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Regulation of Nicotinic Acetylcholine Receptor Channel Function by Acetylcholinesterase Inhibitors in Rat Hippocampal CA1 Interneurons

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are involved in cognition and may play a role in Alzheimer's disease (AD). Known inhibitors of acetylcholinesterase (AChE) are used to treat AD and are known cognitive enhancers; however, their mechanism of action relating to AD is not fully understood. We tested several AChE inhibitors, including huperzine A, tacrine, and 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51), on nAChRs in rat hippocampal CA1 interneurons in slices using patch-clamp techniques. These interneurons express both α7 and non-α7 subunit-containing nAChRs and were activated with pressure applications of acetylcholine (ACh), choline, or carbachol. These AChE inhibitors had no significant effect on either the amplitude or kinetics of α7 nAChRs activated by ACh, but they slowed the rate of recovery from desensitization through an indirect mechanism; responses activated with either choline or carbachol were unaffected. For non-α7 receptors, these inhibitors significantly increased the amplitude and decay phase for responses induced by ACh (but not carbachol), also through an indirect mechanism. Slices preincubated with diisopropylflurophosphate (to permanently inactivate AChE) mimicked the effect of these AChE inhibitors on both α7 and non-α7 nAChRs. In addition, galantamine, which is both an inhibitor of AChE and an allosteric potentiator of nAChRs, had similar effects. Therefore, various AChE inhibitors are having significant and indirect effects on nAChRs through direct inhibition of AChE; this results in an enhanced amount and/or duration of ACh in slices, with no effect on the levels of choline or carbachol. Therefore, drugs that target AChE are likely to be important regulators of cholinergic signaling in the hippocampus.

Acetylcholine (ACh), acting through neuronal nicotinic acetylcholine receptors (nAChRs) and muscarinic G protein-coupled receptors, is an important modulator of electrical activity in the central nervous system and is involved in a variety of physiological processes and synaptic plasticity, including cognition and development (Decker and McGaugh, 1991; Jones et al., 1999; Levin, 2002; Volpicelli and Levey, 2004). The loss of cholinergic function has been implicated in Alzheimer's disease (AD), the leading cause of dementia (Terry and Buccafusco, 2003). However, the specific molecular mechanisms involved in the cholinergic signaling deficits in AD are presently unknown.

ACh is synthesized by choline acetyltransferase and is rapidly hydrolyzed by acetylcholinesterase (AChE), the principal role of which is the termination of impulse transmission at cholinergic synapses. Although AChE is encoded by a single gene, it exists in a variety of functional forms caused by alternative mRNA splicing and association with structural subunits (Massoulié et al., 1993; Descarries et al., 1997; Soreq and Seidman, 2001). Drugs that inhibit AChE, which should increase the presence of ACh, are currently the most promising drugs in the treatment of AD (Terry and Buccafusco, 2003). However, some inhibitors of AChE, such as physostigmine and galantamine, are also known to be allosteric potentiators of nAChRs, an action which has been proposed to explain their usefulness in the treatment of AD (Maelicke et al., 2000; Samochocki et al., 2003).

We examined whether various AChE inhibitors alter the function of native nAChR function in rat hippocampal CA1 interneurons in the slice. These interneurons express diverse subtypes of somatodendritic nAChRs, including α7 and non-α7 subunit-containing subtypes (Jones et al., 1999). There are currently at least 11 different nAChR subunits

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ABBREVIATIONS: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; AD, Alzheimer's disease; AChE, acetylcholinesterase; DFP, diisopropylflurophosphate; ACSF, artificial cerebral spinal fluid; MLA, methyllycaconitine; DHβE, dihydro-β-erythroidine; BW284c51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; MS-222, ethylmetaaminobenzoate.

known to be expressed in the rat nervous system, nine of which (α2–α7 and β2–β4) are expressed in the adult rat central nervous system (McGehee and Role, 1995). Many data suggest that the major subtypes of channels in rat hippocampal interneurons are composed of the α7 and α4β2 subunits (Alkondon and Albuquerque, 1993; Jones and Yakel, 1997; Frazier et al., 1998; McQuiston and Madison, 1999). Tacrine, which was one of the first drugs used to treat AD (Terry and Buccafusco, 2003), was not a potentiator of nAChRs (Zwart et al., 2000; Samochocki et al., 2003) but rather inhibited the function of various nAChRs (Canti et al., 1998; Zwart et al., 2000; Prince et al., 2002). Huperzine A is isolated from the Chinese herb *Huperzia serrata* and enhances cognitive function (Zangara, 2003). BW284c51 is a bis-quaternary nitrogen compound (Austin and Berry, 1953) with unknown effects on either cognition or nAChR function. All three of these compounds (tacrine, huperzine A, and BW284c51) are noncovalent and reversible inhibitors of AChE, and none had significant direct effects on either α7-containing or non-α7 subtypes of nAChRs, but they had indirect effects. For the α7-containing nAChRs activated by ACh, these AChE inhibitors significantly slowed the rate of recovery from desensitization, and for the non-α7 receptors, they significantly increased the amplitude and prolonged the decay phase of responses through an indirect mechanism of action. Galantamine, which is both an inhibitor of AChE and an allosteric potentiator of nAChRs, had similar effects. These inhibitors did not directly affect the amplitude or kinetics of these receptors, and this apparent lack of direct effect was confirmed in *Xenopus laevis* oocytes expressing rat α7 and α4β2 nAChR channels; rather, these inhibitors are affecting the nAChR-mediated responses caused by the prolonged duration of ACh that results from a reduction in breakdown by the inhibition of AChE. These data are consistent with the indirect modulation of nAChRs and suggest that drugs targeting AChE may be important regulators of cholinergic signaling and perhaps will continue to be useful in the treatment of AD.

Materials and Methods

Slice Preparation. Standard techniques were used to prepare 350-μm thick acute hippocampal slices from 14- to 19-day-old rats (Khiroug et al., 2003). In brief, rats were anesthetized with halothane (Sigma Chemical, St. Louis, MO) and decapitated. Brains were quickly removed and placed into an ice-cold, oxygenated artificial cerebral spinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose. Upon dissection, brain chunks were glued to the stage of a vibratome (VT1000S; Leica, Wetzlar, Germany) for slicing while immersed in the cooled, oxygenated ACSF. Slices were then used for recordings within approximately 6 h and after at least 1 h of recovery period.

Electrophysiology. Whole-cell, patch-clamp recordings were performed from CA1 stratum radiatum or stratum oriens interneurons. Patch pipettes (Garner 7052 glass; Garner Glass Company, Claremont, CA) with resistances of 3 to 4 MΩ were filled with a solution that contained 120 mM cesium gluconate, 2 mM NaCl, 4 mM Na_2ATP, 0.4 mM Na_2GTP, 4 mM $MgCl_2$, and 20 mM HEPES, pH 7.3. Slices were superfused at room temperature (18–22°C) with ACSF. Synaptic activity was blocked with tetrodotoxin (1 μM) added to the ACSF. In some experiments, atropine (1 or 10 μM) was also added to block putative muscarinic AChR-mediated responses. Cells were clamped using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) at a holding potential of −70 mV. Currents were recorded and analyzed using pClamp software (Axon Instruments); recordings were analyzed only if the holding current was less than 100 pA. Responses were induced by pressure application (Picospritzer II; General Valve, Fairfield, NJ) of either ACh, choline, or carbachol delivered via a θ glass pipette (Harvard Apparatus Inc. Holliston, MA (outer diameter, 2 mm) pulled to a tip diameter of ~3 μm (P-97 puller; Sutter Instrument Company, Novato, CA) placed 20 to 30 μm from the cell body; in this way, two agonists could be rapidly applied to the same cell. Brief (50–200 ms) duration pulses at 10 to 20 psi were typically used to activate α7-containing nAChRs, whereas longer (5-s) pulses (< 10 psi) were typically used to activate non-α7 receptors. Drugs studied were diluted at final concentrations in ACSF and were delivered to the cell through a gravity-fed multichannel perfusion system ending with a nonmetallic syringe needle (inner diameter, 250 μm; WPI, Sarasota, FL) placed just above the slice surface to ensure the homogenous coverage of the area surrounding the cell being studied.

Data Analysis. To study the rate of recovery from desensitization of α7-containing receptors, the time (10 s) between two consecutive agonist pulses (denoted as P1 and P2) was chosen in a series of preliminary experiments so that the amplitude of the second pulse was significantly (but not completely) reduced because of receptor desensitization (Khiroug et al., 2003). Then, the ratio of response amplitudes (the P2/P1 ratio) was a measure of the rate of recovery from desensitization. Because there was variability in this ratio from cell to cell, as well as changes in amplitudes (either increases or decreases) occasionally during the time course of experiments (we often observed rundown of both α7 and non-α7 nAChRs during recordings), we normalized the changes in the P2/P1 ratio (R_{norm}) during experimental treatments compared with the control mean value for each cell using the equation $R_{norm} = ({}^{ex}P2/{}^{ex}P1)/({}^{contr}P2/{}^{contr}P1)$, where ^{ex}P1 and ^{ex}P2 are experimental and ^{contr}P1 and ^{contr}P2 are control peak amplitude values. Statistical analyses were performed using Origin software (OriginLab Corp, Northampton, MA). Averaged data were presented as mean ± S.E.M., and statistical significance was tested using an analysis of variance.

RNA Preparation, Expression, and Electrophysiological Recordings in *X. laevis* Oocytes. mRNA was transcribed in vitro from plasmids using the mMessage Machine kit (Ambion, Austin, TX) according to conditions suggested by the manufacturer. The rat nAChR α7, α4, and β2 plasmids were kindly provided by J. Patrick (Baylor College of Medicine, Houston, TX). Female *X. laevis* frogs were anesthetized by immersion in ice-cold water containing 0.2% MS-222 (Sigma) for 60 min and decapitated. Oocytes were dissected and defolliculated by treatment with collagenase B (3–4 mg/ml; Roche Diagnostics, Indianapolis, IN) for 2 to 4 h in a solution containing 85.2 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES. The total amount of RNA injected for each nAChR subunit was 25 ng. Experiments were performed 3 to 8 days after injection.

Current responses were obtained by two-electrode voltage-clamp recording at a holding potential of −60 mV (unless otherwise stated) using a Geneclamp 500 and pClamp 8 software (Axon Instruments) in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. Oocytes were maintained in culture in the same solution, with the addition of 2.5 mM sodium pyruvate, 0.5 mM theophylline, 50 μg/ml gentamicin, and 5% horse serum. Traces were typically filtered at 0.2 to 1 kHz and sampled at 0.5 to 5 kHz. Electrodes contained 3 M KCl and had resistances of <1 MΩ. ACh was freshly prepared in bath solution from a frozen stock and applied using a synthetic quartz perfusion tube (inner diameter, 0.7 mm) operated by a computer-controlled valve.

Results

Pharmacological Properties of nAChR-Mediated Currents: Activation of Both α7 and Non-α7 Subtypes. In freshly cut slices of rat hippocampus, the pressure appli-

cation of ACh (2 mM) to voltage-clamped CA1 stratum radiatum and stratum oriens interneurons elicited diverse nAChR-mediated responses (Fig. 1). To determine the molecular makeup of the receptors governing these responses, subunit-selective pharmacological agents were used. The α7-containing nAChRs are rapidly activated and desensitized and are potently and selectively blocked by methyllycaconitine (MLA). In addition, choline has been reported to be a selective agonist of α7-containing nAChRs (Papke et al., 1996). Most of the interneurons studied here (>95%) express α7-containing nAChRs, which can be activated when using short-duration (e.g., 100-ms) agonist pressure applications. Under these conditions, responses induced by maximal doses of ACh (2 mM) or choline (10 mM) (Khiroug et al., 2002) are identical in peak amplitude and time course and are completely blocked by MLA (10 nM) (Fig. 1A). In some α7-containing interneurons, ACh also seems to activate a slower non-α7 nAChR-mediated response. In these cells, choline only induces rapid responses that are completely blocked by MLA, whereas ACh responses peak rapidly and decay with a slower secondary component that is insensitive to block by MLA (Fig. 1B); this slower component is blocked by dihydro-β-erythroidine (DHβE; 10 μM), a competitive antagonist of non-α7 nAChRs (data not shown). In some rare interneurons (<5%), only non-α7 nAChRs seem to be expressed because ACh, but not choline, induces slow-activating responses that are unaffected by MLA (data not shown) but are blocked by DHβE (Fig. 1C).

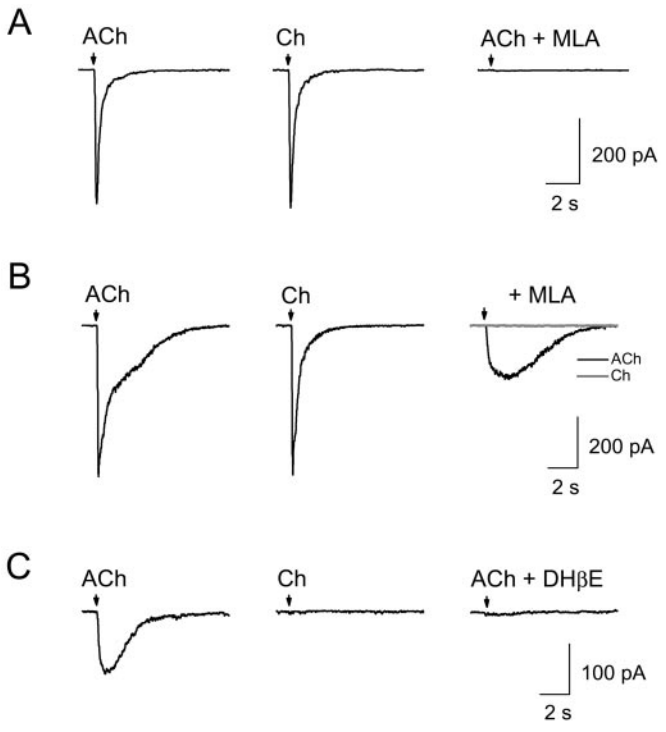

Fig. 1. Pharmacological separation of α7 and non-α7 nAChR-mediated responses. A, in a cell with exclusively α7-containing nAChRs, responses induced by brief (50–200 ms) duration pulses (arrows) of ACh (2 mM; left trace) and choline (Ch, 10 mM; center trace) were similar in amplitude and kinetics, and the ACh response (right trace) was completely blocked by the α7-selective antagonist MLA (10 nM). B, in a different neuron, both α7-containing and non-α7 nAChRs are present. In this cell, ACh activates (left trace) both a fast (MLA-sensitive; right trace) and a slow (MLA-insensitive) component response, whereas choline (center trace) activates only a fast (MLA-sensitive; right trace) response. C, in another neuron, ACh (left trace) activates only a slow component response that is blocked by the non-α7 nAChR antagonist DHβE (10 μM; right trace), whereas there is no response to choline (center trace).

The diverse functional and pharmacological properties of nAChR-mediated responses in these interneurons is consistent with previous reports from various groups showing that these neurons express a diverse array of nAChRs, including α7-containing and a variety of non-α7 subtypes (Jones and Yakel, 1997; Alkondon et al., 1998; Frazier et al., 1998; McQuiston and Madison, 1999; Sudweeks and Yakel, 2000). In addition, by combining differential sensitivity to specific agonists and antagonists, we are able to isolate and effectively study the α7 from the non-α7–containing nAChR populations in individual cells.

Regulation of α7-Containing nAChRs by AChE Inhibitors. In interneurons with predominantly α7-containing nAChR-mediated responses, the various inhibitors of AChE that we have investigated (i.e., huperzine A, tacrine, and BW284c51) had no significant effect on either the amplitude or kinetics of α7 nAChR-mediated responses. However, these inhibitors seemed to have an effect on the rate of recovery from desensitization, which we studied using a dual-pulse protocol.

The α7-containing nAChRs in rat hippocampal CA1 stratum radiatum interneurons in slices desensitize with a biphasic time course (pressure application of 2 mM ACh), with time constants of 117 ms (68% of the fit) and 1.29 s (Khiroug et al., 2003). In addition, these α7-containing nAChRs recovered from desensitization (when activated by the local photolysis of caged carbachol) with a time course of ~20 s (Khiroug et al., 2003). This recovery time is partly caused by the rate of diffusion of ACh away from the receptors and is consistent with the time course of decay of the nondesensitizing non-α7 nAChRs, which had an average half-time of decay of ~4 s (see below). To explore potential changes to the rate of recovery from desensitization caused by AChE inhibitors, a second pulse of ACh (denoted as P2) was applied 10 s after the first (P1; both for 100-ms durations; see *Materials and Methods*). The amplitude of the second ACh pulse (P2) was significantly reduced (relative to the first, P1) because of receptor desensitization to a value of 84 ± 2% (52 cells) (Fig. 2A); this value is defined as the P2/P1 ratio. All three AChE inhibitors were found to significantly and dose-dependently reduce this P2/P1 ratio for ACh-induced responses (Fig. 2A), most likely signifying that they decrease the rate of recovery from desensitization through an indirect mechanism because they would be expected to result in the persistence of ACh because of a reduction in breakdown, which would cause persistent desensitization of the receptors. This is consistent with the significant increase in the time course of decay of the non-α7 nAChRs to ~21 s after treatment with BW284c51 (1 μM; see below). BW284c51 was the most potent of the three, and its effects were the most rapidly reversible (for 10-min washout) (data not shown). The P2/P1 ratio was not affected by DHβE (1–10 μM; 12 cells), which indicates that non-α7 nAChRs were not involved in the alteration in this ratio.

With a fast-desensitizing channel such as the α7 nAChR, inhibiting the function of AChE would be expected to increase desensitization (e.g., to reduce the recovery from desensitization), which is consistent with our data. To further explore this possibility, we used choline (10 mM), a selective activator of α7-containing nAChRs and not a substrate for AChE. Using the dual-pulse protocol mentioned above, the

P2/P1 ratio for choline-induced responses was 65 ± 4% (25 cells) (Fig. 2B), which is significantly less than for ACh; this apparent slower recovery from the desensitization of choline-induced responses is probably caused by the persistence of choline (because it is not broken down by AChE), which causes the persistent desensitization of the receptors. In contrast to ACh, the P2/P1 ratio for choline-induced responses was not significantly affected by exposure to any of the three AChE inhibitors (Fig. 2B). These data are consistent with the fact that these AChE inhibitors are not having a direct effect on the nAChRs themselves; otherwise, choline-induced responses should have been affected. Furthermore, these data suggest that these AChE inhibitors exert their effect selectively on ACh-induced responses because their actions result in an increase in the concentration of ACh (caused by a reduction in breakdown) and that this increase in ACh is altering the P2/P1 ratio for ACh-induced responses by decreasing the rate of recovery from desensitization.

Carbachol is another agonist, similar to ACh, that will activate α7-containing and non-α7 nAChRs, as well as muscarinic ACh receptors. However, unlike ACh, carbachol (like choline) is not a substrate for AChE. Therefore, we tested whether the effects of carbachol might be sensitive to these AChE inhibitors. Brief pressure applications of carbachol (4 mM) induced α7 nAChR-mediated responses with amplitudes and kinetics similar to those of ACh and choline (Fig. 2C). Using the dual-pulse protocol, the P2/P1 ratio for carbachol was 48 ± 5% (20 cells) (Fig. 2C), which is significantly less than for either ACh or choline; this apparent slower recovery from desensitization of carbachol-induced responses is probably caused by the persistence of carbachol (because it is not broken down by AChE), which causes the persistent

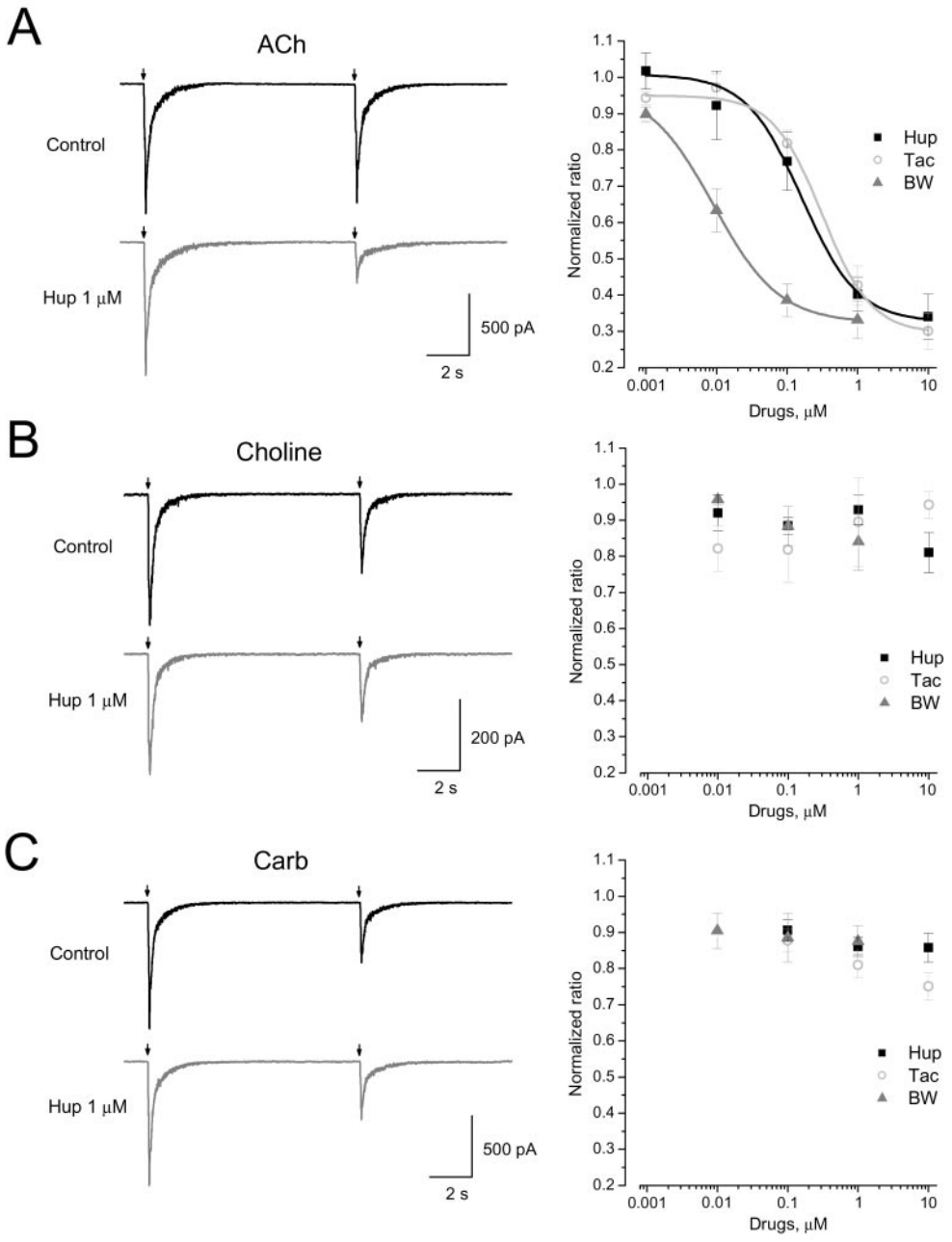

Fig. 2. AChE inhibitors enhance desensitization of α7 nAChRs through an indirect mechanism when activated by ACh but not by carbachol or choline. The rate of recovery from desensitization of α7-containing nAChRs was studied using a dual-pulse protocol whereby a second pulse of agonist was applied 10 s after the first (100-ms duration for both; see *Materials and Methods*). With this protocol, the amplitude of the second response was significantly reduced relative to the first (because of receptor desensitization) for all three agonist studied (top traces, left); A, ACh; B, choline; C, carbachol (Carb). The application of AChE inhibitors [experiments with huperzine A (Hup; 1 μM) have been chosen for demonstration (bottom traces, left)] had no significant effect on either the amplitude or kinetics of the first response to either ACh, carbachol, or choline and no significant effect on the amplitude of the second response to either carbachol or choline. However, AChE inhibitors significantly reduced the amplitude of the second response to ACh. The normalized ratio of the second response relative to the first (see *Materials and Methods*) and the effect of different concentrations of huperzine A, tacrine (Tac), and BW284c51 (BW) on the change in this ratio are shown on the right. All values are averages of 3 to 11 cells.

desensitization of the receptors. This is consistent with the slow time course of decay of non-α7 nAChRs activated by carbachol (see below). In addition, this ratio was unaltered by exposure to any of the three AChE inhibitors studied (Fig. 2C), similar to responses activated by choline but in contrast to those activated by ACh. As with ACh, this P2/P1 ratio for carbachol-induced responses was not affected by DHβE (1–10 μM; nine cells).

Because neither choline nor carbachol is a substrate for AChE, the inhibition of AChE would not be expected to alter choline- or carbachol-induced responses unless these inhibitors have direct effects on the nAChRs themselves. This was not the case, because neither the kinetics, amplitudes, nor recovery from desensitization of α7-containing nAChR responses activated by either choline or carbachol was affected by huperzine A, tacrine, or BW284c51. However, these AChE inhibitors did have an effect on the recovery of desensitization of ACh-induced responses only, which suggests that the effects of these inhibitors on the function of the α7-containing nAChRs is indirect (i.e., by inhibiting the function of AChE and increasing ACh levels).

Preincubation with Diisopropylflurophosphate, an Irreversible AChE Inhibitor, Mimics the Effects of Reversible AChE Inhibitors. Diisopropylflurophosphate (DFP) is an organophosphate that covalently modifies and irreversibly inhibits AChE function and can induce long-term cognitive deficits and alterations in the expression of cholinergic receptors (Upchurch and Wehner, 1987; Bushnell et al., 1991; Prendergast et al., 1997; Stone et al., 2000). Slices were preincubated with high doses (100 μM) of DFP for 30 min and then washed for >1 h. In these DFP-preincubated slices (e.g., without any AChE inhibitors), ACh and choline still activated α7-containing nAChR responses in interneurons (Fig. 3A); however, the P2/P1 ratio for ACh responses was significantly reduced to 33 ± 3% (three cells) compared with the control value of 84% (see above), whereas the P2/P1 ratio for choline responses was not significantly changed (74 ± 4% in three cells preincubated with DFP compared with 65% for controls) (Fig. 3B). Furthermore, the addition of BW284c51 to these preincubated slices had no further effect on the P2/P1 ratio for ACh-induced responses (Fig. 3). Therefore, preincubation with DFP seemed to mimic the effect of bath application of the reversible AChE inhibitors. These data further suggest that the effect of AChE inhibitors is to modulate the function of nAChRs through their interaction with AChE and not via a direct action on the nAChR.

Regulation of Non-α7 nAChRs by AChE Inhibitors. The non-α7 nAChRs are more difficult to study than α7-containing nAChRs in rat hippocampal interneurons for several reasons, including (but not limited to) infrequent occurrence, small amplitude of responses, larger molecular diversity, and more rapid rundown (Shao and Yakel, 2000; Sudweeks and Yakel, 2000; Khiroug et al., 2003). Nevertheless, we tested whether these AChE inhibitors altered the function of these receptors (Fig. 4).

All three AChE inhibitors (huperzine A, tacrine, and BW284c51) potentiated the function of non-α7 nAChRs. In cells with functional non-α7 nAChRs (any α7-containing nAChRs were blocked with MLA), the bath application of BW284c51 (100 nM) potentiated the response to ACh (2 mM for 5 s) by 69 ± 20% (six cells) (Fig. 4A). Furthermore, the rate of decay of these ACh responses (with an average half-time of decay of 4.3 ± 0.2 s; seven cells) was significantly slowed by BW284c51 (to 21 ± 3 s in five cells, with 1 μM BW284c51) (Fig. 4D). In the same cell (Fig. 4B), the activation of these non-α7 nAChRs by carbachol was unaffected by BW284c51. It is noteworthy that the half-time of decay for carbachol-induced responses (17 ± 2 s; five cells) was much longer (i.e., slower decay) than for ACh in control conditions; after BW284c51 application, the decay half-times were similar (Fig. 4D). These data, combined with the fact that the decay of ACh responses was dramatically reduced by AChE inhibition, are consistent with the notion that the rate of decay of ACh-induced responses (mediated by the non-α7 nAChRs) is determined (at least in part) by the breakdown of ACh by AChE. Similar effects on amplitude and decay rate were also observed with both huperzine A and tacrine (data not shown). In slices preincubated with DFP, the decay of ACh responses was much slower (24 ± 2 s; four cells) than in control slices (Fig. 4C). In fact, the time course of decay of ACh responses in DFP-treated slices was not significantly different from the time courses with BW284c51 (for ACh) and for carbachol responses (Fig. 4D). Therefore, as with the data from α7-containing nAChRs, preincubation with DFP seemed to mimic the effect of bath application of the reversible AChE inhibitors. Furthermore, these data also suggest

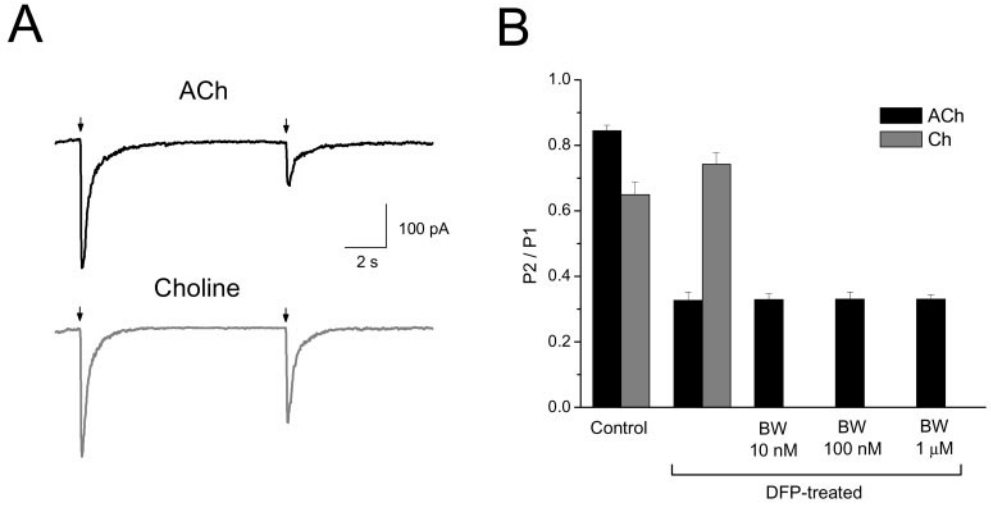

Fig. 3. Preincubation with DFP mimics the effect of reversible AChE inhibitors. A, in slices preincubated with DFP (100 μM for 30 min, then washout for >1 h), an irreversible inhibitor of AChE, ACh and choline still activated α7-containing nAChRs with similar amplitude and kinetics. However, the amplitude of the second ACh response was significantly reduced, whereas the second response to choline was similar to that of the control slice response and was significantly larger than the second response to ACh. B, the ratio of the amplitudes of the second compared with the first pulse under control conditions for ACh and choline (Ch) in DFP-treated slices and with the further addition of different concentrations of BW284c51. All values are averages of 3 to 52 cells.

that these AChE inhibitors are not having a direct potentiating effect on the non-α7 nAChR itself, because carbachol responses were unaffected.

Regulation of α7 and Non-α7 nAChRs by Galantamine. Galantamine is another inhibitor of AChE that has been reported previously to be an allosteric potentiator of nAChRs (Maelicke et al., 2000; Samochocki et al., 2003). We compared the effect of galantamine on both the α7 and non-α7 nAChRs with the other AChE inhibitors that we tested. Similar to the effects of all three AChE inhibitors, the amplitude and kinetics of α7-containing nAChR responses were not significantly affected by galantamine (up to 10 μM), and the P2/P1 ratio for ACh responses was significantly and dose-dependently reduced (although higher doses of galantamine were required to produce the same effect) (Fig. 5, A and B). In addition, similar to the effect of the other AChE inhibitors, the P2/P1 ratio for choline responses was not significantly affected by galantamine (Fig. 5, A and B).

In cells with functional non-α7 nAChRs, galantamine also had effects similar to those of the other AChE inhibitors. The bath application of galantamine (10 μM) potentiated the response to ACh by 87 ± 24% (five cells) (Fig. 5C) and significantly slowed the half-time of decay from 2.8 ± 0.3 to 11 ± 1 s; lower doses of galantamine were less effective. Furthermore, the activation of these non-α7 nAChRs by carbachol was unaffected by galantamine (data not shown). These data suggest that under the current experimental conditions, galantamine, like the other AChE inhibitors that we have tested, affects nAChR function through an indirect mechanism of action.

Effect of AChE Inhibitors on Rat α7 and α4β2 nAChR Channels Expressed in *X. laevis* Oocytes. To further test whether AChE inhibitors may have direct effects on nAChR function, we expressed rat α7 and α4β2 nAChR channels in *X. laevis* oocytes because it has been suggested that these are the major subtypes of nAChRs in rat hippocampal interneurons (Alkondon and Albuquerque, 1993; Frazier et al., 1998; McQuiston and Madison, 1999). As shown previously (Khiroug et al., 2002), the rapid application of ACh (2 mM) to oocytes expressing the rat α7 nAChR subunit induced inward current responses (at a holding potential of −60 mV) that activated and decayed rapidly (Fig. 6A). When using the dual-pulse protocol as mentioned above (slightly modified by applying ACh for 500-ms duration at 6-s intervals), neither the amplitude nor kinetics of the responses nor the P2/P1 ratio was significantly altered by either BW284c51 (100 nM or 1 μM; three cells), tacrine (1 μM; three cells), or huperzine A (1 μM; three cells) (Fig. 6A). These data suggest that these AChE inhibitors do not have a direct effect on α7 nAChR function expressed in *X. laevis* oocytes at the concentrations used in this study.

The application of ACh (1 mM for 3 s) to oocytes expressing the rat α4β2 nAChR channels induces responses that activate and decay much more slowly than α7 nAChRs (Fig. 6B). Furthermore, the bath application of BW284c51 (100 nM; three cells) had no significant effect on either the amplitude or decay of responses caused by activation of the α4β2 nAChRs, which suggests that BW284c51 does not have a direct potentiating effect on these channels expressed in *X. laevis* oocytes.

Discussion

Cholinergic signaling mediated through nicotinic and muscarinic ACh receptors are integral in controlling and regulating hippocampal excitability, synaptic plasticity, and cogni-

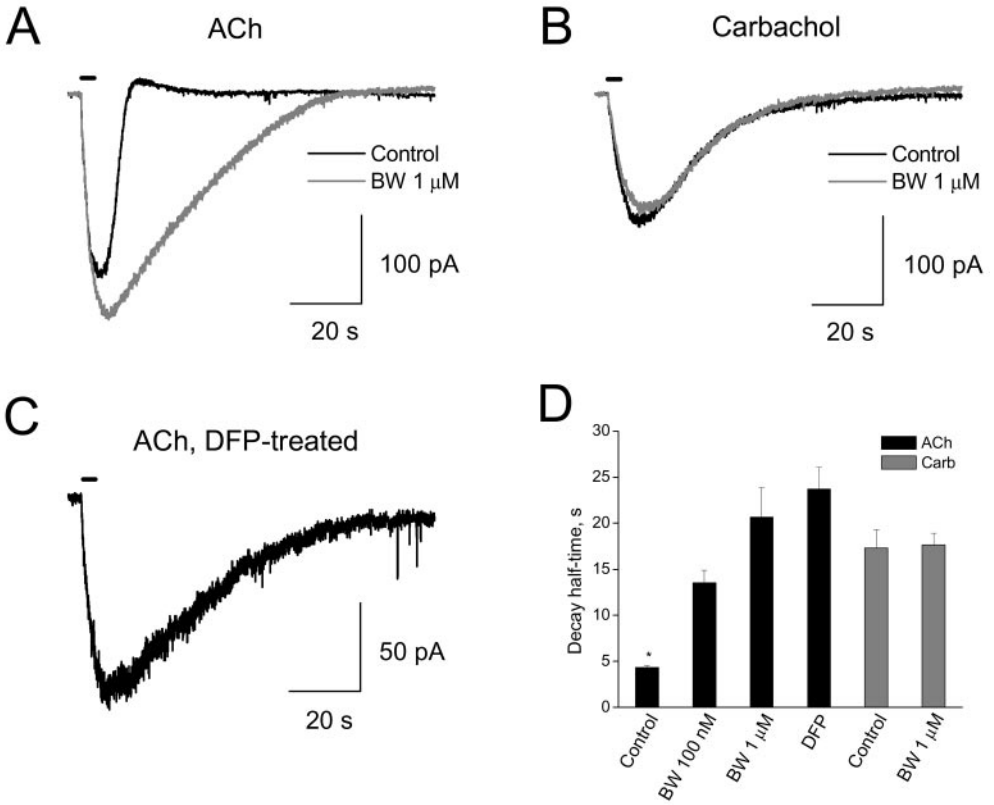

Fig. 4. Effect of AChE inhibitors on non-α7 nAChRs. A, in a cell with functional non-α7 nAChRs (α7-containing nAChRs were blocked with MLA 10 nM), BW284c51 (BW; 1 μM) potentiated the amplitude and slowed the rate of decay of ACh-induced responses activated with longer (5-s) duration pulses (horizontal bars). B, in the same cell, responses activated by carbachol were unaffected by BW284c51. C, representative trace from a cell in DFP-treated slices showing the activation of non-α7 nAChRs caused by ACh application. D, decay half-times for non-α7 nAChRs activated by either ACh or carbachol, in the presence of BW284c51, or in DFP-treated slices. All values are averages of three to seven cells.

tive processing. Furthermore, dysfunctions in cholinergic signaling and nAChR function have been directly linked to AD. Central to this is the regulation of the release and breakdown (via hydrolysis by AChE) of ACh at synapses and extrasynaptic sites. We have shown that the regulation of AChE activity can have direct implications for the function and regulation of nAChRs, both α7-containing and non-α7 receptors, in rat hippocampal interneurons in the slice. The inhibitors of AChE that we have focused on, huperzine A, tacrine, and BW284c51 (huperzine A and tacrine are known cognitive enhancers, and tacrine is presently used to treat AD), do not directly affect the amplitude or kinetics of these receptors. The lack of direct effect was also observed in *X. laevis* oocytes expressing rat α7 and $\alpha4\beta2$ nAChR channels, which have been suggested to be the major subtypes of nAChRs in rat hippocampal interneurons. Instead, these inhibitors slowed the rate of recovery from desensitization of the α7-containing nAChRs in rat hippocampal interneurons through an indirect mechanism (i.e., because they cause the persistence of ACh because of a reduction in breakdown, which causes persistent desensitization of the receptors) and increased both the amplitude and decay time of the non-α7 nAChR-mediated responses through an indirect mechanism involving the inhibition of the breakdown of ACh, resulting in an enhanced amount or duration of ACh exposed to these channels.

AChE is encoded by a single gene; however, it exists in a variety of functional forms (which share similar catalytic properties) caused by alternative mRNA splicing and association with structural subunits (Massoulié et al., 1993; Descarries et al., 1997; Soreq and Seidman, 2001). AChE comprises six molecular forms corresponding to a series of oligomers of a common catalytic subunit; there are three globular forms (monomeric G_1, dimeric G_2, and tetrameric G_4) and three asymmetric forms (A_4, A_8, or A_{12}) consisting of one, two, or three tetramers with a collagen tail. In mammalian brain, the main form of AChE is G_4 (comprising approximately 60–90%), which is predominantly hydrophobic and membrane-bound and is localized within several intracellular sites, as well as on the outer surface of neurons and their processes. AChE is also secreted as a soluble enzyme in hydrophilic form, including in hippocampus, and this secretion is regulatable; this secreted soluble form is believed to hydrolyze ACh in the extracellular space more effectively than the membrane-bound form. In contrast to the neuromuscular junction, no foci of AChE exists at synaptic zones; instead, it is found along pre- and postsynaptic membranes. The significance of these different molecular forms of AChE is not clear at the moment; however, it has been suggested that different isoforms may have different physiological roles (Soreq and Seidman, 2001; Day and Greenfield, 2002).

Some inhibitors of AChE, such as physostigmine and galantamine, are known to also be direct allosteric potentiators of nAChRs because they potentiate responses to low (i.e., submaximal) doses of agonist, an action that has been proposed to explain their cognitive-enhancing abilities and usefulness in the treatment of AD (Maelicke et al., 2000; Samochocki et al., 2003). These groups also found that tacrine was not an allosteric potentiator of nAChRs, which is consistent with our data. Therefore, the beneficial effects of tacrine and huperzine A (which we also found was not an allosteric potentiator of nAChRs in our system) on cognition (and perhaps in the treatment of AD) is most likely not caused by a direct allosteric potentiation of nAChRs but instead is probably caused by the direct regulation of ACh concentration by AChE. Under the experimental conditions used here (i.e., maximal doses of agonists), we found that galantamine produced the same effects on both the α7 and non-α7 nAChRs, effects that were indirect and therefore not likely to be caused by direct binding to and regulation of channel func-

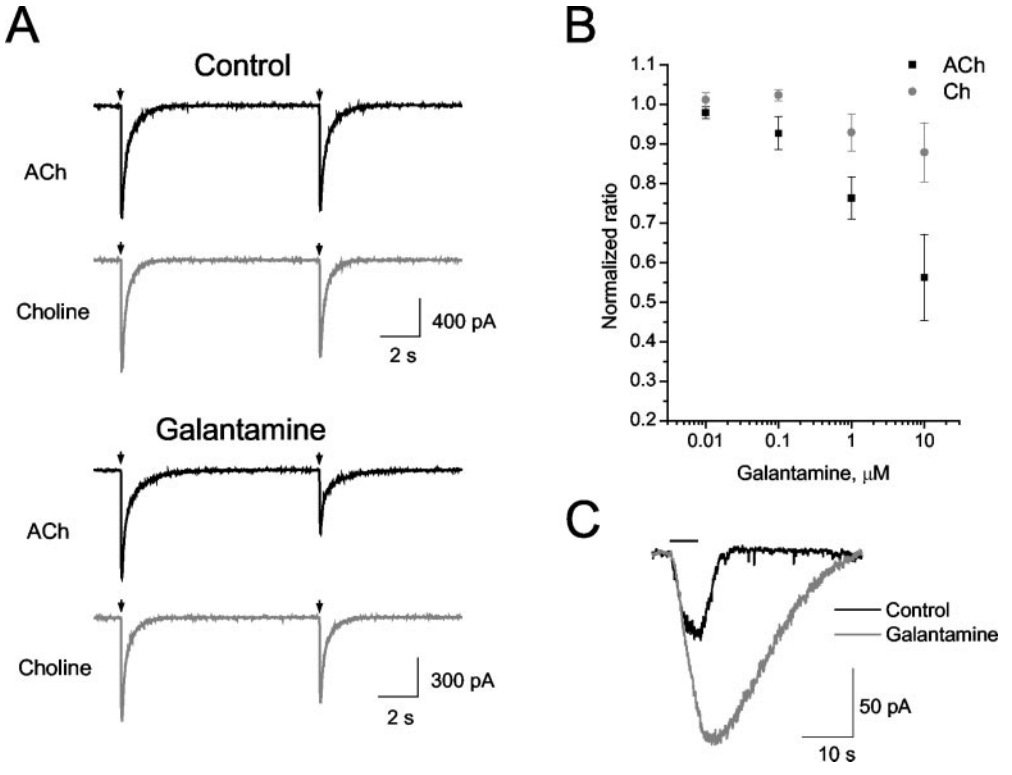

Fig. 5. Effect of galantamine on α7 and non-α7 nAChRs. A, using a dual-pulse protocol to study the rate of recovery from desensitization of α7-containing nAChRs, galantamine (10 μM) was tested and found not to significantly effect either the amplitude or kinetics of the first response to either ACh or choline, but it significantly reduced the amplitude of the second response to ACh but not to choline. In this cell, there was significant rundown of the response amplitude without any significant change in P2/P1 ratio (see *Materials and Methods*). B, the normalized ratio of the amplitudes of the second response relative to the first for ACh and choline, and the effect of different concentrations of galantamine on the change in this ratio. All values are averages of four to six cells. C, in a cell with functional non-α7 nAChRs, galantamine (10 μM) potentiated the amplitude and slowed the rate of decay of ACh-induced responses.

tion. The fact that we did not seem to observe an allosteric potentiating effect of galantamine was also consistent with results from Samochocki et al. (2003), who observed potentiation of nAChR function only with submaximal doses of agonist. Finally, the dose-dependence of the effect of galantamine that we observed was also more consistent with the inhibition of AChE, because concentrations of galantamine >1 μM were required for maximal regulation of nAChR function (consistent with the ability of galantamine to inhibit AChE, with an IC_{50} of ~3 μM), whereas maximal allosteric potentiation was observed at concentrations of galantamine of <1 μM (Samochocki et al., 2003).

The potentiation of non-α7 nAChRs by AChE inhibition is consistent with the well-known link of nAChRs, in particular non-α7 nAChRs, to cognition and AD (Picciotto et al., 1995; Levin, 2002). The non-α7 nAChRs have been shown to pattern network activity in rodent hippocampus via the regulation of θ activity (Cobb et al., 1999), and there is a substantial decrease in the number of nAChRs (in particular α4-containing) in patients with AD (Paterson and Nordberg, 2000; Court et al., 2001). However, it remains unclear how the regulation that we have observed with the α7-containing nAChRs (i.e., the prolongation of the recovery from desensitization) by AChE inhibition is related to cognition and/or AD, because block of α7-containing nAChRs in the hippocampus seems to impair memory performance (Levin, 2002). Altering ACh levels by blocking its hydrolysis would be expected to affect not only nAChRs but also muscarinic ACh receptors as well, whose function has also been linked to cognition and AD (Volpicelli and Levey, 2004). Understanding the precise molecular mechanism whereby cognitive-enhancing drugs and those that are used to treat AD acts is critical to further aid in the development of therapeutic interventions, as well as in the understanding of the mechanisms relating to cognition and the pathology of AD.

Reversible and irreversible inhibitors of AChE are used not only as cognitive enhancers and in the treatment of AD but also as insecticides and chemical warfare agents. Furthermore, AChE has been proposed to have functions other than hydrolyzing ACh to terminate cholinergic signaling (Soreq and Seidman, 2001). For example, AChE has been reported to promote neurite outgrowth in hippocampal neurons (Day and Greenfield, 2002) and primary dorsal root ganglion neurons (Bigbee et al., 2000) to induce aggregation of β-amyloid peptides and to increase their neurotoxicity (De Ferrari et al., 2001), as well as to induce neuronal cell loss, astrocyte hypertrophy, and behavioral deficits in mammalian hippocampus (Chacon et al., 2003). Therefore, it is not surprising that AChE-interacting drugs have a broad array of diverse physiological and pathophysiological effects.

Although the rate of ACh hydrolysis by AChE is extremely fast, Descarries et al. (1997) have suggested that in the brain (as opposed to the neuromuscular junction), the rapid elimination of ACh occurs mainly through diffusion and not hydrolysis by AChE. Therefore, they have suggested that the inhibition of AChE will only have minimal effects on cholinergic synaptic transmission. However, we have shown that endogenous AChE is extremely effective and rapid in hydrolyzing exogenously applied ACh because the decay of the non-α7 nAChR-mediated responses was dramatically slowed by AChE inhibition; the half-time of decay of ACh-induced responses was increased to ~21 s after AChE inhibition as opposed to ~4 s before. The fact that ACh responses decayed in 4 s (when the activity of AChE was not inhibited) demonstrates how fast the hydrolysis of ACh is occurring in these slices. In the future, it will be important to investigate how synaptically activated nAChR-mediated responses (i.e., caused by activation by endogenous ACh) are modified by AChE activity.

Previous reports have shown that α7-containing nAChRs are located at postsynaptic sites on hippocampal interneurons and mediate fast cholinergic excitatory synaptic transmission (Alkondon et al., 1998; Frazier et al., 1998). In addition, it was recently shown that these α7-containing nAChRs were located primarily at perisomatic sites (<70 μm from the soma) (Khiroug et al., 2003). Whether these receptors might also be located and function extrasynaptically remains to be determined. Furthermore, it is not known at the moment whether the non-α7 nAChRs, which because of their slow activation and desensitization might be more suited to an extrasynaptic role, are located at postsynaptic sites and can mediate fast cholinergic excitatory synaptic transmission in the hippocampus. There is evidence in the hippocampus that most of the cholinergic action may be mediated by nonsynaptic, diffuse, so-called "volume transmission" (Descarries et al., 1997). The α7-containing nAChRs have also been found on presynaptic terminals in the hippocampus, on which their activation increased intraterminal Ca^{2+} levels and facilitated transmitter release (Gray et al., 1996); however, others have failed to observe this effect (Vogt and Regehr, 2001). How these various subtypes of nAChRs are normally activated and regulated by endogenous ACh and how AChE activity may regulate this remain to be determined. Despite these questions that remain to explain the multifaceted cholinergic signaling mechanisms, we have shown significant effects of AChE action in hippocampal slices, and thus this enzyme is likely to have a significant impact on cholinergic receptor-mediated synaptic and extrasynaptic signaling pathways. Understanding these various pathways, which are important to hippocampal activity because dysfunctions in these pathways are known to occur in AD (and other neurological disorders), will shed light that will aid in the further development of drugs for the treatment of AD.

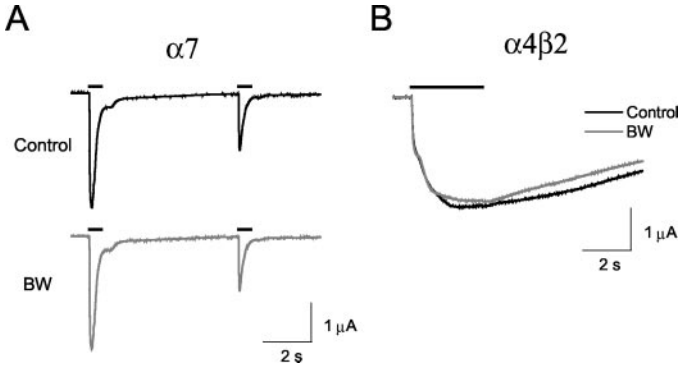

Fig. 6. Effect of AChE inhibitors on α7 and $\alpha4\beta2$ nAChRs expressed in *X. laevis* oocytes. A, in a *X. laevis* oocyte expressing α7 nAChRs, a dual-pulse protocol was used to activate these channels whereby a second pulse of ACh (2 mM) was applied 6 s after the first (500-ms duration for both). The application of BW284c51 (BW; 100 nM) had no significant effect on either the amplitude or kinetics of either the first or second response to ACh. B, in a *X. laevis* oocyte expressing $\alpha4\beta2$ nAChRs that were activated by ACh (1 mM; 3 s), BW284c51 (100 nM) had no significant effect on the amplitude or decay of current responses.

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