Long-Lasting Enhancement of Glutamatergic Synaptic Transmission by Acetylcholine Contrasts with Response Adaptation after Exposure to Low-Level Nicotine

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Attempts to mimic synaptic delivery of acetylcholine (ACh) with brief, repetitive pulses of high concentration ACh at synapses of medial habenula (MHN) and interpeduncular nucleus (IPN) neurons *in vitro* elicited temporally distinct facilitation and inhibition of glutamate secretion via nicotinic and muscarinic ACh receptor-mediated pathways, respectively. ACh-induced nicotinic facilitation was sustained for up to 2 hr, whereas muscarinic inhibition was transient. Prolonged exposure to nicotine inactivated nicotinic receptors selectively, thus decreasing the

relative contribution of the facilitatory versus inhibitory influences of ACh. The net effect of ACh in modulating glutamatergic transmission at MHN–IPN synapses may be determined by pre-exposure to nicotine, because the drug appears to switch the balance between the facilitatory and inhibitory actions of ACh.

Key words: neuromodulation; glutamate; acetylcholine; nicotine; presynaptic; nicotinic acetylcholine receptors; medial habenula; interpeduncular nucleus

The cholinergic system in the brain has been implicated in a variety of behavioral and cognitive functions, such as working memory, aspects of learning, attention, and arousal. These actions underlie the interaction of the endogenous neurotransmitter ACh with a variety of receptors that modulate neuronal excitability in networks that receive cholinergic afferents (Wainer et al., 1993; Sarter and Bruno, 1997). However, the cellular mechanisms underlying this neuromodulation are poorly understood. Although muscarinic acetylcholine receptors (mAChRs) are renowned for their effects in cortical regions affected in Alzheimer's disease, nicotinic acetylcholine receptors (nAChRs) may also contribute to cholinergic signaling in the normal and aging brain. Nicotine alters a variety of cognitive and behavioral functions through its specific interaction with nAChRs found within the diffuse terminal fields of central cholinergic projections (Woolf, 1991; Levin, 1992). Activation of nAChRs in circuits related to behavioral reinforcement may underlie the renowned effects of nicotine as an addictive drug (Stolerman and Shoaib, 1991; Schelling, 1992; Stolerman and Jarvis, 1995; Rose and Corrigan, 1997; Mansvelder and McGehee, 2000).

Nicotinic receptors are found in the cell bodies, dendrites, and within the presynaptic domains of neurons. Recent electrophysiological studies have provided direct evidence that nAChRs mediate synaptic transmission at central synapses (for review, see Jones et al., 1999). In addition, nAChRs are targeted to synaptic terminal and preterminal domains, consistent with demonstrated

effects of ACh and nicotine on the release of a wide variety of neurotransmitters (Rapier et al., 1990; Grady et al., 1992; McGehee et al., 1995; Dani and Heinemann, 1996; Gray et al., 1996; McGehee and Role, 1996; Role and Berg, 1996; Wonnacott, 1997; MacDermott et al., 1999).

Nicotine interaction with nAChRs facilitates the induction of long-term potentiation of glutamatergic neurotransmission in the hippocampus (Fujii et al., 1999), as well as in the ventral tegmental area (Mansvelder and McGehee, 2000). Whether similar mechanisms involving the activation of nicotinic receptors underlie the CNS effects of the endogenous ligand ACh remains to be determined. In addition, little is known of how prolonged exposure to nicotine might alter the net effect of ACh at central synapses in which transmission normally involves activation of both nicotinic- and muscarinic receptor-mediated pathways.

In this study we investigated the modulation of glutamate secretion by ACh and nicotine at synapses between medial habenula (MHN) and interpeduncular nucleus (IPN) neurons *in vitro*. The MHN–IPN pathway is part of the limbic system thought to be involved in arousal and reinforcement (Wilson et al., 1972; Wirtshafter, 1981). Glutamate serves as the excitatory transmitter at MHN–IPN synapses (Brown et al., 1983; McGehee et al., 1995). We used different patterns of nicotinic receptor agonist delivery in an attempt to mimic the modulation of cholinergic MHN–IPN synapses in the brain. Our results suggest that nicotine obtained from tobacco and ACh released at synaptic sites evoke distinct forms of neuromodulation of glutamate release that differ in both duration and magnitude. Furthermore, prolonged exposure to nicotine that mimicked habitual smoking inactivated nAChRs, thus profoundly altering the neuromodulatory effects of the endogenous transmitter.

MATERIALS AND METHODS

Cocultures. MHN and IPN were dissected from embryonic day 11 (E11) to E13 chicken embryos. Dispersed IPN neurons were cocultured with same-age MHN microexplants. Methods were as described previously

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(Role, 1988; Gardette et al., 1991; Brussaard et al., 1994), except that the MHN microexplants were plated 24 hr before the IPN neurons.

Electrophysiology. Cells were visualized on a phase-contrast inverted microscope (Zeiss IM 135 TV; Zeiss, Oberkochen, Germany). The cultures were continuously superfused with recording medium with a four-port perfusion nozzle. The external solution included (in mM): 145 NaCl, 2.5 KCl, 0–1 MgCl₂, 2.5 CaCl₂, and 10 HEPES, pH 7.4. An IPN neuron, visually confirmed to be contacted by processes emerging from the MHN microexplants, was selected and membrane currents were measured using the whole-cell perforated-patch configuration (Hamill et al., 1981) with amphotericin-permeabilized patches as described previously (Horn and Marty, 1988; Rae et al., 1991). The intracellular solution included: 53 mM KCl, 75 mM K₂SO₄, 5 mM MgCl₂, 10 mM HEPES, and 200 μ M amphotericin, pH 7.2. Voltage-clamp recordings were performed using a List EPC-7 patch-clamp amplifier (Medical Systems Corp., Greenvale, NY). The spontaneous activity was recorded in extracellular solution supplemented with tetrodotoxin (TTX) (1 μ M; Calbiochem, La Jolla, CA) to block Na⁺ action potentials. Membrane currents were recorded continuously with a videocassette recorder (Sony, Tokyo, Japan) through a PCM interface (Digidata 1200; Axon Instruments, Foster City, CA). The currents were digitized with the pClamp 7.0 software (Axon Instruments). Spontaneous synaptic currents (minis) were detected from the digitized traces, and their amplitude, rise time, decay time, and frequency were computed with the Mini Analysis Program (Synaptosoft Inc., Leonia, NJ).

Drug application. The agonists and antagonists were applied by switching the perfusion medium with a solenoid valve-activated delivery system. Control experiments were performed by switching the regular medium to a medium diluted 10-fold with water and by measuring the resulting change of junction potential at the tip of a patch electrode immersed in the recording bath. It was found that complete exchange of medium at the site of recording occurred within <0.5 sec. Nicotine and acetylcholine were obtained from Sigma (St. Louis, MO).

Effects of prolonged exposure to nicotine were studied by adding the drug to established synaptic cocultures of MHN–IPN (1 μ M nicotine, 1–3 d after coculture). After 24–72 hr, the treatment media was removed, and electrophysiological recordings were performed in control solution. Results obtained with 24 and 72 hr nicotine treatment did not differ significantly and, as such, these data were pooled. Recovery from prolonged exposure to nicotine examined synaptic cocultures exposed previously to 1 μ M nicotine for 48 hr, after repeated exchange with 10 times the incubation volume by nicotine-free culture medium. Cultures were subsequently maintained for 3–24 hr in control, MHN–IPN conditioned media (Gardette et al., 1991).

Statistical assay of synaptic facilitation. Statistical methods were used to identify synapses that manifested significant changes in transmission after application of either ACh or nicotine and to compute the time course and magnitude of synaptic facilitation. The mini frequency was sampled for each innervated neuron as the number of minis occurring during sequential periods of 10 sec. The frequency bins were normalized to the average frequency calculated from at least 5 min of control recording time before agonist application. The average $\pm$ SEM of three or six of these frequency bins was calculated to measure the average frequency during the initial, 1 min or longer intervals after experimental treatment. Significance of changes in mini frequency were compared for equal sample size and were evaluated by Student's *t* test. Such analysis provided an unbiased assay of whether a synapse was cholinergic, and, if so, of the time at which mini frequency was significantly changed from baseline (delay for onset of facilitation), as well as of the time at which the frequency returned to the basal level (duration of facilitation).

RESULTS

Synaptic facilitation by nicotinic AChRs-mediated pathways

Patch-clamp recordings of IPN neurons were used to monitor synaptic activity at MHN–IPN synapses *in vitro*. The recordings were performed in the presence of TTX (1 μ M), sufficient to block the occurrence of superthreshold activity. The remaining TTX-resistant synaptic activity was abolished by concurrent treatment with the glutamate receptor antagonists CNQX and APV (McGehee et al., 1995). Thus, the synaptic currents recorded in TTX-treated MHN–IPN preparations reflected the spontaneous secretion of glutamate (i.e., minis). Before application of ACh or

nicotine, the basal mini frequency was approximately one to two events per second. The average amplitude of miniature synaptic currents was 10–20 pA (holding potential of -70 mV), with rising phases <3.0 msec and decay rates fit by single exponentials with τ values of ~ 3 –6 msec. Consistent with some well established features of spontaneous release, increasing extracellular tonicity or adjusting extracellular K⁺ to 40 mM enhanced the frequency but not the amplitude of minis recorded (data not shown).

To investigate the neuromodulatory effects of ACh and nicotine on glutamate release, we monitored agonist-induced changes in the frequency of minis at MHN–IPN synapses. After a period of control recording of >5–15 min, the natural delivery of ACh as it likely occurs *in vivo* was approximated by applying short-duration, repetitive pulses of high concentrations of ACh to sites of neuron–neuron contact (100 msec, 50–200 μ M ACh, 7 pulses at 0.2 Hz). ACh was removed between applications by local microperfusion in <0.5 sec. In contrast, the more prolonged and low levels of nicotine, such as those achieved in arterial blood during smoking, were modeled by applying low concentrations of nicotine (0.5–1 μ M) continuously for 1 min (Benowitz et al., 1989). Both application paradigms elicited statistically significant modulation of synaptic transmission at a subset of MHN–IPN synapses (cholinergic synapses). Phasic application of ACh elicited a significant facilitation of TTX-resistant activity at 58% of MHN–IPN synapses (total number of responsive synapses divided by the total number of connections recorded), whereas significant inhibition of transmission was recorded at $\sim 20\%$ of the synapses ($n = 24$). In contrast, the effects of nicotine were strictly facilitatory ($\sim 40\%$ of tested synapses; $n = 44$) (Fig. 1). The observation that ACh and nicotine did not elicit significant changes in mini frequency at all of the MHN–IPN synapses tested, as well as the range of effects elicited by cholinergic agonists at responsive synapses (see below), suggest that the population of synapses assayed is not homogeneous (McGehee et al., 1995). As such, our criteria for significance in cholinergic responses may result in an underestimate in the actual number of responsive synapses.

Synaptic current analysis before and after either nicotine or ACh treatment revealed that they were no significant changes in mini amplitude at the majority of synapses with statistically significant changes in mini frequency (data not shown). This finding is consistent with previous reports of nAChR-mediated presynaptic facilitation rather than a change in postsynaptic responses to glutamate (McGehee et al., 1995; Alkonon et al., 1996; Gray et al., 1996; Guo et al., 1998). Facilitation produced by the phasic application of ACh was typically larger in magnitude and more persistent than the synaptic facilitation elicited by continuous nicotine (Fig. 1, compare *A*, *B*).

Temporal differences between the responses to ACh and nicotine application were the most obvious (Fig. 2). The delay for onset of facilitation (i.e., the time elapsed between the beginning of agonist application and the time at which a statistically significant increase in the mini frequency was detected) was typically longer for ACh than for nicotine. Thus, with nicotine, 83% of facilitated synapses responded during application of the drug. In contrast, with ACh, less than half of the cholinergic synapses exhibited significant changes in mini frequency within 1 min of agonist application (Fig. 2*Ai*). The average delay in onset of facilitation was 0.7 ± 0.2 min with nicotine versus an average delay of 1.5 ± 0.5 min with ACh (Fig. 2*Bi*). The difference in average delay observed with the two agonists was statistically significant.

Figure 1. Facilitation of glutamatergic neurotransmission by synaptic-like pulses of acetylcholine and by “smokers” concentrations of nicotine. *A*, The electrophysiological traces in *Ai* depict glutamatergic minis recorded at an MHN–IPN synapse in culture, before and after the synapse had been challenged with seven 100 msec pulses of 200 μ M ACh applied at 0.2 Hz. The frequency plots in *Aii* and *Aiii* illustrate experiments performed at two distinct MHN–IPN synapses. They show the number of minis per 10 sec intervals, before and after ACh application. Note the different time scales for the two plots. The recordings in *Ai* were taken from the experiment illustrated in *Aiii*. Asterisks indicate the time on the frequency plot at which the electrophysiological traces in *Ai* were sampled. *Aii* illustrates a synapse in which pulsatile ACh elicited immediate increase in the mini frequency, which was followed by a second episode of facilitation that lasted $\sim$ 10 min. *Aiii* depicts a synapse in which facilitation was protracted and lasted up to 2 hr after ACh application. *B*, At two representative MHN–IPN synapses, application of a 1 min pulse of 0.5 μ M nicotine (*Nic*) enhanced the frequency of the minis. In *Bii*, the frequency peaked and then rapidly declined before the end of nicotine application, whereas in *Biii*, the frequency remained elevated during and up to $\sim$ 1 min after the end of the nicotine challenge. No protracted facilitation was seen with nicotine. The electrophysiological traces in *Bi* are taken from the experiment illustrated in *Biii*. Asterisks as in *A*.

The rise to peak facilitation and return to baseline mini frequency was relatively rapid with nicotine. Most nicotine-facilitated synapses reached peak frequency levels within 2 min of drug application (83%) (Fig. 2*Aii*), and the average time-to-peak for nicotine-induced facilitation was 1.3 ± 0.3 min (Fig. 2*Bii*). In contrast, only 50% of ACh-facilitated synapses reached a maximum increase in mini frequency within 2 min (Fig. 2*Aii*), and the average time-to-peak facilitation was 7.0 ± 2.7 min (Fig. 2*Bii*), a value significantly larger than that found with nicotine.

Facilitation produced with ACh lasted much longer than facilitation induced by nicotine. With nicotine, the mini frequency remained elevated for $\sim$ 0.5–10 min after drug application. On average, the increase in mini frequency returned to baseline within 3.2 ± 0.7 min of nicotine application (Fig. 2*Biii*), and the duration of significant synaptic facilitation never exceeded 10 min (Fig. 2*Aiii*). In contrast, ACh-elicited facilitation lasted an average of 27.5 ± 10.6 min (Fig. 2*Biii*). Transmission remained significantly elevated above control levels at $>40\%$ of cholinergic synapses 10 min after ACh application. At 21% of cholinergic synapses, facilitation lasted for >1.5 –2 hr (i.e., as long as we were able to reliably monitor activity) (Fig. 2*Aiii*, *Ciii*).

The facilitation elicited by ACh tended to be somewhat larger

than that evoked by nicotine at most MHN–IPN connections. The average percentage change in frequency for ACh peaked at $557 \pm 159\%$, whereas that for nicotine was $398 \pm 132\%$ above the basal level of secretion (Fig. 2*Biv*). Although differences in the average facilitation elicited by ACh versus nicotine were not statistically significant, the cumulative frequency distributions are broad. Thus, $>70\%$ of MHN–IPN synapses facilitated by nicotine were enhanced by less than twofold, whereas ACh elicited more than fivefold facilitation at the majority of synapses tested (Fig. 2*Aiv*).

Prolonged increases in mini frequency by ACh were also observed at synapses treated with the muscarinic antagonist atropine (Fig. 2*Civ*), consistent with a nicotinic receptor-mediated mechanism of sustained facilitation. Analysis of the subset of synapses that exhibited more rapid facilitation by ACh revealed an initial episode of facilitation, similar in magnitude and duration to that induced by nicotine, preceding the second phase of more sustained increased mini frequency (Fig. 2, compare *Ci*, *Cii*).

Synaptic depression by muscarinic AChRs-mediated pathways

At 21% of synapses assayed, ACh elicited a temporally distinct phase of synaptic depression during which a net decrease in mini

Figure 2. Quantitative analysis of the time course and magnitude of ACh- and nicotine-induced facilitation. **A**, The graphs depict cumulative histogram distributions of time course parameters and maximal value for ACh- and nicotine-induced facilitation. In **A_i**, the delay for onset of facilitation is the time elapsed between the beginning of agonist application and the time when significant increase in mini frequency was observed. Each data point on the plot is the cumulative percentage of synapses for which the delay in minutes was shorter than or equal to a given value indicated on the *abscissa*. The other plots in **A_{ii}–A_{iv}** were constructed in a similar manner. Right shifts of the curves indicate larger values of ACh-induced facilitation compared with nicotine-induced facilitation. These parameters were calculated at individual responsive synapses as described (see Materials and Methods). **B**, The column plots are average $\pm$ SEM of the same parameters as those depicted in **A**. Asterisks indicate significant difference for nicotine versus ACh-induced facilitation. **C**, The graphs depict compound averages of normalized mini frequency measured at synapses that responded either to ACh or nicotine. For each synapse, the frequency was sampled as the number of minis occurring in 20 sec bins in **C_i** and **C_{ii}** and in 1 min bins in **C_{iii}** and **C_{iv}**. Before averaging, the frequency at individual synapses was normalized to the mean frequency observed before agonist application. Each data point depicts average $\pm$ SEM normalized frequency. In **C_i** is shown the average normalized frequency of 18 synapses (of a total of 44 synapses) that responded to 1 min of 0.5–1 μ M nicotine. In **C_{ii}** is shown the average normalized frequency of eight synapses selected for their immediate response to seven 100 msec pulses of 200 μ M ACh (delay for onset of facilitation $\leq$ 2 min). In **C_{iii}** is shown average $\pm$ SEM normalized frequency of all synapses (14 of a total of 24 synapses) that responded to ACh. The value at 90 min is the average of 3 of 14 responsive synapses that were still facilitated at this time. In **C_{iv}** is depicted average $\pm$ SEM normalized frequency of four synapses that responded to ACh (of a total of 8 synapses) in the presence of atropine 0.5–1 μ M.

frequency was evident (Fig. 3). At this subset of synapses, mini frequency was decreased rapidly and repeatedly within seconds of ACh application. Recovery from inhibition was rapid, and activity returned to control within 10–20 sec after the end of ACh application (Fig. 3*A–C*). ACh-elicited inhibition of glutamate release was abolished by low concentrations of atropine (500 nM to 1 μ M) (Fig. 3*A,D*), and inhibition was never observed when nicotine was used as agonist. These data are consistent with inhibition of release by activation of presynaptic muscarinic receptors as reported previously (Marchi and Raiteri, 1989; Segal, 1989; Sheridan and Sutor, 1990; Vidal and Changeux, 1993; Barral et al., 1999; Rouse et al., 1999). At a subset of cholinergic synapses (13%), ACh produced an initial decrease and then an increase in mini frequency (Fig. 3*C*). Concomitant inhibition and facilitation of synaptic transmission may arise from coordi-

nate activation of presynaptic muscarinic and nicotinic AChRs on MHN inputs to IPN.

Effects of prolonged exposure to nicotine

The results presented above are consistent with the involvement of both nicotinic and muscarinic AChRs in the modulatory effects of ACh at MHN–IPN synapses. Hence, the net effect of ACh will depend (at least in part) on the relative contribution of these two general classes of ACh receptors at individual synapses. Continuous exposure to low-level nicotine can cause reversible desensitization, permanent inactivation, and increase in the number of surface nAChRs. The extent of these effects depends on the receptor subtypes and the cell type in which the receptors are expressed (Wonnacott, 1990; Peng et al., 1994; Dani and Heinemann, 1996; Olale et al., 1997; Peng et al., 1997; Wang et al., 1998;

Figure 3. Inhibition of glutamatergic neurotransmission by synaptic-like pulses of acetylcholine. **A**, The frequency plot depicts an experiment in which seven 100 msec pulses of ACh 200 μ M given at 0.2 Hz produced transient inhibition of synaptic transmission at an MHN–IPN synapse. A second round of ACh stimulation applied in the presence of 1 μ M atropine failed to inhibit transmission. **B**, The electrophysiological traces were sampled from the experiment shown in **A**. They illustrate minis recorded before (top 2 traces), during (middle 2 traces), and after (bottom 2 traces) application of ACh in the absence of atropine. **C**, Average normalized mini frequency of four synapses (of a total of 24 synapses examined) that were inhibited by the pulses of ACh. Note that, at the end of the episode of inhibition, facilitation became apparent, indicating the contribution of both nicotinic and muscarinic receptors. Frequency bin, 10 sec. **D**, Average normalized mini frequency of eight synapses that were recorded in the presence of 0.5–1 μ M atropine. None of these synapses exhibited detectable synaptic inhibition and were thus averaged for the plot. Some nicotinic synaptic facilitation was apparent during the pulses of ACh.

Kuryatov et al., 2000). To examine the net effects of continuous nicotine on responses to subsequent application of nicotine and ACh, we exposed MHN–IPN synapses to nicotine for sustained periods of time as described in Materials and Methods. Synaptic transmission in response to subsequent challenges with either acute nicotine or ACh was assayed within the first 3 hr after removal of the drug and replacement in nicotine-free recording media. In this set of experiments, we calculated the average rate of responsiveness to nicotinic agonists within platings as described in Figure 4. In a subset of control cultures, an average of $51 \pm 6\%$ synapses was thus found to respond to acute nicotine (Fig. 4*A,G*; an evaluation of between-sample vs within-sample variance performed on the entire pool of synaptic connections compared with that of this subset of connections revealed no significant difference). After prolonged exposure to nicotine, the average rate of responsiveness was significantly reduced ($19 \pm 8\%$) (Fig. 4*B,G*).

Because recovery of presynaptic facilitation did not occur within the first 3 hr after nicotine treatment, we tested whether longer recovery times in nicotine-free medium would return responsiveness to pretreatment levels. Synaptic cocultures, previously exposed to nicotine for 48 hr, were allowed to recover in control medium for >3–24 hr before assay (Fig. 4*G*). After ~5 hr in a nicotine-free environment, there was little recovery of nicotine-elicited synaptic facilitation or of the percentage of nicotine-responsive synapses. Full recovery to “pretreatment” control levels of nicotine-induced synaptic facilitation required 24–27 hr of “drug-free” conditions (percentage of nicotine-responsive synapses, 48 ± 8 vs $51 \pm 6\%$ in control).

Facilitatory responses to ACh (of both short and long duration) were similarly susceptible to the apparent inactivation of nicotinic AChRs by prolonged exposure to nicotine. ACh-elicited increases in mini frequency were detected at only $19 \pm 12\%$ of MHN–IPN synapses subjected to prolonged nicotine treatment (compared

with $60 \pm 14\%$ of sibling control synapses) (Fig. 4*C,D,H*). In contrast, muscarinic AChR-mediated effects were unaltered by nicotine exposure. Thus, the temporal profile and extent of synaptic inhibition elicited by ACh was equivalent in control and nicotine-treated conditions (Fig. 4*E,F,H*). The net effect of prolonged nicotine exposure, therefore, is a significant decrease in the proportion of synapses that are facilitated versus those that are depressed by subsequent challenges with ACh (Fig. 4*H*).

Sustained nicotine was without significant effect on other parameters of glutamatergic synaptic transmission at MHN–IPN synapses. Neither the basal frequency, average amplitude, amplitude distribution, nor rise or decay time of glutamatergic minis were altered by prolonged exposure to nicotine (Table 1). Likewise, nicotine did not significantly affect either the amplitude or kinetics of currents recorded in IPN neurons in response to direct application of glutamate (300 μ M) (Table 1).

DISCUSSION

ACh, delivered in repetitive high-concentration pulses, can elicit both facilitatory and inhibitory neuromodulation of glutamatergic synapses between MHN and IPN neurons. Initially, ACh-elicited facilitation matches the typically monophasic and short-lived effects of nicotine delivered as when inhaled through smoking. Second, and perhaps most importantly, ACh can elicit sustained enhancement of glutamate transmission, an effect that also appears to be initiated by interaction with nicotinic AChRs. At most cholinergic synapses ACh elicited facilitation that lasted for ~30 min. At 21% of responsive synapses facilitation was persistent, lasting for >1 hr after application of a single set of ACh pulses. Finally, phasic application of ACh, but not nicotine, also elicits significant inhibition of synaptic transmission at a subset of MHN–IPN synapses. Synaptic inhibition appears to involve the activation of muscarinic AChRs and was relatively transient.

Figure 4. Prolonged exposure to nicotine inactivates presynaptic nicotinic AChRs but does not affect muscarinic receptors. The frequency plots illustrate recordings performed in control conditions and after prolonged exposure to nicotine. Note the different time scales in these graphs. They depict average normalized frequency: *A*, 12 control synapses that responded to acute nicotine; *B*, 19 synapses in which acute nicotine failed to elicit synaptic modulation after prolonged treatment with nicotine (in the inset are depicted 5 treated synapses that responded to acute nicotine); *C*, 10 control synapses that responded to pulsatile application of ACh; *D*, 12 treated synapses that were unresponsive to ACh (the inset illustrates 3 treated synapses that exhibited responsiveness to ACh); *E*, four control synapses that were inhibited by ACh; and *F*, three synapses treated with prolonged nicotine that were inhibited by ACh. Note that, in contrast to control synapses (*E*), some level of facilitation did not follow inhibition after prolonged exposure to nicotine. In *G* and *H*, the data are shown as percentage of synapses that were modulated by nicotine or ACh, respectively. Columns depict the average $\pm$ SEM percentage of synapses facilitated by nicotine and either facilitated or inhibited by ACh under the following conditions: in control coculture (*Contr*); after 24–72 hr treatment with 1 μ M nicotine (*Nic*); after 48 hr treatment with the drug followed by 5 hr recovery in nicotine-free media (*R 5 hr*); and after 48 hr treatment with the drug, followed by 24 hr recovery (*R 24 hr*). To calculate the average percentage of responsive synapses, the number of synapses that responded to nicotine or ACh was divided by the total number of recorded synapses in each culture plating. Then, the ratios in three to six different culture platings for each condition were averaged. For convenience, upward columns illustrate the percentage of synapses that were facilitated in each condition, whereas downward columns illustrate the percentage of synapses that were inhibited.

Nicotinic ACh receptor-mediated facilitation

Our protocol for ACh delivery was intended to mimic secretion of ACh from active cholinergic nerve endings. Obviously, the exact profile of agonist exposure experienced by cholinergic receptive sites in the IPN is unknown and our protocol reflects a “best-guess” based on limited *in vivo* data on central cholinergic activity. Phasic, rather than continuous, application of ACh is likely to better approximate ACh release during activation of cholinergic neurons. Different subsets of cholinergic neurons have been found to discharge intermittent bursts of action potentials (Khateb et al., 1992; Alonso et al., 1996; Manns et al., 2000), and ACh released from cholinergic nerve endings is rapidly degraded

by acetylcholinesterase. Morphological evidence consistent with a presynaptic or perisynaptic release of ACh has been provided in cortical and limbic areas of the brain (Woolf, 1991; Lubin et al., 1999). Pulsatile delivery of ACh producing persistent facilitation of glutamatergic transmission likely occurs at sites of MHN–IPN contact *in vivo*, because the IPN receives the most abundant cholinergic innervation in the brain, arising from cholinergic nuclei located within the basal forebrain, the brainstem, and from cholinergic neurons within the MHN per se (Woolf, 1991).

This study also attempted to model the effects of nicotine because it may be delivered with both “acute” (i.e., when smoking a single cigarette) and prolonged (i.e., in habitual smokers) ad-

Table 1. Prolonged nicotine did not alter basal spontaneous glutamate secretion

	Basal secretion of glutamatergic minis				Response to 300 μ M glutamate
	Average frequency (minis/5 sec)	Average amplitude (pA)	Average rise time (msec)	Average decay time (msec)	Average peak current (pA)
Control	10.1 $\pm$ 2.1	13.0 $\pm$ 0.8	1.2 $\pm$ 0.1	3.6 $\pm$ 0.7	319 $\pm$ 43
Prolonged nicotine	6.6 $\pm$ 1.4	13.2 $\pm$ 0.9	1.3 $\pm$ 0.1	4.2 $\pm$ 0.6	272 $\pm$ 44
Recovery 5 hr	6.3 $\pm$ 2.3	12.3 $\pm$ 1.7	1.1 $\pm$ 0.1	3.1 $\pm$ 0.6	ND
Recovery 24 hr	6.8 $\pm$ 1.9	13.8 $\pm$ 1.6	1.2 $\pm$ 0.2	2.7 $\pm$ 0.3	ND

The average frequency of minis recorded before the challenge with nicotine or ACh (basal minis), their average amplitude, rise time, and decay time were unchanged by prolonged exposure with 1 μ M nicotine or during washout in drug-free media (recovery). Peak currents measured in voltage-clamped IPN neurons in response to 3–5 sec pulses of 300 μ M glutamate were not affected by prolonged nicotine exposure. ND, Not determined.

ministration by cigarette smoking. Smoking results in gradual increases and decreases of arterial nicotine concentration, which are likely to sum with repeated administrations (i.e., with each “puff” and with multiple cigarettes) because the turnover of nicotine is relatively slow. The maximal drug concentrations achieved are thought to be in the range of 0.5–1 μ M (Benowitz et al., 1989; Russell, 1989; Henningfield et al., 1993). Acute smoking was modeled by applying 0.5–1 μ M nicotine to MHN–IPN synapses continuously for 1 min. The drug elicited synaptic facilitation that was relatively short lived and that subsided within minutes of drug removal (McGehee et al., 1995; Alkonon et al., 1996; Gray et al., 1996; Guo et al., 1998; present study).

The most parsimonious interpretation of our results is that acute nicotine and the pulses of ACh initiate facilitation by similar cellular mechanisms, that is, by activating presynaptic nAChRs expressed at MHN nerve terminals (McGehee et al., 1995). The following observations support this view: (1) ACh enhanced the frequency of minis without changing their amplitude; (2) sustained ACh-induced facilitation persisted in the presence of the muscarinic antagonist atropine; (3) both ACh- and nicotine-induced facilitation were depressed to a similar extent when nAChRs were deactivated by preexposure with nicotine; and (4) ACh can elicit an initial episode of facilitation with time course and magnitude that closely resemble those of the short burst of facilitation induced by nicotine.

Based on these findings and those of numerous other studies, activation of presynaptic nAChRs, by either ACh or nicotine, appears to promote calcium influx, indirectly through voltage-gated calcium channels and/or directly through the nicotinic receptors (Mulle et al., 1992; McGehee et al., 1995; Gray et al., 1996; Lena and Changeux, 1997; Girod et al., 2000). The nAChR-elicited increase in intraterminal calcium thereby initiates presynaptic facilitation, whereas the more prolonged facilitation observed with “synaptic-like” application of ACh might arise from downstream effects, such as calcium-induced calcium release and subsequent activation of second-messenger cascade(s). In addition, other mechanisms involved in long-term potentiation of glutamatergic synapses (Mansvelder and McGehee, 2000) may account for the prolonged changes in synaptic transmission reported here. If the effects of ACh involve the activation of NMDA receptors, then inhibition of these receptors as a result of nAChR activation (Fisher and Dani, 2000) may work as a negative feedback mechanism to limit synaptic responses to ACh.

Pulsatile application of high concentrations of nicotine recapitulates some of the more long-lasting effects of ACh, inducing facilitation for 5–10 min in hippocampal cultures (Radcliffe and Dani, 1998). Persistent enhancement of glutamate release lasting

1 hr or more, initiated by ACh and mediated via nicotinic receptor activation, has not, to our knowledge, been reported previously. Although our results are consistent with ACh causing prolonged enhancement of evoked secretion of glutamate, such long-term effects could not be investigated in the present system because stable recordings of evoked transmission (i.e., >10 min) are rare.

Recent reports have suggested that nicotine can facilitate the induction of long-term potentiation of glutamatergic transmission in the hippocampus (Fujii et al., 1999) and in the midbrain dopaminergic reward pathways (Mansvelder and McGehee, 2000). The present results show that the endogenous neurotransmitter ACh may use similar cellular mechanisms to promote sustained facilitation of glutamate secretion via activation of presynaptic nAChRs.

Muscarinic ACh receptor-mediated inhibition

Our observation that synaptic-like pulses of ACh elicited muscarinic receptor-mediated inhibition of glutamate release at MHN–IPN synapses is consistent with data obtained in other brain regions, including the hippocampus (Marchi and Raiteri, 1989; Segal, 1989; Sheridan and Sutor, 1990; Rouse et al., 1999), striatum (Barral et al., 1999), and cortex (Vidal and Changeux, 1993). Results obtained in the hippocampus (Egorov et al., 1996; Qian and Saggau, 1997) and in the striatum (Barral et al., 1999) indicate that synaptic depression results from the inhibition of voltage-gated calcium channels by presynaptic mAChRs. A direct interaction of mAChRs with the secretory machinery has also been proposed (Scanziani et al., 1995; Linial et al., 1997).

Prolonged exposure with nicotine may alter the balance between the facilitatory and inhibitory effects of ACh

ACh may facilitate or inhibit transmission at MHN–IPN synapses, depending on whether the ligand interacts with presynaptic nicotinic and/or muscarinic AChRs. Opposing effects of ACh by activation of nAChRs versus mAChRs have also been reported in studies of cortical synapses (Vidal and Changeux, 1993). At such sites, the balance of synaptic facilitation versus depression, and hence, the net effect of ACh, will be determined by the relative contribution of functional nicotinic versus muscarinic AChRs. Shifts of this balance may occur in habitual smokers in which receptive sites are exposed to sustained, low levels of nicotine that are expected to selectively desensitize or inactivate certain subtypes of nAChRs (Wonnacott, 1990; Peng et al., 1994; Dani and Heinemann, 1996; Olale et al., 1997; Peng et al., 1997; Wang et al., 1998; Kuryatov et al., 2000). Habitual smokers seem to adjust

their cigarette consumption so as to achieve stable plasma concentration of nicotine throughout the day (Benowitz et al., 1989; Russell, 1989; Henningfield et al., 1993). We modeled habitual smoking by exposing MHN–IPN cultures to nicotine for extended periods of time (24–72 hr). Prolonged exposure to nicotine depressed both nicotine- and ACh-mediated presynaptic facilitation, without altering muscarinic receptor-mediated synaptic depression, consistent with a functional inactivation of presynaptic nAChRs. Full recovery of nicotinic receptor-mediated synaptic facilitation required ~24 hr, suggesting an approximate half-time for recovery of ~10–12 hr. Such slow kinetics are consistent with proposed models of sustained deactivation of nAChRs, requiring new receptor synthesis and insertion for full recovery of function. Similar effects of prolonged exposure to nicotine were found for nAChRs expressed in reconstituted systems (Whiting et al., 1991; Peng et al., 1994, 1997). Because muscarinic receptor activation and ACh-elicited presynaptic inhibition are unaltered by prolonged exposure to nicotine, the treatment shifts the balance toward muscarinic AChR-mediated inhibition and decreases the contribution of nicotinic AChR-mediated presynaptic facilitation.

Our protocol for prolonged exposure to nicotine only partially recapitulates habitual smoking. In contrast to continuous treatment with the drug, a typical day of smoking is interrupted by several hours of smoke-free sleep. Nevertheless, indirect evidence suggests that, despite nocturnal interruption of nicotine self-administration, smoking induces significant inactivation of brain nicotinic receptor. Indeed, the number of nicotinic receptor binding sites in postmortem brains increases in habitual smokers (Breese et al., 1997; Perry et al., 1999), and surface receptor upregulation has been proposed to be initiated by inactivation of receptors by chronic nicotine (Marks et al., 1983; Schwartz and Kellar, 1985; Fenster et al., 1999). Our results suggest that inactivation of certain subtypes of nicotinic receptor by nicotine inhaled through smoking may cause a switch between presynaptic facilitatory and inhibitory influences of central cholinergic projections.

Overall, these results suggest that activation of central cholinergic pathways may regulate neuronal networks by producing a combination of long-lasting presynaptic nicotinic AChR-mediated facilitation and shorter-term, presynaptic muscarinic AChR-mediated inhibition of glutamatergic neurotransmission. Furthermore, aspects of nicotine dependence may involve selective disruption of the nicotinic component of cholinergic signaling by prolonged exposure to nicotine.

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