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Regulation of Neuronal Function by Choline and 4OH-GTS-21 Through α7 Nicotinic Receptors

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Uteshev, Vladimir V., Edwin M. Meyer, and Roger L. Papke. Regulation of neuronal function by choline and 4OH-GTS-21 through α7 nicotinic receptors. *J Neurophysiol* 89: 1797–1806, 2003. First published December 4, 2002; 10.1152/jn.00943.2002. A unique feature of α7 nicotinic acetylcholine receptor physiology is that, under normal physiological conditions, α7 receptors are constantly perfused with their natural selective agonist, choline. Studying neurons of hypothalamic tuberomammillary (TM) nucleus, we show that choline and the selective α7 receptor agonist 4OH-GTS-21 can regulate neuronal functions directly, via activation of the native α7 receptors, and indirectly, via desensitizing those receptors or transferring them into a state "primed" for desensitization. The direct action produces depolarization and thereby increases the TM neuron spontaneous firing (SF) rate. The regulation of the spontaneous firing rate is robust in a nonphysiological range of choline concentrations $>$200 μM. However, modest effects persist at concentrations of choline that are likely to be attained perineuronally under some conditions (20–100 μM). At high physiological concentration levels, the indirect choline action reduces or even eliminates the responsiveness of α7 receptors and their availability to other strong cholinergic inputs. Similarly to choline, 4OH-GTS-21 increases the TM neuron spontaneous firing rate via activation of α7 receptors, and this regulation is robust in the range of clinically relevant concentrations of 4OH-GTS-21. We conclude that factors that regulate choline accumulation in the brain and in experimental slices such as choline uptake, hydrolysis of ACh, membrane phosphatidylcholine catabolism, and solution perfusion rate influence α7 nAChR neuronal and synaptic functions, especially under pathological conditions such as stroke, seizures, Alzheimer's disease, and head trauma, when the choline concentration in the CSF is expected to rise.

INTRODUCTION

Choline is an essential physiological component of the cerebral spinal fluid (CSF) and is important for the structural integrity of cell membranes, acetylcholine (ACh) synthesis, and lipid and cholesterol transport and metabolism. Neurons grown in culture have an absolute requirement for choline (Eagle 1955). Choline is accumulated in all tissues via simple diffusion or specific carrier mechanisms (Zeisel et al. 1980). Under normal physiological conditions, the brain concentration of choline varies within a range of 10–20 μM and can rise to over 100 μM in a number of pathophysiological conditions attributed to abnormal phospholipid metabolism, such as neural trauma and chronic degenerative disorders, including Alzheimer's disease (Farooqui and Horrocks 1994; Jope and Gu 1991; Klein et al. 1997; Scremin and Jenden 1991).

Recently, choline has been identified as a selective agonist of α7 nicotinic acetylcholine receptors (nAChR) (Albuquerque et al. 1997; Mandelzys et al. 1995; Papke et al. 1996). Selective nicotinic α7 receptor activation has been shown to exert a neurotrophic function in several systems, including nerve growth factor (NGF)-differentiated PC12 cells that otherwise undergo significant degeneration when serum and NGF are removed (Martin et al. 1994). Choline exerted a similar neuroprotective activity in these cells, as well as in sympathetic ganglion cultures that express pharmacologically defined α7 nicotinic receptors (Koike et al. 1989). The nicotinic nature of the neuroprotection was demonstrated with the antagonist, mecamylamine, and the neuroprotective role of intracellular calcium was indicated by block with BAPTA (Koike et al. 1989). Choline derived from membrane phosphatidylcholine metabolism may protect α7-containing neurons selectively, accounting for the relative sparing of these receptors that has been observed in Alzheimer's disease compared with other types of nicotinic receptors (e.g., $\alpha4\beta2$; Lang and Henke 1983). Therefore choline generation during the hydrolysis of membrane phospholipids may provide a general mechanism for local cytoprotective actions that are important for maintaining the integrity of α7 nAChR-containing pathways in the brain during pathological conditions. Conversely, choline deficiency expected in a typical electrophysiological experiment due to a rapid solution perfusion may both reduce the ACh synthesis to a nonphysiologically low level and also impede intrinsic cytoprotective mechanisms.

The TM nucleus of the posterior hypothalamus represents one of the major brain centers involved in regulating multiple functions such as the arousal state; brain energy metabolism; endocrine, autonomic, and vestibular functions; locomotor activity; feeding; drinking; sexual behavior; and analgesia (Schwartz et al. 1991; Wada et al. 1991). The physiological role of α7 nAChR expression in TM neurons is not known, but the fact that the α7 subtype of nAChRs represents the only class of nicotinic receptors natively expressed in these neurons (Papke et al. 2000a; Uteshev et al. 1996) makes TM neurons unique and suggests a possible functional link between cho-

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linergic and histaminergic systems in the posterior hypothalamus.

Previous studies have shown that TM neurons are natural pacemakers, demonstrating spontaneous firing (SF) with a frequency of 1–8 Hz in the absence of synaptic inputs in slices (Haas and Reiner 1988; Llinas and Alonso 1992) or after acute dissociation (Taddese and Bean 2002; Uteshev et al. 1995). A persistent sodium current component as well as the slow calcium prepotentials were analyzed and are thought to be responsible for the TM neuron SF (Llinas and Alonso 1992; Stevens and Haas 1996; Taddese and Bean 2002; Uteshev et al. 1995). However, other sources such as prolonged α7 nAChR activation may be involved in facilitation of the SF. In the present set of experiments, TM neurons were studied in slices and in acutely dissociated form to compare how exposure to choline and 4OH-GTS-21, an α7-selective agonist, affect neuronal excitability and α7 receptor availability to subsequent cholinergic inputs.

METHODS

Chemicals

4OH-GTS-21 was synthesized and provided by Taiho Pharmaceuticals (Tokushima, Japan). All other chemicals were obtained from Sigma (St. Louis, MO).

Tissue preparation and solutions

Sprague-Dawley rats (Charles Rivers, Wilmington, MA) were used in all experiments: 2- to 4-wk-old rats in slice patch-clamp experiments and 2- to 6-wk-old in experiments with acutely dissociated neurons. The level of expression of the functional α7 nAChRs in TM neurons obtained from 2- to 6-wk-old rats was estimated by comparing response amplitudes to applications of 0.5–1 mM ACh and was found to be stable among the age groups used. The brains were removed after decapitation and placed for 1–2 min in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 126 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.3 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, and 10 glucose (pH 7.4) when bubbled with carbogen (95% O_2-5% CO_2). Two to three 300- to 400-μm-thick slices containing the TM nuclei were prepared as described previously (Uteshev et al. 1995, 2002). Slices were then transferred to the storage chamber, where they were perfused with oxygenated ACSF for $\leq$10 h. For patch-clamp slice experiments, slices were transferred to the recording chamber just before the experiment. During the patch-clamp slice experiment, slices were perfused with the oxygenated ACSF at the rate of 1.5 ml/min. Slices prepared for the acute dissociation of TM neurons were transferred to 20 ml of oxygenated ACSF, and 1–2 mg/ml papain (papaya latex in crude form, 1.9 units/mg, Sigma) was added for 50–60 min at room temperature. After papain treatment, slices were washed using the ACSF and were maintained in the ACSF at room temperature for $\leq$10 h (bubbled with carbogen).

Patch-clamp experiments with acutely dissociated neurons

Slices were placed in the experimental physiological solution composed of the following composition (in mM): 150 NaCl, 3.5 KCl, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4). Neurons from the TM nucleus were isolated and identified as described previously (Uteshev et al. 1995, 2002). Recording patch-clamp pipettes with the resistance of 2–3 MΩ were polished and filled with the following intracellular solution (in mM): 40 CsCl, 100 CsF, and 10 HEPES (pH 7.3). Data were acquired at 2–5 kHz with a sampling rate of 50–100 μs and analyzed using pClamp8 software (Axon Instruments, Union City, CA).

Patch-clamp experiments in slices

Slices were transferred to the recording chamber just before the experiment. Whole cell recordings were conducted at room temperature (22–24°C). A perfusion pump (INSTECH, Plymouth Meeting, PA) was used to perfuse slices in the recording chamber with an adjustable rate (0–1.5 ml/min). Syringe pumps were used to add experimental drugs to the perfusion flow before it entered the recording chamber. The final concentrations of drugs in the chamber were calculated based on the rates of the pumps. Typically, the 1.5 ml/min rate of the main perfusion flow was used, and a typical dilution factor for the drug delivered by the syringe pump was 100. The time delay (1–1.5 min) necessary to equilibrate solutions in the recording chamber was determined in a set of separate experiments by a slice patch-clamp recording from a TM neuron during bath applications of 100–300 μM *N*-methyl-D-aspartate (NMDA) with or without 1 mM Mg^{2+} (data not shown). A picospritzer with application pressure 10–30 PSI (Parker Instrumentation, Cleveland, OH) was used to deliver drugs to neurons via a pipette (3–7 MΩ) identical to those used for patch-clamp recordings. The intracellular electrode solution contained the following (in mM): 125 K-Glu, 1 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 2 Mg-ATP, 0.3 Na-GTP, and 10 K-HEPES (pH 7.3). The data were recorded using pClamp 9 (beta) software and a MultiClamp 700A amplifier (Axon Instruments).

High affinity [3H]methyllycaconitine binding

Hypothalami and hippocampi were rapidly dissected from killed 4- to 5-mo-old Sprague-Dawley albino rats and assayed for nicotine-displaceable, high-affinity [3H]methyllycaconitine (MLA) binding using a modification of the procedure of Davies et al. (1999). The MLA concentration used in this study was 2.3 nM, a concentration that is selective for α7 receptors. Tissues were homogenized in 20 volumes of ice cold Krebs Ringer buffer (KRH; 118 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 20 mM HEPES; pH 7.5) with a Polytron (setting 4 for 15 s). After two 1-ml washes with KRH at 20,000*g*, the membranes (10 or 90 μg protein for hypothalamus or hippocampus, respectively) were incubated in 0.5 ml KRH with 2.3 nM [^{3}H]MLA (Tocris, Ellisville, MO) for 60 min at 4°C with specified choline concentrations, $\pm$5 mM nicotine. Tissues were washed three times with 5 ml cold KRH by filtration through Whatman GF/C filters that had been preincubated for 30 min with 0.5% polyethylenimine. They were assayed for radioactivity using liquid scintillation counting. Nicotine-displaceable binding was calculated for each choline concentration in triplicate in each experiment, from which K_i values were determined using the Prizm program, using a K_d value of 1.8 nM for MLA (Davies et al. 1999).

RESULTS

Regulation of the TM neuron spontaneous firing by choline and 4OH-GTS-21

The TM neuron SF and its regulation by choline and 4OH-GTS-21 were studied in whole cell patch-clamp slice experiments. We found that bath applications of 80–320 μM choline and 3–9 μM 4OH-GTS-21 increased the TM neuron SF frequency (Figs. 1 and 2). In the presence of 1.5 μM TTX, a similar choline treatment produced a slow, sustained depolarization (Fig. 2, *A* and *B*). Note that the slow depolarization was not observed without TTX, when TM neurons exhibited a robust SF (Figs. 1 and 2). Therefore it appeared that under physiologically relevant conditions, TM neurons translate what would be a slow depolarizing cholinergic signal into a sustained increase in the SF frequency. Bath perfusion of slices with muscarine (1–3 μM) did not generate any detectable

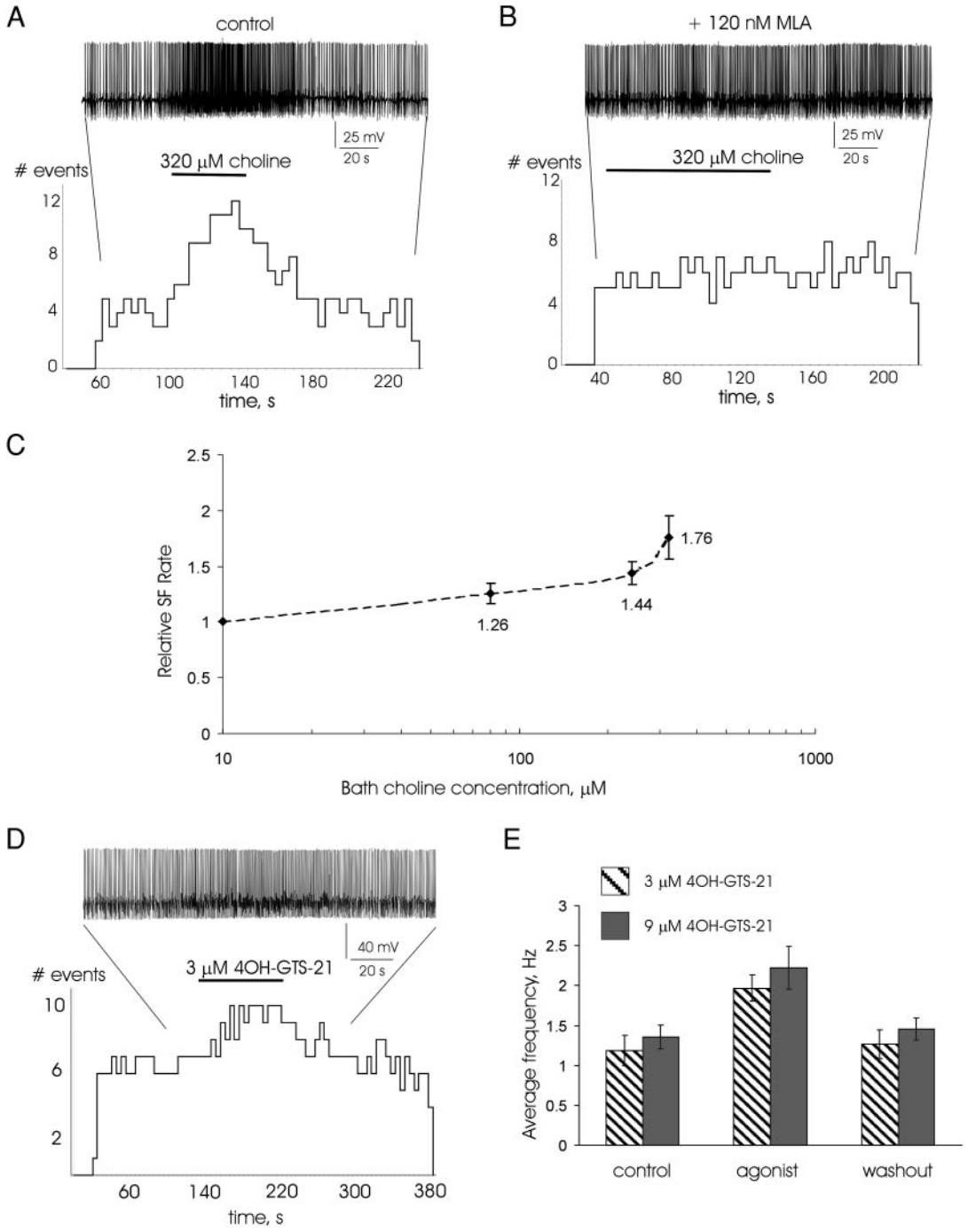

FIG. 1. Choline and 4OH-GTS-21 increase the tuberomammillary (TM) neuron spontaneous firing (SF) rate in the hypothalamic slices. Bath applications of choline (80–320 μM) and 4OH-GTS-21 (3–9 μM) reversibly increased the SF rate of TM neurons. *A*: typical trace is presented showing a TM neuron spontaneous firing under control and washout conditions and during a choline (320 μM) application. A frequency histogram that corresponds to the presented trace is shown below. The bin size was 4 s. *B*: after a 3-min washout of choline, [3H]methyllycaconitine (MLA; 120 nM) was added to the bath perfusion solution for 3–5 min. The following choline (320 μM) application did not increase the SF rate, suggesting that α7 receptors were involved. *C*: summary of results is shown. Each graph point represents an average value obtained from at least 7 experiments (4 experiments were conducted to collect data for choline concentration, 240 μM). A considerable increase in the SF rate can be observed as a result of bath choline applications when choline concentration exceeds 240 μM. A slight increase of the SF rate is expected for physiological concentrations of choline, i.e., 20–50 μM (dashed line). Choline concentrations below 10 μM did not produce any effect on the SF rates (data not shown) and therefore 10 μM choline was referred to as the effective threshold concentration. Values next to the averaged data points define the increased ratios relative to the control SF rate. *D*: similarly to choline, bath applications of 3 μM 4OH-GTS-21, a concentration equivalent to 300 μM choline in terms of activation potency, produced a reversible increase of the TM neuron SF rate. *E*: summary of several experiments is shown where 3 μM ($n = 5$) or 9 μM ($n = 4$) 4OH-GTS-21 was bath applied and the SF frequency (in Hz) was measured before and during application and after the washout.

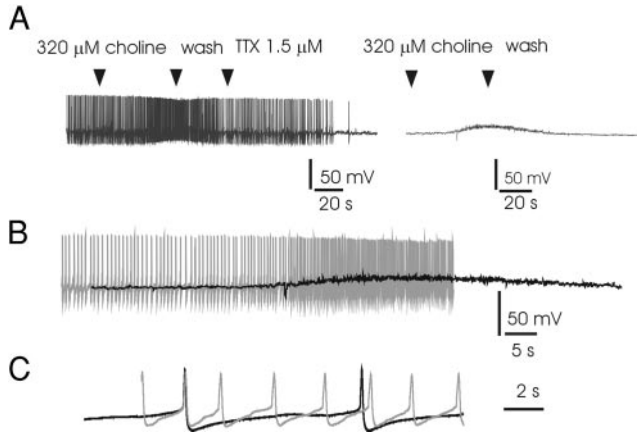

FIG. 2. The α7 receptor-mediated depolarization in TM neurons is translated into the increase in SF frequency. *A*: applications of 320 μM choline evoked an increase in the SF rate in the absence of TTX. After the application of 1.5 μM TTX, the identical 320 μM choline application evoked a slow depolarization that was not observed in the absence of TTX. *B*: traces with and without TTX shown in *A* are overlaid in *B* to emphasize that TM neurons translate the slow choline-mediated depolarization into the increase in SF rate. *C*: examples of the action potentials before (thick line) and during (thin line) 320 μM choline applications are overlaid using an expanded scale. The amplitude of action potentials is slightly reduced as the SF frequency increases, presumably due to a residual sodium channel inactivation.

depolarizing effects or changes in SF (data not shown), consistent with the results reported previously in this preparation (Uteshev et al. 1996). Since TM neurons exclusively express α-bungarotoxin- and MLA-sensitive α7 nAChRs (Papke et al. 2000a; Uteshev et al. 1996) and do not require any synaptic inputs to fire spontaneously (Haas and Reiner 1988; Llinas and Alonso 1992; Taddese and Bean 2002; Uteshev et al. 1995), and both the effect of choline on SF and the slow depolarizations were MLA-sensitive (Fig. 1, *A* and *B*), we conclude that the observed effects were most likely mediated by α7 nAChRs.

Preincubation of TM neurons in physiologically relevant concentrations of choline

Patch-clamp experiments were conducted in hypothalamic slices using brief pulses (5–10 ms) of 200 μM ACh or 2 mM choline delivered to selected TM neurons via a picospritzer while the slice was perfused with or without supplemental choline, and the resulting responses were blocked by 60–120 nM MLA (Fig. 3*A*). (Note that 200 μM ACh and 2 mM choline are equivalent in terms of α7 nAChR activation capacity; Uteshev et al. 2002.) The duration and the inter-stimulus interval of ACh applications were optimized in the beginning of each experiment to generate stable responses, prior to the addition of bath choline. Perfusion of slices with different concentrations of choline or 4OH-GTS-21 reduced or completely desensitized control responses to higher concentrations of ACh or choline. Specifically, while perfusion of slices with 10 μM choline did not affect the shape and amplitude of responses to 200 μM ACh (data not shown), preincubation in choline 20–80 μM for 2–5 min decreased responses to 200 μM ACh in a concentration-dependent manner (Fig. 3, *B* and *C*). Recovery from this inhibition was full and rapid and occurred within the first 3–5 min of perfusion of the slice with choline-free solution (Fig. 3*B*).

Since a picospritzer was used, the final agonist concentration that reached the selected TM neuron in a slice could not be determined precisely due to dilution, leak, and diffusion of agonist during and between applications. Therefore to better quantify the effects of choline inhibition, we conducted parallel experiments using a rapid agonist application system and acutely dissociated TM neurons, where agonist concentration in the vicinity of a selected neuron between and during agonist application, as well as the application durations themselves, could be well controlled and monitored.

Acutely dissociated TM neurons were patch-clamped and exposed to rapid solution exchanges as described previously (Papke et al. 2000a; Uteshev et al. 2002). We determined the fraction of a full α7 nAChR response to relatively high agonist concentrations, such as 50 μM, 200 μM, and 1 mM ACh, under conditions of prolonged or even tonic receptor activation/desensitization that produced by physiologically relevant low choline concentrations (10–100 μM). Consistent with the observations made in hypothalamic slice recordings, preincubation of TM neurons in low choline concentrations for 2–5 min reduced the current responses to 0.05–1 mM ACh (Fig. 4).

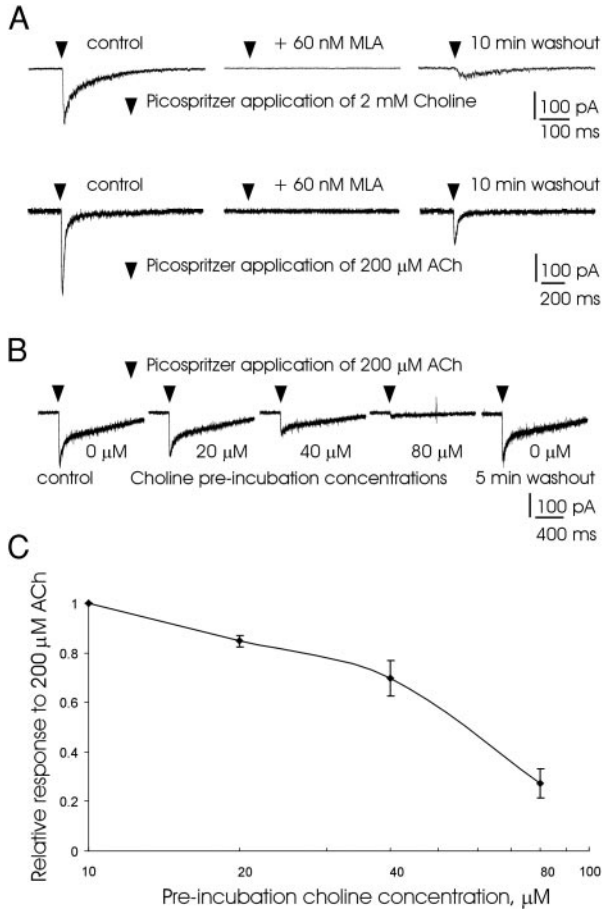

FIG. 3. Choline preincubation reduces the α7 receptor responsiveness in the hypothalamic slices. A picospritzer was used to deliver 200 μM ACh to a selected TM neuron in slices. *A*: picospritzer applications evoked rapid responses, sensitive to 60–120 nM MLA and slowly reversible. *B*: typical example of an experiment where 200 μM ACh-mediated responses were reduced by 2–5 min perfusion of slices with physiologically relevant concentrations of choline. *C*: summary of data obtained from 5 experiments is shown. Choline concentrations below 10 μM did not produce any effect on the α7 receptor responses (data not shown), and therefore 10 μM choline was referred to as the effective threshold concentration.

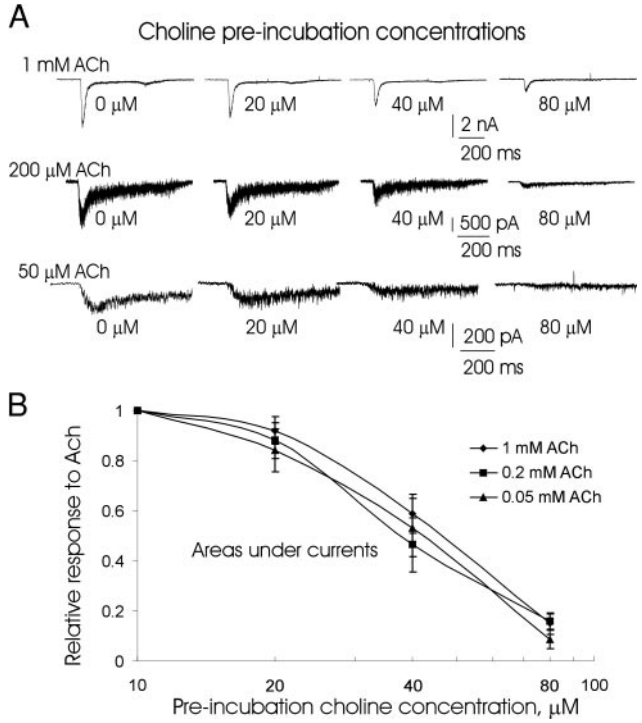

FIG. 4. Choline preincubation reduces the α7 receptor responsiveness in experiments with acutely dissociated TM neurons. Rapid applications of 1 mM, 200 μM, and 50 μM ACh were employed to determine quantitatively the inhibitory effects of physiological concentrations of choline on the responsiveness of α7 receptors and their availability to cholinergic inputs of varying strengths. *A*: typical examples of responses to rapid applications of 1 mM, 200 μM, and 50 μM ACh in the absence or presence of physiologically relevant choline concentrations are shown. Choline preincubation lasted for 2–5 min before the preincubation solution was rapidly replaced (approximately 6–8 ms) (Uteshev et al. 2002) by the application solution containing ACh. *B*: graph represents a summary of results where current response areas (net charge) over a 200-ms-long interval between 30 and 230 ms after the beginning of application were measured. Each graph point represents an average of results obtained from at least 4 experiments. Similar curves have been obtained when current peaks were measured (data not shown).

Figure 4*B* summarizes experimental results showing that low concentrations of choline (20–80 μM) would be sufficient to create a sustained low level of receptor occupancy and a state primed for desensitization that would make α7 nAChRs less effective in generating whole cell responses or excitatory postsynaptic currents (EPSCs). Interestingly, the degree of inhibition of control ACh responses during preincubation of TM neurons in 20–80 μM choline has been relatively insensitive to ACh concentrations used for the control application, i.e., 50 μM, 200 μM, or 1 mM. However, while it is reasonable to suggest that responses to lower ACh concentrations ($<$50 μM) may be more susceptible to 20–80 μM choline-mediated inhibition, such responses would be difficult to quantify because of the low signal-to-noise ratio.

We have previously reported that when high agonist concentrations are applied, the α7 nAChR-mediated current reaches its peak before the completion of the agonist exchange process (Uteshev et al. 2002). Moreover, the response of α7 nAChRs to low agonist concentrations is slow and prolonged and may represent an important physiological modality of α7 nAChR function associated with a considerable calcium influx (Papke et al. 2000a; Uteshev et al. 2002). Therefore as we suggested previously, it may be advantageous to characterize α7 nAChR responses by evaluating net charge under the late current phase (Uteshev et al. 2002). However, the results of the present study indicate that both current net charge (Fig. 4*B*) and peak (data not shown) are equally informative in how they describe the choline-mediated inhibition of receptor responsiveness.

Preincubation of acutely dissociated TM neurons in low clinically relevant concentrations of 4OH-GTS-21

The low range for choline in CSF has been estimated to be approximately 10 μM. This concentration would correspond roughly to 50 nM of 4OH-GTS-21, in terms of relative potencies estimated by the current peak (i.e., nonequilibrium conditions) (Uteshev et al. 2002). For 4OH-GTS-21, the range of 100–200 nM might also correspond to the threshold for seeing therapeutic effects. Therefore we sought to address the question of what fraction of a full α7 nAChR response to a high agonist concentration would remain under conditions of a constant presence of prolonged α7 nicotinic receptor activation, comparable with what might be produced by a low borderline therapeutic concentration of 4OH-GTS-21. Typical examples of a control α7 nAChR response evoked by the application of 200 μM 4OH-GTS-21 and a response after preincubation for 10 min or longer in 200 nM of 4OH-GTS-21 are shown in Fig. 5, *A* and *B*. These estimates are consistent with the results reported previously that demonstrated a 40–60% inhibition of 1 mM ACh-mediated transient responses after TM neurons were preincubated in 1–3 μM ACh (Uteshev et al.1996).

Note that the effects of 4OH-GTS-21 preincubation were different on the transient peak current (45% inhibition) than on the late current, which rises and then decays after the washout of the 200 μM 4OH-GTS-21 and return to 200 nM 4OH-GTS-21 (only 24% inhibition). This suggests that the preincubation effects of low agonist concentrations are most active at attenuating transient currents mediated by α7 nAChRs and yet may be relatively ineffectual at reducing currents in the late phase of an evoked response, as the channels equilibrate between open and desensitized states, even in the presence of high concentrations of agonist.

We conducted parallel experiments to determine what fraction of a full α7 nAChR response to low and high concentrations of ACh (i.e., 50 μM and 1 mM) would remain during a preincubation of TM neurons in low borderline therapeutic concentrations of 4OH-GTS-21, i.e., 200–400 nM (Fig. 5, *D* and *E*). The results show that 200–400 nM 4OH-GTS-21 produced inhibition essentially equivalent to that produced by 40–80 μM choline (compare Figs. 5*E* and 4*B*).

Effects of preincubating TM neurons in choline on the increase in TM neuron excitability

Figure 4 shows that preincubation of TM neurons in low concentrations of choline reduces the responsiveness of α7 nAChRs and their availability to subsequent cholinergic inputs, and therefore, may alter the effects of nicotinic agonists on the neuronal excitability (Fig. 1). Here we investigated how α7 nAChR desensitization, induced by low concentrations of choline, adjusts the sensitivity of TM neuron SF to high concen-

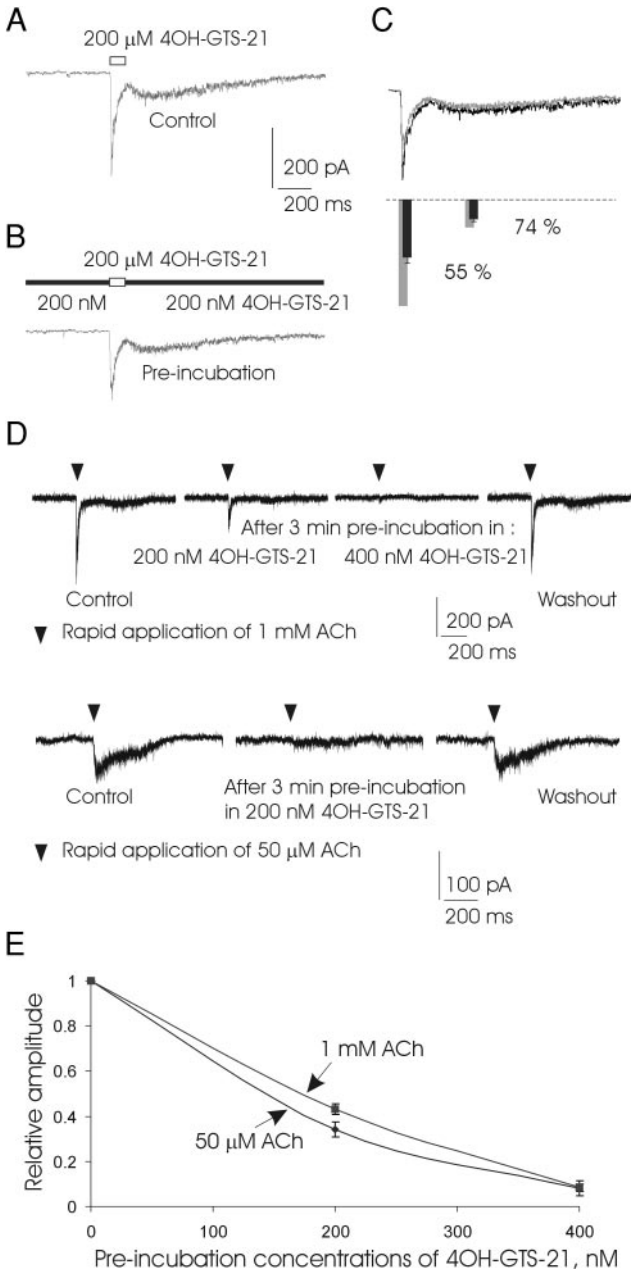

FIG. 5. Preincubation in clinically relevant concentrations of 4OH-GTS-21 reduces the α7 receptor responsiveness in acutely dissociated TM neurons. Examples of control responses to applications of 200 μM 4OH-GTS-21 before (*A*) and 15 min after (*B*) preincubation of TM neurons in 200 nM 4OH-GTS-21. *C*: preincubation-mediated reduction in the response amplitude. The current peak decreased by 45%, while the current rebound decreased by 26% (n = 6). *D*: preincubation of TM neurons in 200 and 400 nM 4OH-GTS-21 reduced the response amplitudes to 1 mM and 50 μM ACh. *E*: summary of results obtained from at least 3 experiments at each of the indicated combinations of 4OH-GTS-21 preincubation and rapid ACh application.

trations of choline (300 μM). We bath-applied choline (20–200 μM) for 1–3 min before bath-applying 300 μM choline to evoke the control increases in SF (Fig. 6). The results show that the 20–80 μM choline-mediated reduction of α7 nAChR responses did not significantly alter the 300 μM choline-mediated increase of the SF rate (Fig. 6*B*). However, higher choline concentrations, such as 200 μM choline, which themselves affected SF rate, did significantly reduce the increase in SF rate (Fig. 6*B*) produced by applications of 300 μM choline. This observation suggests that although 20–80 μM choline reduced the transient whole cell α7 nAChR responses to brief applications of ACh (0.05–1 mM), it preserved a sufficient activation capacity of the receptors to modulate the basic excitability of TM neurons.

Choline displacement of radiolabeled MLA

The concentration response studies for the activation of rat α7 receptors by choline and 4OH-GTS-21 have been previously published (Papke and Papke 2002). However, it is well documented that the equilibrium affinities of nAChR for agonist may be significantly higher than agonist potency for activation. Therefore equilibrium binding experiments with hippocampal or hypothalamic tissues were conducted to estimate the degree to which agonist binding sites would be occupied by choline under our preincubation conditions. The data (data not shown) indicated that choline displaced radiolabeled MLA with K_i values of 59 ± 12 and 42 ± 6 μM in the hippocampal and hypothalamic tissues, respectively.

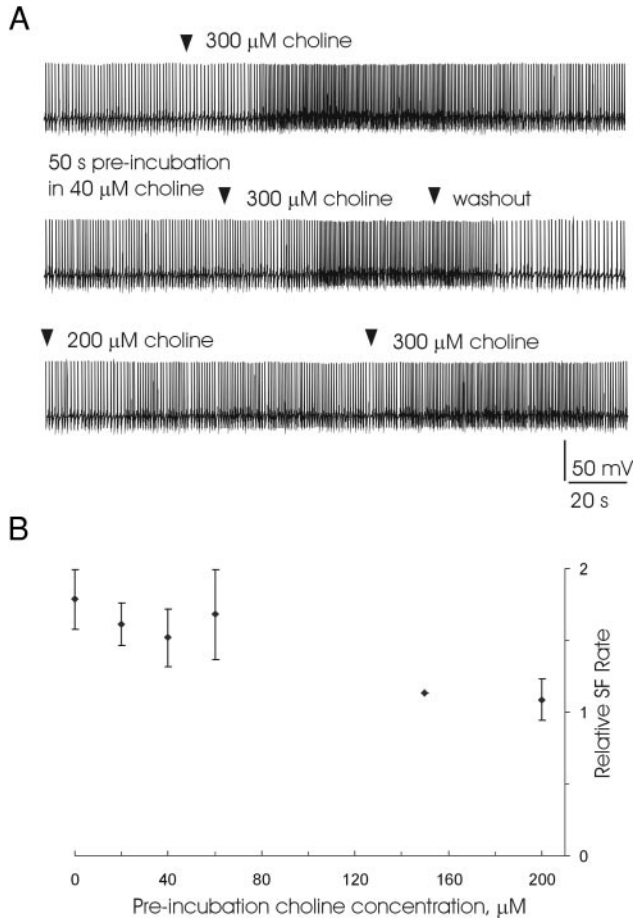

FIG. 6. Choline preincubation reduces the sensitivity of TM neuron SF to choline in slices. *A*: influence of preincubation in 20–60 μM choline on the 300 μM choline-mediated increase in the SF rate has been investigated. Bath applications of 300 μM choline led to an increase in the TM neuron SF rate in slices ($n > 16$). *B*: this increase was not significantly altered by preincubations in 40–60 μM choline (n = 8). Preincubation in 200 μM choline significantly reduced the increase in the SF rate evoked by 300 μM choline (n = 3). A single data point at 150 μM choline corresponds to 1 experiment.

DISCUSSION

In this study, we show that choline and 4OH-GTS-21 can regulate TM neuron function by activating or desensitizing α7 nAChRs. Prolonged or phasic α7 nAChR activation may be involved in a direct regulation of the SF. Strong facilitation of SF is associated with the depolarization of TM neurons, which might occur in the brain under a number of pathological conditions that raise the extracellular choline concentration above 80–100 μM or conditions where there is a relatively high level of an exogenous agonist. Presumably, choline and 4OH-GTS-21 modulate TM neuron SF by augmenting the excitatory action of the persistent sodium channels, which are primarily responsible for maintaining the firing of TM neurons (Llinas and Alonso 1992; Taddese and Bean 2002; Uteshev et al. 1995). The excitation of TM neurons, seen as an α7 receptor-mediated depolarization, can be obtained in agonist-concentration ranges where α7 nAChR desensitization is apparently not limiting. The distinct modality of α7 nAChR activation associated with low agonist concentrations is characterized by minimal slow receptor desensitization and thus also significant net charge (Uteshev et al. 2002). Moreover, our results indicate that slight increases in the TM neuron SF rate produced by low agonist concentrations are expected to last longer than robust increases in excitation evoked by high agonist concentrations. This is consistent with other reports of synaptic modulation by α7 nAChRs at low agonist concentrations persisting over prolonged periods of time (Mansvelder et al. 2002).

α7 nAChR agonists can modulate neuronal function indirectly by promoting α7 nAChR desensitization or transferring receptors into a state primed for desensitization. Each of these actions would be predicted to alter the fraction of potential α7 nAChRs available for information processing via fast synapses. The effects of prolonged exposure to low concentrations of agonist (i.e., preincubation), which inhibit the α7 nAChR responses to rapid applications of high agonist concentrations, are likely to be physiologically important because any strong cholinergic signals received by α7 nAChRs, either in the form of diffuse volume transmission (Descarries et al. 1997) or fast cholinergic synapses (Alkondon et al. 1998; Frazier et al. 1998; Gray et al. 1996; Hatton and Yang 2002; Ji and Dani 2000; Radcliffe and Dani 1998), will necessarily be received over background effects of choline. It is important to note that the effects that we observed with TM neurons in hypothalamic slices can be generalized to other cell populations expressing high levels of α7 nAChRs, such as the interneurons of the hippocampus (C. J. Frazier, personal communication).

α7 nAChRs have sometimes been characterized as "low-affinity" neuronal nAChRs. In part, this is because the unique fast desensitization of α7 nAChRs requires the use of high concentrations of agonist to evoke large transient currents. However, using net charge analysis, we have recently shown that effective concentrations for channel activation are 10-fold lower than previously believed (the EC_{50} values for choline and 4OH-GTS-21 are 415 and 1.6 μM, respectively; Papke and Papke 2002; Uteshev et al. 2002). Additionally, α7 receptors have been characterized as low affinity because they do not show the same large increase in affinity on desensitization that has been detected in equilibrium binding experiments with other subtypes of nAChR. For example, while nicotine has an EC_{50} of 50 μM for the activation of $\alpha4\beta2$ receptors (Papke et al. 2000b), in equilibrium binding experiments, nicotine binds with a K_d of about 10 nM (Cairns and Wonnacott 1988; Reavill et al. 1988). It has been previously reported that the K_i of 4OH-GTS-21 for the displacement of radiolabeled α-bungarotoxin was 170 nM (Meyer et al. 1998), 9.4-fold lower than the EC_{50} for receptor activation, suggesting that the binding did not undergo the same affinity increase during membrane preparation as seen with other receptor subtypes. To determine whether this phenomenon was seen with other selective α7 nAChR agonists and antagonists, similar equilibrium binding experiments were conducted with choline and labeled MLA. These results indicated that choline displaced radiolabeled MLA with K_i values of approximately 50 μM, a value which is also about eightfold lower than the EC_{50} for α7 receptor activation, suggesting that the relatively modest increase in affinity seen during equilibrium binding studies with α7 nAChRs is independent of the ligands selected.

The preincubation concentrations of choline and 4OH-GTS-21 that inhibit transient (i.e., nonequilibrium) agonist-evoked responses roughly correspond to the concentrations at which 50% equilibrium binding would be expected. There are several potential hypotheses that would account for this observation, including one that we proposed previously to account for a variety of properties of α7 nAChRs. This hypothesis states that (Papke et al. 2000a) the open probability of α7 nAChRs is greatest for intermediate levels of fractional occupancy (e.g., 2 or 3 of 5 possible agonist binding sites occupied), and that receptors with higher levels of agonist occupancy are more likely to be in closed or desensitized states than in the open state. This model accounts for the fast transient current that occurs during the jump to a saturating concentration of agonist (Papke et al. 2000a; Uteshev et al. 2002). It may also be used to explain the effects of choline and 4OH-GTS-21 preincubations. We can use the equilibrium affinity to predict the relative levels of agonist occupancy among the receptors following preincubation. With 50% of all agonist binding sites occupied, the predicted percentages of receptors with 0, 1, 2, 3, 4, or 5 agonist binding sites occupied are 3%, 16%, 31%, 31%, 16%, and 3%, respectively. If this is the steady-state occupancy before a jump to a higher (i.e., saturating) concentration of agonist, then based on the model, approximately 50% (i.e., $31 + 16 + 3$) of the receptors would already be in a state where more agonist binding would not make them more likely to open. This could account for the decrease in the amplitude of the transient current evoked by the jump to high agonist concentration. Likewise, with 50% site occupancy, 20% of the receptors would be at the two highest levels of fractional agonist occupancy already, and may therefore represent the percentage of receptors preequilibrated into the slow desensitized state. This value corresponds to the inhibition of the late current which arose and then decayed with the washout of the 200 μM 4OH-GTS-21 following preincubation with 200 nM 4OH-GTS-21 (Fig. 5*C*).

Numerous groups have used brain slice preparations to investigate the role of α7 receptors in synaptic function (Alkondon et al. 1998; Frazier et al. 1998; Gray et al. 1996; Hatton and Yang 2002; Ji and Dani 2000; Radcliffe and Dani 1998). Ambient levels of choline in the tissue has largely been an ignored parameter in these experiments. Our data indicate that fluctuations in free choline concentrations will modulate some

α7 nAChR-mediated effects and consequently, their physiological importance, in subtle manners, with more dramatic effects likely under conditions that elevate extracellular choline. At typical bath perfusion rates (e.g., 2 ml/min), choline levels in the slice may fall to as low as one-third normal (V. V. Uteshev, R. L. Papke, and L. Prokai, unpublished observation), so that in a typical electrophysiological experiment, in which brain slices are perfused with choline-free ACSF, the basal α7 nAChR occupancy may be particularly low. Adding physiologically relevant concentrations of choline to the perfusion ACSF solutions may be appropriate in studies concerned with the potential effects of decreases in receptor availability due to desensitization.

Choline is essential in the CNS for the biosynthesis of both ACh and some membrane phospholipids. The level of ambient choline in the CSF is normally below 20 μM due to a dynamic equilibrium involving availability from the bloodstream and removal by low- and high-affinity uptake mechanisms (Klein et al. 1992). Our experiments show that low basal choline levels induce no more than 20% inhibition of α7 nAChR responsiveness to subsequent strong (i.e., 0.05–1 mM ACh) cholinergic inputs. While, weaker cholinergic inputs ($<$50 μM ACh) may be more sensitive to inhibitory effects of physiological choline, alternatively, there might be synergistic effects between physiological levels of choline and low levels of ACh resulting from volume transmission (Descarries et al. 1997) or the decaying phase of strong cholinergic inputs. One function of baseline choline levels might therefore be to tune cholinergic information transfer among neurons in multiple ways. In some cases, basal choline may have the effect of reducing cholinergic background noise and enhancing the effects of volume transmission, and in other cases, making α7 nAChRs less available for low-intensity cholinergic signals, yet preserving the responsiveness of α7 nAChRs to strong cholinergic inputs.

In addition to the low ambient concentrations of choline in the brain, rapid transient increases in extracellular ACh concentrations and thus proportionate increases in choline levels are expected as a result of multiple synchronous releases of ACh from cholinergic varicosities in the vicinity of synaptic or nonsynaptic specializations in the brain areas packed with cholinergic fibers. Under basal conditions, in the presence of a choline esterase inhibitor, the level of ACh detected by microdialysis in the hippocampus is only about 10-fold lower than the ambient choline level (Koppen et al. 1997). This level of ACh corresponds to the ACh that derives directly from the synaptic activity in the hippocampus since when the synaptic release of ACh is augmented by the inhibition of muscarinic autoreceptors with atropine, ACh levels increase fivefold, and choline levels show a further twofold increase (Koppen et al. 1997).

While the significance of physiological fluctuations in choline concentrations relative to α7 nAChR function remains to be more carefully elucidated, our data do suggest several possibilities. The tonic influx of low concentrations of calcium ions associated with either nicotinic receptor activation or activation of other receptors can be neuroprotective, so it is feasible that the basal activity provided by extracellular choline may be additive with other calcium-elevating systems to modulate cell survival, especially in complex, multiple-receptor systems such as the brain. Additionally, while, the desensitization induced by low choline concentrations would probably not impact most strong cholinergic signals by more than 10–20%, this effect would be significantly greater if choline concentrations were somehow elevated, either from hydrolysis of ACh or from pathological conditions described below. Regarding ACh-hydrolysis, any choline that diffused within or from synaptic sites would be likely to modulate α7 nAChR function to a much greater extent than would be seen with circulating choline concentrations. In particular, two possibilities regarding this residual, high level of choline suggested from our data are *1*) transmission from rapid, repeated cholinergic firing could be reduced by nAChR desensitization induced by residual synaptic choline; and *2*) intracellular calcium concentrations would likely be modulated both by choline that diffused from the synapse to extrasynaptic nAChR receptors as well as by cholinergic synaptic processes, making this a particularly complex system.

There are also a variety of other processes that have been found to increase extracellular choline concentrations dramatically, including NMDA receptor activation (Zapata et al. 1998), cellular dysfunction, energy deprivation (Djuricic et al. 1991), and cell death by excitotoxicity (Gasull et al. 2000) or ischemia (Rao et al. 2000). Cell death is well known to lead to the breakdown of phosphatidylcholine, the principle plasma membrane phospholipid, to choline and diacylglycerol, providing a large source of the α7 nAChR agonist. All of these conditions that create local elevations in choline may impact neuronal function through α7 nAChR receptors. It is intriguing to hypothesize that this choline may be sufficient to protect other, local neurons from the same toxic insult, although it should be noted that $\geq$3 h of α7 receptor activation is required prior to most insults for neuroprotection to be observed (Li et al. 1999); this accounts for the inability of localized choline to protect against ischemic damage in stroke since there is no pretreatment interval in that condition (Shimohama et al. 1998).

It is interesting to note that α7 nAChR expressing neurons in the hippocampus appear to be mostly spared in Alzheimer's disease, a slowly progressive disorder, despite widespread and profound neuronal loss in that region, as well as the loss of ascending cholinergic synaptic inputs. Amyloid-induced neuronal dysfunction is hypothesized to be a component of this disease, based on the accumulation of plaques containing this peptide. Agonist induced activation of α7 receptors prevents neurotoxicity induced by amyloid peptides in several model systems in a manner that is blocked by selective α7 antagonists (Kihara et al. 2001; Shimohama and Kihara 2001). Whether activation by physiologically relevant choline concentrations, or, more likely, choline from dying neurons, provides similar protection against Alzheimer's disease is a possibility that remains to be ascertained. However, it should be noted that evaluating the physiological significance of α7 receptor activation is complicated by a variety of observations. For example, α7 antagonists and agonists respectively interfere with and improve memory related behaviors (Rezvani and Levin 2001), yet α7 receptor knockout mice appear to have few behavioral deficits (Paylor et al. 1998). Amyloid peptides that accumulate in Alzheimer's disease have been reported to block α7 receptors at nanomolar concentrations (Liu et al. 2001). However, it has also been suggested that amyloid peptides may activate α7 receptors at picomolar concentrations (Dineley et al. 2002). Therefore it is not clear how choline-induced activation or

desensitization of α7 receptors would interact with these possibly dose-dependent actions of amyloid peptides, or whether this interaction would even remain constant throughout the course of the disease as amyloid load increases. Nonetheless, our results allow us to hypothesize that high levels of extracellular choline found near dying neurons in this disease may protect nAChR-expressing neurons in two manners, both involving these receptors: *1*) through inactivation of nAChRs that are otherwise potential targets for amyloid; and *2*) tonically activating the few remaining nondesensitized receptors, permitting a small calcium influx that is neuroprotective.

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