory tone remains to be explained. One simple explanation is that the loss of presynaptic nAChRs in nicotine-exposed mice may alter GABA release at LH synapses (Cohen et al., 2005).

Activation of presynaptic nAChRs by ambient ACh strongly enhances GABA release in control preparations. If prenatal nicotine exposure downregulates the expression of presynaptic $\alpha 7^{\star}$ nAChRs, then such increased inhibitory tone may reflect compensatory upregulation in the release of GABA per se. Additional mechanisms may also contribute to the increased GABAergic transmission. For example, perinatal exposure to nicotine may upregulate the number of $GABA_A$ receptors on postsynaptic neurons, unmask silent GABAergic synapses, and/or increase the number of GABAergic release sites.

Numerous studies demonstrate that chronic nicotine exposure results in an upregulation of nAChRs in several preparations (Yates et al., 1995; Olale et al., 1997; Peng et al., 1997; Ke et al., 1998; Molinari et al., 1998; Wang et al., 1998; Fenster et al., 1999; Buisson and Bertrand, 2001; Slotkin et al., 2002). In contrast, we find that pups subject to prenatal nicotine exposure were unresponsive to the effects of postnatal nAChR activation. Together, it appears that prenatal exposure to nicotine can alter the synaptic strength and plasticity of GABAergic inputs to PF LH neurons by increasing inhibitory tone and by attenuating cholinergic modulation of LH synapses.

Potential physiological significance

These studies initiate tests of the hypothesis that appetite-related circuits within the hypothalamus are subject to modulation via the activation of presynaptic nicotinic AChRs. Despite the well known appetite-suppressant effect of nicotine self-administration and the appetite-stimulating effects of nicotine cessation, the mechanisms underlying nicotine effects on feeding remain elusive. We propose that the effects of nicotine administration and its withdrawal may result, at least in part, from nAChR-mediated changes in the balance of synaptic excitability of the LH/MCH neurons for several reasons. First, nicotine administration into the LH significantly decreases food intake (Miyata et al., 1999; Yang et al., 1999; Miyata et al., 2001). Second, nicotine alters the expression of feeding-related neuropeptides and their receptors within the LH (Frankish et al., 1995; Kane et al., 2000; Li et al., 2000a,b; Kane et al., 2001). Third, nicotine treatment elicits both short- and long-term changes in the release of a variety of transmitters in LH (Miyata et al., 1999; Meguid et al., 2000; Li and Pan, 2001; Zhang et al., 2001). Current studies reveal that either direct application of nicotine or increased levels of ACh in the LH facilitates GABAergic transmission. Finally, there is robust enhancement of GABAergic transmission after *in vivo*, prenatal exposure to nicotine. Activation of presynaptic nAChRs on GABAergic interneurons may offset the pro-appetite, orexigenic signaling of endocannabinoids (Jo et al., 2005), favoring a net inhibition of MCH-positive neurons. Such synaptic tuning of LH neurons would explain, at least in part, the anorexigenic activity of nicotine.

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