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Nicotinic Receptor-mediated Biphasic Effect on Neuronal Excitability in Chick Lateral Spiriform Neurons

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Abstract

Local neuronal circuits integrate synaptic information with different excitatory or inhibitory time windows. Here we report that activation of nicotinic acetylcholine receptors (nAChRs) leads to biphasic effects on excitability in chick lateral spiriform (SPL) neurons during whole cell recordings in brain slices. Carbachol (100 μ M in the presence of 1 μ M atropine) produced an initial short-term increase in the firing rates of SPL neurons ($125 \pm 14\%$ of control) that was mediated by postsynaptic nAChRs. However, after 3 min exposure to nicotinic agonists, the firing rate measured during an 800 msec depolarizing pulse declined to $19 \pm 7\%$ (100 μ M carbachol) or $26 \pm 8\%$ (10 μ M nicotine) of the control rate and remained decreased for 10–20 min after washout of the agonists. Similarly, after 60 sec of electrically-stimulated release of endogenous acetylcholine (ACh) from cholinergic afferent fibers, there was a marked reduction ($45 \pm 5\%$ of control) in firing rates in SPL neurons. All of these effects were blocked by the nAChR antagonist dihydro- β -erythroidine (30 μ M). The inhibitory effect was not observed in Ca^{2+} -free buffer. The nAChR-mediated inhibition depended on active G-proteins in SPL neurons and was prevented by the GABA_B receptor antagonist phaclofen (200 μ M), while the GABA_B receptor agonist baclofen (10 μ M) decreased firing rate in SPL neurons to $13 \pm 1\%$ of control. The inhibitory response thus appears to be due to a nAChR-mediated enhancement of presynaptic GABA release, which then activates postsynaptic GABA_B receptors. In conclusion, activation of nAChRs in the SPL initiates a limited time window for an excitatory period, after which a prolonged inhibitory effect turns off this window. The prolonged inhibitory effect may serve to protect SPL neurons from excessive excitation.

Keywords

synaptic transmission; central integration; GABAergic receptors; Nicotinic receptors

Integration of synaptic information is essential for brain function (Egger et al., 1999; Froemke and Dan, 2002; Nishiyama et al., 2000; Pouille and Scanziani, 2004). Since the action potential is the output signal of neurons, changes in firing frequencies and firing patterns represent integration of neuronal information (Chang and Berg, 1999; Hoffman and McNaughton, 2002; Pape and McCormick, 1989; Zhang and Poo, 2001). For example, Froemke and Dan (2002) examined spike-timing-dependent plasticity in visual cortical slices and found that a time window of several tens of milliseconds for the first spike in each burst has a crucial role in determining the sign and magnitude of synaptic modification. Pouille and Scanziani

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(2004) report that in hippocampal slices two distinct inhibitory circuits adjust neuronal excitability: one, time-locked with submillisecond precision to the onset of the action potential series, transiently inhibits the somatic and perisomatic regions of pyramidal cells; the other, activated in proportion to the rate of action potentials in the series, durably inhibits the distal apical dendrites.

In this work, we examine changes in neuronal excitability over time as a result of activation of nicotinic acetylcholine receptors (nAChRs). Nicotinic receptors are widespread in the brain and are important for many physiological functions such as learning, memory, and cognition. Changes to cholinergic pathways occur in several major diseases including nicotine addiction and neurodegenerative diseases (For reviews see Changeux et al., 1998; Dani and Heinemann, 1996; Role and Berg, 1996). The chick lateral spiriform nucleus (SPL), a part of the basal ganglia that is important for visually-guided movements (Doupe et al., 2005; Reiner, 2002), is a model system for examining various roles of nAChRs in the brain. Postsynaptic nAChRs are densely expressed on the somata and dendrites of essentially all SPL neurons, and mediate fast excitatory responses that can be large enough to generate action potentials (Nong et al., 1999; Sorenson and Chiappinelli, 1990). Presynaptic and preterminal nAChRs are also present in the SPL, and when activated enhance the release of GABA from terminals within the SPL (McMahon et al., 1994; Tredway et al., 1999; Zhu and Chiappinelli, 2002). To date, however, no study has focused on the integration of both pre- and postsynaptic nAChRs in this nucleus in a time-dependent manner.

Experimental Procedures

Chick embryos (16 to 19 days of incubation) were decapitated and their brains removed quickly and immersed in cold (0–2 °C), 5% CO₂/95% O₂-saturated N-Methyl-D-glucamine (NMDG)-artificial cerebrospinal fluid (ACSF). The composition of normal ACSF in mM was: 126 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃ and 10 glucose. The differences between NMDG-ACSF and normal ACSF were the replacement of NaCl with equal molar NMDG and the adjustment of MgCl₂ to 10 mM in the NMDG-ACSF. 200–300 µm thick coronal brain slices were cut using a Vibratome tissue slicer (TPI, St. Louis, MO). The slices were then maintained in an incubation chamber filled with NMDG-ACSF at room temperature for about 20 min and then transferred to an incubation chamber filled with normal ACSF at room temperature for at least 40 min before using for recording. All experiments were performed at room temperature (22–24 °C). Atropine (1 µM) was routinely added to ACSF to completely block muscarinic AChRs. Slices were fully submerged in the recording chamber and were perfused continuously with ACSF (4 ml/min). The SPL neurons were visualized with an Axioskop microscope (Zeiss, Jena, Germany) for whole-cell recording. Patch pipettes were pulled with a Flaming-Brown Micropipette Puller (P-87) and had a tip resistance between 5–7 MΩ measured with a filling internal solution composed in mM: 140 K-gluconate, 2 MgCl₂, 1 EGTA-Na₄, 10 HEPES, 3 Mg-ATP and 3 phosphocreatine-Na₂.

After the membrane was ruptured, series resistance was less than 10 MΩ and was constant during the entire experiment so that series resistance compensation was not used. Criteria for a healthy cell for long-term recordings (over 1 hr) were as follows: 1) Resting membrane potential just after rupture of the membrane more negative than –45 mV; 2) Holding current at –70 mV in voltage clamp mode more positive than –250 pA; 3) Input resistance constant under normal conditions; 4) DC drift after pulling the pipette from the cell at the end of the experiment less than ±5 mV. Recordings not meeting these criteria were not used. Electrophysiological signals were amplified in voltage- or current-clamp modes with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and were sampled with pClamp7 software through a Digidata 1200 DMA interface.

Measurement of firing rates

The standard protocol used to measure firing rates under various conditions was to first adjust the membrane potential of SPL neurons in current clamp mode to -70 mV through the use of continuous hyperpolarizing current. Next, a depolarizing current pulse of 800 msec was applied, with the intensity of the pulse adjusted such that the membrane potential during the pulse matched the previously determined plateau potential for that neuron (see Fig. 3C1). The firing rate was then determined by counting the number of action potentials that were elicited during the 800 msec depolarizing current pulse. In some experiments, a second method for measuring firing rates was also used. In this case, the intensity of the pulse current was kept constant, and the amount of continuous current injected was adjusted so that membrane potential during the pulse was at the plateau potential (see Fig. 3C2). Both methods gave similar results (Fig. 3F).

For agonist applications, initial firing rates were determined within the first 15 sec of exposure to the agonist. Firing rates measured during prolonged agonist exposures were obtained 3 – 4 min after the start of agonist exposure, except where noted otherwise. For conditioning stimulation experiments, firing rates were obtained within 1 sec after the end of the conditioning stimulation train.

Choice of nicotinic agonists

Nicotine (1 μ M and 10 μ M) was chosen as an agonist that can activate all subtypes of nicotinic receptors at a concentration where it is also selective (i.e., does not activate muscarinic or other receptors). Nicotine generally requires extended washing out for full recovery in brain slice recordings in part due to its affinity for the receptors, but also because it can pass into and be retained within cell membranes due to its hydrophobicity. For this reason, carbachol (100 μ M) was also chosen as an agonist that can activate all nicotinic receptors. When 1 μ M atropine is present, carbachol does not activate muscarinic receptors at the concentrations used. Atropine was required in these experiments in any case because we also examined the effects of endogenously released ACh, which like carbachol can activate both nicotinic and muscarinic receptors. We selected carbachol over ACh for the exogenously applied agonist because with exogenously applied ACh it is difficult to control for the rapid enzymatic breakdown of ACh by acetylcholinesterase, an enzyme that does not break down carbachol. Carbachol (100 μ M) was perfused with a fast perfusion system (PLA Scientific Instruments, NY) through a glass micropipette (400 μ m in diameter) placed close to the recorded neurons so that onset of drug effects was relatively quick and constant. Nicotine was applied by bath perfusion.

In experiments examining the effects of endogenously released ACh, a stimulating electrode was placed with micromanipulators onto the lateral side of the SPL, where the cholinergic afferent tract is visible in the slices (Nong et al., 1999). This electrode was used to provide conditioning stimulation trains (10–60 sec @ 20 Hz, 50–800 μ A intensity, 200 μ s duration). Since little is known regarding actual *in vivo* frequencies of transmission in the SPL, we chose 20 Hz because it is in the middle range of SPL neuronal firing, if one assumes that this range falls within the sensitive region of membrane excitability (see Fig. 1C–D).

Choice of nicotinic antagonists

We selected dihydro- β -erythroidine (DH β E) as a nicotinic antagonist that blocks essentially all subtypes of nAChRs at a concentration of 20–50 μ M. At these concentrations DH β E has little or no effect on GABAergic or other receptors, unlike d-tubocurarine which blocks GABA_A receptors at concentrations required to block some nAChRs (Wotring and Yoon, 1995). Two major subgroups of nAChRs are the α 2- α 6-containing receptors and the α 7- α 8-containing receptors (reviewed in Dani and Bertrand, 2007). Particularly for presynaptic nAChRs it is useful to pharmacologically distinguish between these two subgroups, since α 7-

α 8-containing nAChRs have the highest permeability for Ca^{2+} . Methyllycaconitine (MLA) was chosen as an additional antagonist because at 50 nM it blocks α 7- α 8-containing nAChRs while having little effect on other subtypes of nAChRs (Chang and Berg, 1999; Yum et al., 1996). DH β E and MLA were applied by bath perfusion.

Statistical analysis

The Repeated Measures One-Way ANOVA with a Tukey post-test (Prism 4.0, GraphPad Software) was used to test for statistical differences between 3 or more groups. In experiments where only 2 groups were being compared, the two-sided *t*-test was used. For all statistical analyses, $p < 0.05$ was chosen as the level meeting statistical significance. Data are expressed as mean $\pm$ S.E.M.

All efforts were made to minimize both the suffering and the number of animals used in these experiments. All experiments conformed to National Institutes of Health guidelines on the ethical use of animals and were approved by the George Washington University Medical Center IACUC.

All drugs were purchased from Sigma (St. Louis, MO) except methyllycaconitine (MLA) from Research Biochemicals, and phaclofen and MCPG from Tocris.

Results

Characteristics of tonic and weakly adapting SPL neurons

Since our goal was to study output patterns in SPL neurons, we first determined their responses to a prolonged (800 ms) depolarizing current pulse. Of the 229 neurons recorded from, 57% were categorized as tonic firing or weakly adapting neurons, 30% were completely adapting neurons, 7% were bursting neurons, and the rest of the neurons (6%) were grouped as irregularly firing neurons. Tonic and weakly adapting neurons fired continuously during a relatively strong (150 to 200 pA) depolarizing current pulse of 800 ms duration (Fig. 1B, insert). For neurons in this category, the interspike interval of the last two action potentials was less than 3 times that of the initial two spikes. In contrast, the firing of a completely adapting neuron usually ceased during the first half of the pulse and the last interspike interval was much longer than the first one (data not shown). Similar criteria have been used in other studies (D'Angelo et al., 1998; Kröner et al., 2000; Sivaramakrishnan and Oliver, 2001). In the present work we selected only tonic and weakly adapting neurons for further study because their firing rates in response to repeated 800 ms depolarizing current pulses were consistent throughout 60 min recording sessions (Fig. 1A and 1B), indicating that the fidelity of information transmission, including firing frequency and pattern, was maintained for prolonged periods. The membrane properties of a sampling of tonic and weakly adapting neurons are summarized in table 1.

The two major factors determining the firing rate of a neuron in response to depolarizing current are the intensity of the depolarizing current and the plateau potential underlying each action potential. In order to determine which factor best reflected the observed changes in firing rates, we examined the relationship between firing rate and current intensity, and that of firing rate and plateau potential. Under control conditions, the plateau potential had a linear relationship with firing rate (Fig. 1C). In contrast, the control firing rate increased towards an asymptote as current intensity was raised (Fig. 1D).

Both endogenous and exogenous nicotinic agonists elicit biphasic effects in a time-dependent manner

The activation of postsynaptic nAChRs located on the somas and dendrites of SPL neurons elicits a large excitatory inward current (Sorenson and Chiappinelli, 1990; McMahon et al.,

1994;Nong et al., 1999) but does not produce a slower onset hyperpolarizing response such as is seen in neurons of the dorsolateral septal nucleus (Sorenson and Gallagher, 1996). During a 1 min exposure to 100 μ M carbachol (in the presence of 1 μ M atropine to block all muscarinic responses), nAChRs partially desensitized and the inward current decreased to $64\pm4\%$ ($p<0.01$; $n=6$) of the initial peak amplitude, remaining at this level until the end of agonist application (Fig. 2A). Altering membrane potential may change membrane excitability and influence firing rate or firing pattern in some brain regions. For instance, the resting membrane potentials of neurons in rat thalamus vary between waking and sleeping states and neuronal firing patterns are consequently altered (Avanzini et al., 1989;McCormick, 1992;Mukhametov et al., 1970). In one series of experiments, we therefore held the membrane potential steady by manually adjusting the continuous depolarizing current at just above action potential threshold for several minutes. In normal ACSF, this produced spontaneous firing at a stable rate (Fig. 2B). After current clamping the membrane potential of the same neuron back to the control level (-70 mV), 100 μ M carbachol was added through our fast perfusion system. Carbachol depolarized the membrane potential, and elicited action potentials when the membrane potential was more depolarized than threshold potential. Soon after the onset of carbachol application the firing rate was higher than in normal ACSF, but with prolonged perfusion of carbachol the firing rate decreased (Fig. 2C). The inhibitory effects of carbachol were slowly reversible on washing out the agonist (Fig. 2D). The prolonged inhibitory effect does not appear to be caused by the desensitization of nAChRs because when additional depolarizing current was applied in order to maintain the same plateau potential as that of control, the firing rate was still markedly reduced (see Fig. 3C). The duration (17.8 ± 6.8 sec, $n=6$) of the initial excitatory firing rate period was measured from the onset of firing to the time point where the observed firing rate began to decrease in experiments such as the one shown in Fig. 2C. Activation of nAChRs in the SPL thus opened a limited time window of excitation, after which prolonged activation of nAChRs turned off this facilitation of informational transmission and significantly depressed the following information flow.

Two questions need to be addressed according to the above results. The first question is how to qualitatively measure excitatory and inhibitory rates. The second question is: does endogenous activation of nAChRs result in the same consequence? The number of action potentials produced by an 800 ms depolarizing current pulse was used to quantify the effects of carbachol. Two methods were employed to match the plateau potentials underlying the action potentials during control, carbachol, and agonist washout measurements. In the first approach, membrane potential was held at -70 mV and the intensity of the depolarizing current pulses was adjusted until the plateau potentials were matched. The second method was to maintain a fixed pulse current intensity while adjusting membrane potential with continuous current (Fig. 3). The initial effect of carbachol was to increase membrane excitability so that the firing rate significantly increased to $125\pm14\%$ of control ($p<0.05$; $n=5$; Fig. 3B and 3E). In contrast, after prolonged perfusion with carbachol (3 min or longer) under both methods used, membrane excitability was inhibited and firing rate significantly decreased to $19\pm7\%$ or $18\pm5\%$ of control, respectively ($p<0.001$ for each; $n=8$; Fig. 3C and 3F). The inhibitory effects of prolonged carbachol could be washed out after 10 min of perfusion with normal ACSF (Fig. 3E–F). The magnitude of the decrease in firing rates observed during prolonged perfusion with carbachol was similar for both methods used ($p>0.05$; $n=8$), indicating that under our experimental conditions the plateau potential determined the firing rate and the change in firing rate was independent of resting membrane potential.

During prolonged perfusion with carbachol, the relationship between firing rate and current intensity no longer regressed to a Boltzman fit ($R^2=0.45$), and the membrane appeared to become rectifying to input current (Fig. 1D). At the same time, the relationship between firing rate and plateau potential was still linear but shifted to the right, indicating that the intrinsic neuronal output still functioned at more depolarized levels of membrane potential (Fig. 1C).

In one series of experiments, a stimulation electrode was placed on the cholinergic afferent pathway to the SPL (Nong et al., 1999). A stimulus train (10 or 60 sec @ 20 Hz, pulses of 60–800 μ A and 200 μ s) was applied to the cholinergic tract and then the firing frequency of SPL neurons was measured within 1 sec after the end of the stimulus train during an 800 msec depolarizing current pulse. There was no significant change in firing frequency after the 10 sec stimulus train (Fig. 4A; $p>0.05$, $n=5$). However, there was a marked reduction to $43\pm5\%$ of the control firing rate measured after 60 sec of cholinergic tract stimulation (Fig. 4B, $p<0.01$, $n=5$). In the presence of the nAChR antagonist dihydro- β -erythroidine (DH β E; 30 μ M), the stimulation on firing frequency was abolished (Fig. 4B, $p>0.05$, $n=5$). Thus, activation of nAChRs either by exogenous agonist or by release of endogenous ACh results in similar information integration.

Effects of nicotine and nicotinic antagonists

Nicotine (1 and 10 μ M) produced biphasic effects on firing rate similar to those observed with carbachol (Fig. 5A–B). The initial short-term excitatory effects of nicotine at 1 and 10 μ M were significantly different from control ($115\pm6\%$ and $116\pm8\%$ of control, respectively; $p<0.05$ for each; $n=5$ for each group) but the percentage increase was similar at these two concentrations. During prolonged perfusion (3 min) with nicotine, the lower concentration of nicotine decreased firing rate less than the higher concentration ($71\pm6\%$ and $26\pm8\%$ of control, respectively, $p<0.001$ for each; $n=5$ for each group). While the inhibitory effects of nicotine could be washed out, the washout time required was longer (over 20 min) than that required for carbachol.

DH β E blocks essentially all subtypes of nAChRs at 20–50 μ M. Following pretreatment of brain slices with 30 μ M DH β E for 5 min, carbachol (100 μ M) no longer altered the firing rates of SPL neurons, confirming that activation of nAChRs is required for both the initial excitatory response ($100\pm3\%$ of control; $p>0.05$; $n=5$) and the delayed inhibitory response ($100\pm3\%$ of control; $p>0.05$; $n=5$; Fig. 4C). The nicotinic antagonist methyllycaconitine (MLA) was used to examine the possible role of $\alpha 7$ - and/or $\alpha 8$ -containing nAChRs in these responses. At 50 nM MLA, all nAChRs composed solely of $\alpha 7$ and/or $\alpha 8$ subunits should be blocked, while blockade of most other nAChR subtypes in chick brain requires higher concentrations of MLA (Chang and Berg, 1999; Yum et al., 1996). In the presence of 50 nM MLA, the firing rates of SPL neurons during prolonged exposure to carbachol (100 μ M) were still significantly reduced ($61\pm4\%$ of control, $p<0.01$, $n=5$; Fig. 4D), but the magnitude of the decrease was less than that observed when MLA was not present (see Fig. 3F and 5C).

Mechanism of the prolonged inhibitory effect of nicotinic agonists

While the initial short-term excitatory effect of nicotinic agonists was an expected consequence of activation of postsynaptic nAChRs, the mechanism behind the prolonged inhibitory effect was initially unclear. The delayed nature of the inhibitory effect suggested that a second messenger might be involved in this response. GTP- γ -S is a non-hydrolysable GTP analog which persistently combines with the α subunits of G proteins to interfere with their normal functions (Fein and Corson, 1981; Liu et al., 1993). When 50 μ M GTP- γ -S was diffused into SPL neurons from the patch pipette solution the inhibitory effect of nicotine (10 μ M; 3–8 min perfusion) was completely prevented. Under these conditions, prolonged nicotine actually increased the firing rate to $113\pm3\%$ of control ($p<0.05$; $n=5$; Fig. 6).

Taking this result into account, a possible mechanism for the inhibitory effect would be that nicotinic agonists were acting on presynaptic nAChRs to enhance the release of an inhibitory transmitter that then activated a G-protein-dependent metabotropic receptor located on the SPL neurons. As a first approach to this question, synaptic transmission would be crucial to the prolonged inhibitory effect. The ion channels of neuronal nAChRs are permeable to Ca^{2+} , and

$\alpha 7$ -containing receptors exhibit particularly high Ca^{2+} flux (Dajas-Bailador and Wonnacott, 2004; Role and Berg, 1996; Vernino et al., 1994). Experiments with Ca^{2+} -free ACSF were therefore done to test this possibility. Perfusion with Ca^{2+} -free ACSF decreased the postsynaptic nAChR-mediated inward currents measured in SPL neurons to $73 \pm 4\%$ of control ($p < 0.01$; $n = 5$) and completely blocked synaptic transmission evoked by electrical stimulation of afferent fibers (data not shown). The firing rates of SPL neurons in response to depolarizing current pulses increased slightly during perfusion with Ca^{2+} -free ACSF, reaching a stable rate after 7 min ($130 \pm 4\%$ of the rate in ACSF; $p < 0.05$; $n = 5$; Fig. 7A–B). Similar results reported by other laboratories (Madison and Nicoll, 1984; Storm, 1989; Zhou and Hablitz, 1996) were attributed to decreased accommodation caused by blockade of Ca^{2+} -activated K^+ conductances in rat brain slices. In Ca^{2+} -free ACSF, prolonged perfusion with nicotine (5 min at $10 \mu\text{M}$) had no effect on the firing rate of SPL neurons ($101 \pm 4\%$ of the rate in Ca^{2+} -free ACSF; $p > 0.05$; $n = 5$; Fig. 7B).

The above data are consistent with the hypothesis that the prolonged inhibitory response was the result of activated presynaptic nAChRs causing increased release of an inhibitory transmitter. To further examine this mechanism, antagonists of several inhibitory metabotropic receptors were included in the perfusion solution. A cocktail solution of $200 \mu\text{M}$ phaclofen, $500 \mu\text{M}$ α -methyl-4-carboxyphenylglycine (MCPG), and $2 \mu\text{M}$ naloxone was perfused for 10 min before $100 \mu\text{M}$ carbachol was added. Under this condition, prolonged carbachol had no significant effect on firing rate ($88\% \pm 6\%$ of control; $p > 0.05$; $n = 5$; Fig. 7C). When phaclofen alone was tested, the prolonged inhibitory effect was similarly blocked in 5 of the 6 cells tested ($92\% \pm 4\%$ of control; $p > 0.05$; $n = 5$; Fig. 7D), indicating that GABA was most likely to be the neurotransmitter released by prolonged exposure to nicotinic agonists, and which then bound to GABA_B receptors on postsynaptic SPL neurons to produce a G protein-dependent postsynaptic inhibition of firing rate. The effects of baclofen, a GABA_B receptor agonist, were then examined. Baclofen ($10 \mu\text{M}$) markedly reduced the firing rates of SPL neurons in response to depolarizing current pulses to $13 \pm 1\%$ of the control rate ($p < 0.01$; $n = 3$), confirming that inhibitory GABA_B receptors are located on SPL neurons.

Discussion

The results demonstrate that activation of nAChRs biphasically alters the excitability of SPL neurons in a time-dependent manner. The initial excitatory effect is due to activation of postsynaptic nAChRs on the SPL neurons. The longer-term inhibitory effect appeared only after nAChRs were activated for at least 1 min by either endogenous ACh or exogenous nicotinic agonists, and was due to nAChR-mediated enhanced GABA release leading to increased activation of GABA_B receptors.

The present study was limited to the tonic-firing or weakly adapting neurons, representing 57% of all SPL neurons recorded from. Since recordings with prolonged agonist exposures were carried out on a relatively small number of these neurons, it is possible that they may not have been representative of all tonic-firing SPL neurons. However, the central finding of a delayed inhibitory response following prolonged exposure to nicotinic agonists was consistently observed under a variety of recording conditions with 2 different exogenous nicotinic agonists as well as endogenously released ACh. For each of these separate conditions 5–8 individual neurons were examined, and in each case a statistically significant inhibition in firing rate was observed.

Although the delayed inhibition in firing rate was observed with exogenous nicotinic agonists and endogenously released ACh, the initial excitatory effect on firing rate was confirmed in the present work only with nicotine and carbachol. For the endogenous release experiments, a relatively long stimulus train of either 10 or 60 sec was applied to the cholinergic tract, and the

firing rate was measured within 1 sec after the end of the conditioning train. Significant inhibition was present only after the 60 sec conditioning train, and this is consistent with the delayed nature of the inhibition seen after exogenous agonists. We have previously shown (Nong et al., 1999) using single conditioning pulses that electrical stimulation of the cholinergic fiber tract adjacent to the SPL initiates fast excitatory postsynaptic responses and initiates action potentials in SPL neurons, demonstrating the short term excitatory effects of this stimulation.

Modulation of excitatory actions of nAChRs also occurs in two CNS regions in mammals where postsynaptic nAChRs are prominent. In the hippocampus, postsynaptic nAChRs are present on both interneurons and pyramidal cells, and presynaptic nAChRs enhance the release of GABA (Alkondon and Albuquerque, 2001; Alkondon et al., 1999; Frazier et al., 1998; Hefft et al., 1999). Ge and Dani (2005) have recently shown that action potentials mediated by postsynaptic nAChRs on CA1 pyramidal neurons can lead to either long term potentiation or long term depression, depending on the time relationship between this nicotinic excitation and electrical stimulation of presynaptic fibers in the Schaffer collateral pathway. In neurons of the rat ventral tegmental area (VTA), nicotine acts on postsynaptic nAChRs to initially produce marked excitation, but in the continued presence of nicotine (0.1 – 0.5 μ M) this response is greatly reduced or disappears, due to receptor desensitization (Pidoplichko et al., 1997). In the VTA neurons, a further modulation of nicotine's overall effect is due to differences in desensitization between presynaptic nAChRs located on GABAergic and glutamatergic nerve terminals (Mansvelder et al., 2002). The result of these influences is that even though postsynaptic nAChRs are rapidly desensitized, there is a long-term shift to a more excited state due to the actions of presynaptic nAChRs.

We find that desensitization of postsynaptic nAChRs does occur in SPL neurons, so this might play a role in some of the effects we observe with nicotinic agonists. However, the extent of desensitization of nAChRs in the SPL is much less than in the VTA. In the present study the inward current in SPL neurons in response to 100 μ M carbachol was 64% of the initial amplitude after 1 min of exposure to agonist. Furthermore, when the inhibitory effects of presynaptic nAChRs were blocked by intracellular GTP- γ -S, the excitatory effects of postsynaptic nAChRs persisted even after 8 min at levels comparable to those measured at 3 min in the continued presence of 10 μ M nicotine (compare Fig. 5B and 6B).

Several lines of evidence indicate a role for postsynaptic GABA_B receptors in the delayed inhibitory response to nicotinic agonists. First, the inhibition was completely prevented following perfusion of only the postsynaptic SPL neuron with GTP- γ -S through the patch pipette, indicating that postsynaptic receptors were involved in the phenomenon. Second, the inhibition was blocked by the GABA_B antagonist phaclophen. These two results imply that presynaptically released GABA acts on postsynaptic GABA_B receptors to mediate the inhibition, similar to the role of postsynaptic GABA_B receptors in several other brain regions (Lin and Dun, 1998; K. Yamada et al., 1999). Since nicotinic agonists are known to enhance GABA release in the SPL (McMahon et al., 1994; Tredway et al., 1999), this is consistent with the results observed following prolonged exposure to these agonists. Further evidence for this model is that exogenously applied baclofen mimics this effect. There is also the possibility that presynaptic GABA_B receptors are located on GABAergic nerve terminals in the SPL. In other brain regions, activation of such receptors leads to an inhibition of GABA release (Lin and Dun, 1998; J. Yamada et al., 1999; Chen and Pan, 2006), which would be expected to partially counteract the enhanced GABA release seen in response to nAChR activation. GABA_A receptors do not appear to be involved in the inhibition since the effect is blocked by a selective GABA_B receptor antagonist.

The long-term fidelity of membrane properties in tonic-firing SPL neurons is evident in the consistency of the firing frequencies and patterns observed during one hour recordings in control neurons (e.g., Fig. 1). The normal to slightly increased excitabilities of SPL neurons during initial activation of nAChRs would likely allow information flow during this time window to be faithfully reproduced, with the novelty or specificity of the information maintained. When neuronal membrane potential is depolarized the excitability of the cell usually increases, resulting in an increase in action potential frequency. Consequently the strength of the neuronal influences on downstream targets increases. On the other hand, if the increase in excitability is too large and lasts too long it may result in an excitotoxic effect (Sofroniew and Pearson, 1985; Vecsei et al., 1998). This excitotoxic effect will cause cell injury and eventually cell death. When nAChRs are activated, cations pass through the channels and cause membrane depolarization to increase firing frequency. However, a number of reports indicate that activation of nAChRs can have a protective effect on cell survival and maintenance of normal cellular functions (Laudenbach et al., 2002; Li et al., 2002; Maggio et al., 1998). Our experimental results suggest one cellular mechanism that could help to account for the protective functions of the nAChRs. Even though membrane potential is persistently depolarized in the SPL in the presence of nicotinic agonists, activation of presynaptic nAChRs decreases membrane excitability through a synaptic integrative mechanism which may serve to protect the neurons from an excitotoxic effect.

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Abbreviations

ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
DHβE	

	dihydro- β -erythroidine
MCPG	α -methyl-4-carboxyphenylglycine
MLA	methyllycaconitine
nAChR	nicotinic acetylcholine receptor
NMDG	N-methyl-D-glucamine
SPL	lateral spiriform nucleus
VTA	ventral tegmental area

Fig 1.

Characteristics of tonic-firing neurons in SPL. A and B: Firing rates during 800 msec depolarizing current pulses were stable for at least 1 hour in tonic-firing SPL neurons. Each neuron is represented by a different symbol in A, which shows the number of action potentials per 800 msec pulse at various times after recording was begun. In B, the number of action potentials was normalized to that observed during the first depolarizing pulse, and the mean $\pm$ S.E.M. were plotted against time. Insert in B shows the membrane responses of a typical tonic-firing neuron to depolarizing and hyperpolarizing currents. C and D: The relationship between firing rate and plateau potential (C) and firing rate and current intensity (D). The plateau potential data was sorted into 5 mV bins and the current intensity was sorted into 50 pA bins. Control conditions, open circles; after 3 min in 100 μ M carbachol, closed circles; $n=15$ for both groups; mean $\pm$ S.E.M.

Fig 2.

Activation of nicotinic receptors elicits biphasic effects on firing rates in SPL neurons. A: Carbachol (100 μ M; bar above record) in the presence of 1 μ M atropine (to block all muscarinic responses) produced an inward current mediated by activation of postsynaptic nAChRs on an SPL neuron maintained at -70 mV. The inward current desensitized to 60% of the initial value in this neuron after 30 sec. B. Recording (1 min) of the firing rate of an SPL neuron in normal ACSF. Initial membrane potential is -70 mV, then it is adjusted with depolarizing current to a level just above the threshold for activation of firing. C: Activation of nAChRs by 100 μ M carbachol (applied at start of trace and present throughout recording) elicits an initial excitatory response (depolarization and action potentials), followed by a prolonged reduction in the initial rate of firing (3 min recording). As the nAChR-induced depolarizing current decreased due to desensitization, extra depolarizing current was injected through the electrode so that the plateau potential was matched with that of the control in B. D: After washout of carbachol for 10 min, membrane potential is again adjusted with depolarizing current to just above threshold for activation of firing for 3 min to match recording time in C. Recordings in B–D are from the same SPL neuron, and initial membrane potential is set at -70 mV throughout.

Fig 3.

Quantitative analysis of the biphasic nicotinic effect. A–D show methods used for the measurement of firing rates in SPL neurons. Dashed line indicates membrane potential of –40 mV, and plateau potential in this neuron was slightly below –40 mV. A: Firing was activated by a depolarizing current pulse (800 ms) in ACSF. B: At the onset of carbachol (100 μ M) application the same procedure as in A was repeated. C: After 3 min exposure to carbachol, care was taken to match the plateau potential in two different ways (C1 and C2) and firing rates were measured. D: Response 10 min after washing out carbachol. E–F: Plots of the initial (E) and prolonged (F) effects of carbachol (mean $\pm$ S.E.M., $n=5$ in E, $n=8$ in F). The initial effect (within 15 sec) of carbachol is a significant ($p<0.05$; asterisk) increase in firing rate to $125 \pm 14\%$ of control (E), and the prolonged effect (after 3 min) is a significant ($p<0.001$; double asterisk) reduction to either $19 \pm 7\%$ or $18 \pm 5\%$ of the control rate of firing (F).

Fig 4.

Stimulation of cholinergic afferents can reduce firing rates in SPL neurons via activation of nicotinic receptors. Trains of conditioning stimulation (10 or 60 sec @ 20 Hz) were applied by an electrode at the site of the afferent cholinergic fiber tract. Firing rate in SPL neurons was then measured during a depolarizing current pulse within 1 sec after the end of the stimulus train. A: After the 10 sec stimulus train, there was no change in firing frequency in SPL neurons ($p > 0.1$, $n = 5$). B: After the 60 sec stimulus train, firing frequency was significantly reduced to $43 \pm 5\%$ of control ($p < 0.01$, $n = 5$). The inhibitory effect of 60 sec conditioning stimulation trains was completely abolished when 30 μ M DH β E was present ($p > 0.05$, $n = 5$). All plots are mean $\pm$ S.E.M.

Fig 5.

Effects of nicotine and nicotinic antagonists on firing rates of SPL neurons. A and B: 1 μ M and 10 μ M nicotine had biphasic effects similar to those of carbachol. The initial (first 15 sec) increase in firing rate was $115 \pm 6\%$ of control ($p < 0.05$, $n = 5$) for 1 μ M nicotine and $116 \pm 8\%$ of control ($p < 0.05$, $n = 5$) for 10 μ M nicotine. Prolonged perfusion (3 min) with 1 or 10 μ M nicotine decreased the firing rates to $71 \pm 6\%$ or $26 \pm 8\%$ of control, respectively ($p < 0.001$; $n = 5$). C: The inhibition in firing rate seen during prolonged exposure to 100 μ M carbachol (second bar; $26 \pm 11\%$ of control; $p < 0.01$, $n = 5$) was completely blocked in the presence of 30 μ M DH β E (last bar; $99 \pm 8\%$ of control after wash; $p > 0.05$, $n = 5$). D: Pretreatment of SPL neurons with 50 nM MLA partially prevented the inhibition in firing rate seen after prolonged exposure to 100 μ M carbachol ($61 \pm 4\%$ of control, $p < 0.01$, $n = 5$). All plots are mean $\pm$ S.E.M.

Fig 6.

Activated postsynaptic G-proteins are required for the prolonged nAChR-mediated inhibitory effect. A: After 50 μ M GTP- γ -S was diffused into the neurons via the patch pipette, prolonged bath-perfusion with 10 μ M nicotine (bar above record) for up to 8 min elicited only an excitatory effect on firing rates in SPL neurons ($113 \pm 3\%$ of control; $p < 0.05$; $n=5$). Negative numbers on x-axis in A indicate time before perfusion with nicotine. Positive numbers indicate time of nicotine perfusion. Different symbols in A represent firing rates of different neurons during prolonged exposure to nicotine. B: Plots of firing rate data collected from the five neurons indicated in panel A (mean $\pm$ S.E.M.). These neurons had been exposed to nicotine for between 4 to 7.5 min, as illustrated in panel A.

Fig 7.

External Ca²⁺ and activated GABA_B receptors are necessary for the prolonged nAChR-mediated inhibitory effect. A: Firing rates increase during perfusion (15 min) with Ca²⁺-free ACSF solution (bar above record), compared with firing rates in normal ACSF recorded before Ca²⁺-free ACSF ($130 \pm 4\%$, $p < 0.05$; $n = 5$; mean $\pm$ S.E.M.). B: Plots indicating that the nAChR-mediated inhibitory effect normally produced by prolonged exposure (5 min) to 10 μ M nicotine is blocked in the presence of Ca²⁺-free solution ($101 \pm 4\%$ of rate in Ca²⁺-free ACSF, $p > 0.05$, $n = 5$; mean $\pm$ S.E.M.). C: A cocktail solution containing 200 μ M phaclofen, 500 μ M MCPG, and 2 μ M naloxone blocked essentially all of the inhibitory effect normally seen following prolonged exposure (3 min) to 100 μ M carbachol ($88 \pm 6\%$ of control; $p > 0.05$; $n = 5$ neurons). D: Phaclofen (200 μ M) alone blocked the inhibitory effect of prolonged carbachol (3 min) to the same extent ($92 \pm 4\%$ of control; $p > 0.05$; $n = 5$) as the cocktail solution in C. All plots are mean $\pm$ S.E.M.

TABLE 1

Membrane properties of tonic-firing neurons in SPL

	RMP (mV)	IR (MΩ)	τm (ms)	Action Potential	
				Peak (mV)	Half Width (ms)
Mean	-53	305	4.4	109	1.6
S.D.	4	81	1.4	13	0.3
S.E.M.	1	21	0.4	3	0.07
N	15	15	15	15	15

RMP, resting membrane potential; IR, input resistance; τm, membrane time constant.