ABSTRACT

Choline is an essential nutrient and a precursor of neurotransmitter acetylcholine (ACh) and is produced at synapses during depolarization, upon hydrolysis of ACh via acetylcholinesterase, and under conditions of injury and trauma. Animal studies have shown that supplementation with choline during early development results in long-lasting improvement in memory in adults; however, the mechanisms underlying this effect are poorly defined. Previous studies revealed that choline interacts with type IA (α7*) nicotinic acetylcholine receptors (nAChRs) as a full agonist and as a desensitizing agent and is a weak agonist of type III (α3β4*) nAChRs. Because nAChRs play a role in learning and memory and are generally inhibited by agonists at low concentrations, we investigated in this study the inhibitory effects of choline on non-α7 nAChRs such as type II (α4β2*) and type III nAChRs. Using whole-cell patch-clamp recordings from neurons of rat hippocampal and dorsal striatal slices, we demonstrate that choline inhibited type III nAChR-mediated glutamate excitatory postsynaptic currents (EPSCs). Choline inhibited ACh-induced N-methyl-D-aspartate (NMDA) EPSCs in CA1 stratum radiatum (SR) interneurons of rat hippocampal slices with an IC50 of ~15 μM. Choline did not inhibit NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor EPSCs in CA1 SR interneurons. Choline inhibited type II nAChRs in CA1 SR interneurons with an IC50 of ~370 μM. The present results reveal an order of inhibitory potency for choline type III > type IA > type II nAChRs. It is concluded that brain nAChRs, but not glutamate receptors, are the primary targets for the regulatory actions of choline.

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ABBRévIATIONS: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; AChE, acetylcholinesterase; NMDA, N-methyl-D-aspartate; ACSF, artificial cerebrospinal fluid; SR, stratum radiatum; EPSC, excitatory postsynaptic current; MLA, methyllycaconitine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SLM, stratum lacunosum moleculare; M.P., membrane potential.
mentioned conditions result in approximately 3- to 4-fold increase in the level of choline from that of control. Thus, the free extracellular concentration of choline is likely to reach a minimum of 9 to 20 μM under various pathological and physiological depolarizing conditions. Another major source of choline is at central cholinergic synapses during the breakdown of ACh by AChE. Because ACh is released at cholinergic synapses at millimolar concentrations, it can be surmised that similar concentrations of choline can also be generated at microdomains near central cholinergic synapses, particularly during high-frequency firing of cholinergic neurons. Furthermore, nutritional supplements with choline enhance the levels of choline in the brain and have been recommended for memory enhancement (Klein et al., 1998; Zeisel, 2000). Because the high-affinity choline uptake system is not fully matured in the developing brain (Klein et al., 2002), it is likely that during the fetal and early postnatal period excess free choline will be present in the brain. All these conditions favor an active role for choline at sensitive neuroreceptors and compel the need to identify various choline-sensitive receptors.

Because cholinergic and glutamatergic terminals remain apposed to each other in the brain (Garzón et al., 1999) and glutamate axons are enriched with AChE (Schlaggar and O’Leary, 1994), choline produced at the synapses can interact with type III nAChR present on the glutamate axons. Because both hippocampus and dorsal striatum are regions innervated by cholinergic afferents (Frotscher and Léránt, 1985; Holt et al., 1996), in the present experiments, we tested the possibility that choline inhibits type III nAChRs in hippocampal and dorsal striatal slices from Sprague-Dawley rat brain.

Materials and Methods

Hippocampal Slices. Slices of 250-μm thickness were obtained from the hippocampus of 10- to 23-day-old rats according to the procedure described earlier (Alkondon et al., 2003). Sprague-Dawley rats (Zivic-Miller Laboratories, Newcastle, PA) of both genders were used. Animal care and handling were done strictly in accordance with the guidelines set forth by the Animal Care Committee of the University of Maryland, Baltimore. Slices were stored at room temperature in artificial cerebrospinal fluid (ACSF), which was bubbled with 95% O2 and 5% CO2 and composed of 125 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 2 mM CaCl2, 1 mM MgCl2, and 25 mM glucose. Stratum radiatum (SR) interneurons in the CA1 field of the slices were visualized by means of infrared-assisted videomicroscopy for patch-clamp recordings. In addition, biocytin labeling was used to identify the neurons morphologically.

Dorsal Striatal Slices. Slices of 275-μm thickness were obtained from coronal sections of the brain. Regions of the sections containing dorsal striatum were used. Sprague-Dawley rats (Zivic Miller) of both genders were used. Handling of the slices and identification of the neurons were done by the procedure similar to those for hippocampal slices. Electrophysiological recordings were performed on bipolar-shaped or large rectangular-shaped neurons.

Electrophysiological Recordings. Excitatory postsynaptic currents (EPSCs) and agonist-evoked whole-cell currents were recorded from the soma of various neurons according to the standard patch-clamp technique using an LM-EPC7 amplifier (List Electronic, Darmstadt, FRG). Agonists were applied to the slices via a U-tube, and antagonists were applied via either bath perfusion or via both U-tube and bath perfusion (Alkondon et al., 2003). In some experiments, synaptic NMDA currents were evoked by stimulating Schaffer collaterals using a bipolar electrode. The bipolar electrode was placed in the SR region toward CA3 200 to 300 μm away from the CA1 SR interneuron being recorded. Signals were filtered at 3 kHz and either recorded on a videotape recorder for later analysis or directly sampled by a microcomputer using the pClAMP 9 program (Axon Instruments, Foster City, CA). Neurons were superfused with ACSF at 2 ml/min. Atropine (0.5 μM) was added to the ACSF to block the muscarinic receptors. Bicuculline (10 μM) was added to ACSF to block GABA A receptor activity. Methyllycoclamine (MLA; 10 nM) was included in the ACSF while studying nontype IA nAChR responses. Patch pipettes were pulled from borosilicate glass capillary (1.2-mm outer diameter) and, when filled with internal solution, had resistance between 3 and 5 MΩ. The series resistance ranged from 8 to 20 MΩ. At −68 mV, the leak current was generally between 50 and 150 pA, and when it exceeded 200 pA, the data were not included in the analysis. The internal pipette solution contained 0.5% biocytin in addition to 10 mM ethylene-glycol bis(β-amino-ethyl ether)-N,N’,N’-tetraaetic acid, 10 mM HEPES, 130 mM cesium methane sulfonate, 10 mM CaCl2, 2 mM MgCl2, and 5 mM lidocaine N-ethyl bromide (pH adjusted to 7.3 with CsOH; 340 mosm). Membrane potentials were corrected for liquid junction potentials. All experiments were carried out at room temperature (20–22°C).

Data Analysis. The frequency, peak amplitude, 10 to 90% rise time, and decay-time constant of AMPA EPSCs were analyzed using WinEDR V2.3 (Strathclyde Electrophysiology Software, Glasgow, UK). The peak amplitude of nicotinic currents and the net charge of NMDA receptor-mediated EPSCs and nicotinic currents were analyzed using the pCLAMP9 software (Axon Instruments). Typically, the net charge of agonist-evoked responses was calculated for the duration of the agonist pulse starting from the valve opening. Results are presented as mean ± S.E.M. and compared for their statistical significance by Student’s t test or analysis of variance. Inhibition concentration-response curves were fitted to a Hill equation

\[ I = I_{\text{max}} \cdot A^n / (A^n + IC_{50}^n), \]

where \( I \) is the measured current amplitude or net charge, \( I_{\text{max}} \) is the maximum current amplitude or net charge, \( A \) is the inhibitor concentration, \( n_4 \) is the Hill coefficient, and \( IC_{50} \) is the inhibitor concentration that results in half-maximal response to the agonist.

Drugs Used. ACh chloride, atropine sulfate, (−)-bicuculline methiodide, choline chloride, glycine, lidocaine N-ethyl bromide, NMDA, and trimethylamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). (−)-Mecamylamine.HCl was a gift from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ). MLA.HCl was a gift from Professor M. H. Benn (Department of Chemistry, University of Calgary, AB, Canada). Stock solutions of all drugs were made in distilled water.

Results

Choline Inhibits Type III nAChRs: Evidence from ACh-Induced AMPA EPSCs in Rat Hippocampal Slices. In the presence of muscarinic receptor antagonist atropine, GABA A receptor antagonist bicuculline, and α7 nAChR antagonist MLA, U-tube application of ACh to CA1 SR interneurons induced a burst of AMPA EPSCs at −68 mV (Fig 1A). This response seems to originate from activation of type III nAChRs, which are sensitive to block by low concentrations of mecamylamine (Alkondon et al., 2003). The frequency of AMPA EPSCs during application of ACh was significantly higher than that observed during resting conditions (Fig. 1G). Bath exposure of the slices to choline (300 μM) did not change the frequency of spontaneous events; however, it resulted in a significant reduction in the frequency of ACh-induced AMPA EPSCs (Fig. 1, B and G). Furthermore, bath application of choline did not change the peak amplitude of spontaneous AMPA EPSCs, indicating that choline does not inhibit AMPA receptors. For example,
Choline Inhibits Type III nAChRs: Evidence from ACh-Induced NMDA EPSCs in Rat Hippocampal Slices. U-tube application of ACh to CA1 interneurons at +40 mV induced outward-going NMDA EPSCs in the presence of atropine, bicuculline, and MLA (Fig. 2, A–D). Like the AMPA EPSCs, the NMDA EPSCs induced by ACh result from the activation of mecamylamine-sensitive type III nAChRs (Alkondon et al., 2003). A 10-min bath exposure of hippocampal slices to various concentrations of choline (10–1000 μM) resulted in a concentration-dependent reduction in the magnitude of ACh-induced NMDA EPSCs (Fig. 2, A–F). The inhibitory effect of choline was reversed upon a 10- to 20-min wash with choline-free ACSF. On average, approximately 70% of the response returned to control levels after the wash (Fig. 2E). Because incomplete reversal in some experiments could be accounted for by a slow rundown of responses with time, the inhibitory effect of choline was calculated based on the average response from control and wash. The plot of the mean net charge of ACh-induced NMDA EPSCs against various concentrations of choline revealed a concentration-dependent inhibition (Fig. 2F) with an IC₅₀ of 14.6 μM and a Hill coefficient of 1.29. However, it was also noticed that at concentrations of choline ≤ 3 μM, there was a slight enhancement in the magnitude of ACh-induced NMDA EPSCs (Fig. 2F).

To verify that the inhibitory effect of choline occurred at the type III nAChR and not at the NMDA receptors, we tested the effect of choline on NMDA responses evoked via three different mechanisms in CA1 SR interneurons. For this purpose, outward currents evoked by U-tube application of NMDA in the presence of glycine, sucrose-induced NMDA EPSCs (see Alkondon and Albuquerque, 2005), and Schaffer-collateral-stimulated NMDA EPSCs were tested at +40 mV. A 10-min bath exposure of the slices plus U-tube application of choline (300 μM) had minimal inhibitory effect of both NMDA-evoked currents and sucrose-evoked NMDA EPSCs (Fig. 3, A, B, and E). Likewise, bath exposure of hippocampal slices to 100 μM and 1 mM choline had only a marginal inhibitory effect on Schaffer-collateral-evoked NMDA EPSCs (Fig. 3, C, D, and E). These results confirmed that the inhibitory effect of choline at type III nAChR-triggered EPSCs did not result from blockade of either NMDA receptors or presynaptic sites controlling transmitter release.

Next, we tested the mode of interaction of choline with type III nAChR. If choline interacted by competing with ACh for the agonist-binding site, increasing the concentration of ACh would result in a decrement in the inhibitory response to choline. To test this assumption, we induced NMDA EPSCs against various concentrations of choline (10–1000 μM) in the presence of glycine and sucrose-evoked NMDA EPSCs using a U-tube application of ACh at 30 μM or 1 mM and compared the results with that obtained at 100 μM ACh. The degree of inhibition by choline did not significantly differ from each other at various ACh concentrations used (Fig. 4). These results ruled out a competitive mode of interaction between choline and ACh at type III nAChR. Therefore, it is likely that choline being a weak agonist of type III nAChR binds to the receptor, and such choline-bound receptor undergoes transitions to a desensitized state that cannot be activated by subsequent applications of ACh.

Next, we analyzed the structural features that are important for the inhibitory action of choline on type III nAChRs.
Betaine, a metabolic product formed by the oxidation of choline, retains the trimethyl ammonium moiety of choline but differs by having an acetate moiety instead of an alcohol group (see structure in Fig. 5A). The close structural similarity between betaine and choline allowed us to test the specificity of inhibitory action of choline at type III nAChR. A bath-exposure of hippocampal slices to betaine (1 mM) for 10 min failed to inhibit ACh-induced NMDA EPSCs (Fig. 5, B–D). Instead, exposure to betaine resulted in a slight enhancement in the magnitude of ACh-induced NMDA EPSCs (Fig. 5H). The enhancing action of betaine on type III nAChR response can be attributed to a glycine-like action because bath perfusion of hippocampal slices with glycine (10 μM) also caused an enhancement in ACh-induced NMDA EPSCs (Fig. 5, E–H). Interestingly, a 10-min exposure of the slices to trimethylamine hydrochloride produced approximately 90% inhibition of ACh-induced NMDA EPSCs (results not shown). Thus, the present results suggested that the trimethyl ammonium headgroup of choline is sufficient to induce an in-
hibitory activity on type III nAChRs. The results also suggest that the presence of acetate moiety in betaine hinders the inhibitory effect of trimethylammonium group on type III nAChR.

Type II nAChR Response Is Less Sensitive to the Inhibitory Action of Choline. Our previous studies have shown that ACh is able to activate a slowly desensitizing nicotinic current, namely type II nAChR current (mediated by α4β2* nAChR), which is sensitive to blockade by dihydro-β-erythroidine, in CA1 stratum lacunosum moleculare (SLM) and some SR interneurons (Alkondon and Albuquerque, 2005). Here, we examined the sensitivity of type II nAChR to the inhibitory actions of choline by recording from CA1 stratum lacunosum moleculare (SLM) interneurons. Bath exposure of hippocampal slices to choline up to 100 μM had no noticeable effect on ACh-induced type II currents. However, choline at 200 μM to 10 mM produced a concentration-dependent inhibition of the amplitude and net charge of type II currents (Fig. 6). The plot of the mean net charge of type II currents versus choline concentration yielded an IC50 of 372 μM and a Hill coefficient of 1.51 (Fig. 6). This weak inhibitory effect of choline on type II currents was reversible upon washing the slices with choline-free ACSF for 10 to 20 min.

Dorsal Striatal Neurons Exhibit Choline-Sensitive Type III nAChR Responses. We examined the putative nAChR responses present in the neurons of dorsal striatal slices, because this region receives rich innervation from cholinergic interneurons (Holt et al., 1996). In the absence of MLA, U-tube application of choline (10 mM) evoked rapidly decaying type IA currents in less than 43% of the neurons tested (three of seven neurons). U-tube application of ACh (0.1 mM) to neurons of dorsal striatal slices induced slowly decaying (type II-like) currents (Fig. 7A) in approximately 38% of neurons tested (five of 13 neurons). These slow currents were observed even when 10 nM MLA was included in the ACSF in some of the experiments. In contrast, U-tube application of ACh (0.1 mM) at +40 mV was able to evoke NMDA EPSCs (type III nAChR response; Fig. 7) in approximately 83% of the dorsal striatal neurons tested (10 of 12 neurons). Likewise, U-tube application of ACh...
(0.1 mM) induced AMPA EPSCs at −68 mV (Fig. 7A) in approximately 75% of dorsal striatal neurons tested (nine of 12 neurons). Bath exposure of dorsal striatal slices to choline (300 μM for 10 min) resulted in more than 75% inhibition of ACh-induced NMDA EPSCs (Fig. 7). Dorsal striatal neurons that responded to ACh with AMPA EPSCs and NMDA EPSCs had different morphologies. Figure 8 illustrates neurolucida drawings of two biocytin-filled neurons from dorsal striatal slices. Although both neurons had type III nAChR responses, only the neuron to the left (Fig. 8) showed type II-like nicotinic current as well (see Fig. 7A).

**Discussion**

The present study demonstrates that in hippocampal slices choline is a potent inhibitor of type III nAChR (α3β4* nAChR). In contrast, it is only a weak inhibitor of type II nAChR (α4β2* nAChR) in the same preparation. The strong inhibitory action on type III nAChRs is also observed in dorsal striatal slices. The inhibition of type III nAChR by physiologically and pharmacologically relevant concentrations of choline (0.01–1 mM) suggests that the activity of type III nAChR can be regulated by levels of choline in the brain. Since the nAChRs in the brain are exposed to choline for prolonged periods, their density and/or activity can be increased by such exposure, particularly during early development when the choline uptake system is not fully matured. As the nAChRs are important for learning and memory, they could mediate the memory-enhancing effects of choline observed after prenatal and postnatal administration.
Choline Is a Potent Inhibitor of Type III nAChRs. Our results reveal that type III nAChR is the most sensitive receptor target known thus far for the actions of choline. Choline at <3 μM had a marginal facilitatory action on ACh-induced NMDA EPSCs, and this can be attributed either to a glycine-like action on the NMDA EPSCs (see Fig. 5) or to an allosteric potentiating effect of choline at type III nAChRs. At concentrations higher than 3 μM, choline induced a concentration-dependent inhibition of type III nAChR with an IC50 around 15 μM with noticeable inhibition occurring at 10 μM. It is estimated that the free extracellular concentration of choline reaches between 9 and 20 μM under various pathophysiological depolarizing conditions (see Introduction). Interestingly, the IC50 of choline to inhibit type III nAChR is lower than those reported to inhibit α7 nAChR-mediated type IA currents in cultured hippocampal neurons (IC50 = 37 μM; Alkondon et al., 1997), in SR interneurons of rat hippocampal slices (25–100 μM, Alkondon et al., 1999), and in neurons of hypothalamic tuberomammillary nucleus (20–80 μM; Uteshev et al., 2003), or to inhibit [3H]quinuclidinyl benzilate binding to muscarinic receptors (IC50 = 2 mM; Palacios and Kuhar, 1979). Furthermore, choline up to 100 μM does not seem to inhibit in cultured hippocampal neurons type II nicotinic currents (Alkondon et al., 1997). In fact, in the CA1 interneurons of hippocampal slices, choline was less potent in inhibiting type II currents as the IC50 for such an effect was around 370 μM, which is more than an order of magnitude higher than that needed to inhibit type III nAChR responses. Thus, choline exerts an inhibitory effect on various hippocampal nAChRs with an order of potency type III > type IA > type II. Choline differs from nicotine in the inhibitory profile as nicotine has the order of inhibitory potency type III > type II > type IA (see Alkondon and Albuquerque, 2005). Unlike nicotine, choline is the least potent at inhibiting type II nAChR that is consistent with its inability to activate this receptor. Because type III nAChR responses are present in regions other than the hippocampus such as the dorsal striatum (the present results) and possibly other brain areas, it can be predicted that physiological and pharmacological concentrations of choline are likely to affect several brain functions through this nAChR subtype. It should be noted that neither NMDA receptors nor AMPA receptors are inhibited by choline.

Significance of Choline-Induced Inhibition of Type III nAChR. Choline has many known actions in the brain. Choline supplementation in adult rats causes behavioral hyperactivity (Wecker et al., 1987). On the other hand, prenatal and postnatal choline administration produces enduring changes in brain function in the offspring (Meck et al., 1989). For instance, supplementation of choline during 11 to 17 days of gestation improved memory performance of rats at 24 to 26 months (Meck and Williams, 1997). Furthermore, hippocampal long-term potentiation is enhanced in young rats prenatally supplemented with choline (Pyapali et al., 1998). These cellular and behavioral effects have been linked to various effects of prenatal choline observed in the hippocampus such as enhanced ACh release (Cermak et al., 1998), decreased AChE activity (Cermak et al., 1999), enhanced cholinergic tone (Montoya et al., 2000), enhanced NMDA-receptor mediated transmission (Montoya and Swartzwelder, 2000), and, more recently, an altered structure and function of hippocampal pyramidal neurons (Li et al., 2004). However, it is not clear whether any of these long-term effects of choline result from its action at the nAChRs. Because nicotine is able to enhance memory in rats (Bettany and Levin, 2001) and is able to alter the structure and function of neurons in the brain (Robinson and Kolb 2004; McDonald et al., 2005), it is conceivable that choline may exert some of its above-described actions via brain nAChRs. In fact, it has been reported that dietary choline supplementation selectively increases the density of nicotine binding sites in the rat brain in a manner similar to that seen with nicotine administration (Coutcher et al., 1992). Therefore, similar to the effects of nicotine administration (Alkondon and Albuquerque, 2005), choline administration could increase the density/ activity of type III nAChRs in the brain. Activation of various nAChRs, including type III nAChR, increases the excitability of CA1 interneurons (Alkondon et al., 2003). The activity of

![Fig. 8. Sample neurolucida drawings of biocytin-filled dorsal striatal neurons in slices. These neurons had medium-large cell somata. Dendrites are in black and axon in gray. Calibration bar, 100 μm.](image)

![Fig. 9. Scheme representing the relationship between cholinergic terminal, glutamate terminal, interneuron postsynaptic site, various nAChRs, and AChE.](image)
in interneurons controls pyramidal cell firing and various types of hippocampal rhythms, and thereby contribute to the process of learning and memory (Paulsen and Moser, 1998; Cobb et al., 1999). Thus, choline, by regulating the density and/or activity of various hippocampal nAChRs can produce an enhancement in memory. Because the choline uptake system is not fully matured in the developing brain compared with adult rat brain (Klein et al., 2002), enhanced levels of choline available at extracellular sites in the brain could have profound effects on the developing brain.

Relevance to Brain Cholinergic Signaling
The susceptibility of type III and type IA nAChRs to choline-mediated desensitization has relevance to brain cholinergic signaling. Ultrastructural studies in the hippocampus have revealed a close apposition between cholinergic axon terminals and other unidentified excitatory terminals (Umbriaco et al., 1995). In the ventral tegmental area, direct evidence for the presence of apposing glutamatergic and cholinergic terminals has been shown (Garzón et al., 1999). Since glutamate axons contain AChE as it has been demonstrated in thalamocortical fibers (Schlaggar and O'Leary, 1994), cholinergic neurotransmitter ACh after acting on various nAChRs will be rapidly hydrolyzed to choline. Such synthetically generated choline, if not removed efficiently by the uptake system, is likely to desensitize the nAChRs, particularly the type III and type IA nAChRs that are associated with glutamate axons (see Fig. 9). In such a scenario, a cholinergic stimulus will be initially effective in activating type III and type IA nAChRs on glutamate axons; however, any next stimulus arriving within a short time will be ineffective because the nAChRs are desensitized by the presence of choline. Thus, choline can play an important self-regulatory modulatory role on glutamate transmission via selective inhibition of various nAChRs. The precision of such a mechanism will be determined by factors such as cholinergic impulse frequency, rate of ACh hydrolysis, and choline uptake mechanism.

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References
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