Acetylcholinesterase inhibitors activate septohippocampal GABAergic neurons via muscarinic but not nicotinic receptors.

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List of non-standard abbreviations: AChE, acetylcholinesterase; AChEI, acetylcholinesterase inhibitor; AD, Alzheimer’s disease; Amb, ambenonium; □-BGT, □-bungarotoxin; d-TC, d-tubocurarine; DHBE, dihydro-beta-erythroidine; DFP, diisopropylfluorophosphate; ED, edrophonium; IR-DIC, infrared, differential interference contrast imaging; ISH, in situ hybridization; MSDB, medial septum/diagonal band of Broca; MLA, methyllycaconitine; MT-7, muscarinic toxin 7; musc, muscarine; NS, neostigmine; THA, tacrine/tetrahydroaminoacridine
Abstract

Acetylcholinesterase (AChE) inhibitors, which increase synaptic levels of available ACh by preventing its degradation, are the most extensively prescribed drugs for the treatment of Alzheimer’s disease. In animals, AChE inhibitors improve learning and memory, reverse scopolamine-induced amnesia and produce hippocampal theta rhythm. The medial septum/diagonal band of Broca (MSDB), which maintains hippocampal theta rhythm and associated mnemonic functions via the septohippocampal pathway, is considered a critical locus for mediating the effects of AChE inhibitors. Using electrophysiological recordings and fluorescent labeling techniques to identify living septohippocampal neurons in rat brain slices, we report that AChE inhibitors, in the absence of exogenous ACh, produce a profound excitation in 94% of septohippocampal GABAergic neurons and an inhibition in 24% of septohippocampal cholinergic neurons. The inhibitory and excitatory effects of AChE inhibitors, presumably, occur due to accumulation of ACh that is released locally within the MSDB via axon collaterals of septohippocampal cholinergic neurons. The excitatory effects of AChE inhibitors on septohippocampal GABAergic neurons were blocked by muscarinic but not nicotinic receptor antagonists, especially by the M₃ receptor antagonist, 4-DAMP mustard, but not by M₁, M₂/M₄ muscarinic receptor antagonists. M₃ muscarinic receptor mRNA co-localized with the calcium-binding protein, parvalbumin, a marker of septohippocampal GABAergic neurons. These findings may be useful in designing therapeutic strategies that do not depend on endogenous ACh and may therefore be effective in situations where AChE inhibitors cease to be effective such as in progressive neurodegeneration.
Current therapeutic strategies for the treatment of cognitive deficits associated with Alzheimer’s disease (AD) and other neurodegenerative disorders are based on the cholinergic hypothesis of learning and memory (Bartus et al., 1982). Both the cortex and the hippocampus progressively lose their supply of ACh in AD as well as in Parkinson’s disease, Lewy body dementia and Down’s syndrome, due to a continuing atrophy of their ACh source - the cholinergic cell bodies of the nucleus basalis and the medial septum/diagonal band of Broca (MSDB), respectively (Whitehouse et al., 1982; Mufson et al., 1989; Arendt et al., 1995).

Acetylcholinesterase (AChE) inhibitors, which increase synaptic levels of available ACh by preventing its degradation, are currently the most prescribed drugs for the treatment of AD, albeit of limited effectiveness. The AChE inhibitors, physostigmine and tacrine (tetrahydroaminoacridine, THA) slow the decline of cognitive function and memory in patients with mild or moderate AD (Weinstock, 1995). Beneficial effects of tacrine on the slowing of the electroencephalogram, have also been reported in mild cases of AD patients both after a single dose and up to 3 months of treatment, following which they may become ineffective (Alhainen et al., 1991; Perryman and Fitten, 1991; Jelic et al., 1998). The limited effectiveness of AChE inhibitors has been attributed to several factors that include reduced availability of ACh as the neurodegeneration progresses as well as to molecular changes that may occur following long-term treatment with AChE inhibitors, such as an up-regulation of the AChE enzyme and a down-regulation of the ACh-synthesizing enzyme (Kaufer et al., 1998).

In animals, systemically administered AChE inhibitors produce hippocampal theta waves (Teitelbaum et al., 1975; Olpe et al., 1987; Valjakka et al., 1991; Yamamoto, 1998; Ikonen and Tanila, 2001) and improve learning and memory. These effects are blocked by the muscarinic receptor antagonist, scopolamine, as well as by lesions of the MSDB (Teitelbaum et al., 1975; Olpe et
al., 1987; Ennaceur and Meliani, 1992; Ennaceur, 1998; Ikonen and Tanila, 2001), thereby indicating a critical role for muscarinic receptors and the MSDB in mediating the effects of AChE inhibitors.

The MSDB, which via the septohippocampal pathway maintains hippocampal learning and memory functions and the associated theta rhythm, is also considered a critical locus for the well-described memory-impairing effects of scopolamine (Stewart and Fox, 1990; Givens and Olton, 1994; Givens and Olton, 1995). Scopolamine-induced amnesia has been likened to early symptoms of Alzheimer’s disease and is reversed by AChE inhibitors in rats as well as in subhuman and human primates (Ye et al., 1999). At the cellular level, scopolamine has been shown to act within the MSDB by disrupting impulse flow in the septohippocampal GABAergic pathway. Moreover, the effects of scopolamine can be observed in vitro in a brain slice preparation of the MSDB (Alreja et al., 2000), suggesting that the muscarinic tone, which is critical for learning and memory, originates locally within the MSDB, presumably via axon collaterals of septohippocampal cholinergic neurons (Bialowas and Frotscher, 1987; Leranth and Frotscher, 1989).

Based on the above, we hypothesize that AChE inhibitors, by preventing destruction of available ACh within the MSDB, would lead to an enhancement of the muscarinic tone within the MSDB, a mechanism that may contribute or even be critical to the mnemonic effects of AChE inhibitors. In addition, in a recent study we found that exogenous ACh also excites septohippocampal GABAergic neurons via nicotinic cholinergic receptors (Wu et al., in press), a second goal of this study was to test for the presence of a nicotinic tone in septohippocampal neurons. A knowledge of the cellular mechanisms by which AChE inhibitors work may help design new treatments that could restore cognitive functions when AChE inhibitors cease to be effective.
Methods

Slice preparation for electrophysiological recordings

Brain slices containing the MSDB were prepared from 2-3 weeks old male Sprague-Dawley albino rats using methods detailed previously (Alreja and Liu, 1996). Briefly, rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and killed by decapitation. The ACSF (pH 7.35-7.38), equilibrated with 95%O₂-5% CO₂, contained (in mM): NaCl, 126; KCl, 3; NaH₂PO₄, 1.25; D-glucose, 10; NaHCO₃, 25; CaCl₂, 2 and MgSO₄, 2. Following decapitation, the brain was removed and placed in a petri dish containing ACSF and trimmed to yield a small block containing the MSDB. Two coronal slices of ~300 µm thickness containing the MSDB were cut with a vibrating-knife microtome (Frederick Haer, ME, USA) and transferred to a Plexiglas recording chamber (1.5 ml volume) on the fixed stage of an Olympus BX50WI microscope. The slice was kept in place with a grid and maintained at 33 ± 0.5°C. One to two hours later the slice was used for recording. The chamber was continuously perfused with normal ACSF at a rate of 1-2 ml/min.

Labeling of septohippocampal cholinergic neurons using Cy3-192IgG

Rats were anesthetized using the following cocktail: ketamine -75 mg/kg; Xylazine - 4 mg/kg and acepromazine – 0.075 mg/kg. Cy3-192-IgG (3-5 µl; 0.4 mg/ml) was stereotaxically injected unilaterally or bilaterally into the lateral ventricle of each rat with a Hamilton syringe (22 gauge needle) at a rate of 0.5 µl/min. The coordinates used were: 0.8 mm posterior from bregma, 1.2 mm lateral from midline and 3-4 mm below the dura. Two to five days later, slices were prepared from Cy3-192IgG injected rats and used for electrophysiological recordings. Recordings from unlabeled neurons were restricted to slices taken from rats injected biventricularly with the marker.
Retrograde labeling of septohippocampal neurons

Retrograde labeling of septohippocampal neurons was performed by pressure-injecting 50-100 nl of rhodamine-labeled fluorescent latex microspheres (Lumafluor Inc., Naples, FL, USA) at several sites within the hippocampus using a glass micropipette (40-50 μm tip diameter). Rhodamine-microspheres (0.02-0.2 micron diameter) show little diffusion and consequently produce small, sharply defined injection sites. Once transported back to neuronal somata, the label persists for at least 10 weeks in vivo and 1 yr after fixation. Microspheres have been reported to possess no obvious cytotoxicity or phototoxicity as assessed by intracellular recording and staining of retrogradely labeled cells in brain slice preparation (Katz et al., 1984). The stereotaxic coordinates were (antero-posterior, lateral and ventral): (-2.8, -1.4, -2.8), (-4, -1.4, -2.8) and (-5.8,-4.5 and -3.5 to -6 mm track). 2 or more days later, the injected rats were used to prepare brain slices. Injection sites were confirmed for each experiment.

Fluorescence and infrared imaging

Infrared, differential interference contrast imaging (IR-DIC) was performed to visualize neurons for extracellular or patch-clamp recording using an Olympus Optical (Tokyo, Japan) BX-50 microscope equipped with a 60x water immersion objective (numerical aperture, 0.9; Olympus). Images were detected with a CCD-300-RC camera (DAGE-MTI, Michigan City, IN) and displayed on a standard black and white video monitor (DAGE-MTI, HR 120). The images were transferred to the hard disk of a PC using an LG-3 scientific frame grabber (Scion Image Corpn.; Frederick, MD, USA) and processed further with Adobe Photoshop. Cy3-192IgG-labeled and rhodamine-labeled neurons were visualized using the appropriate fluorescence filters. A neuron viewed with infrared optics was considered to be the same as that viewed with fluorescence optics when the infrared image
and the fluorescent image of the neuron had the same position and orientation with the two imaging systems.

**Electrophysiology recordings**

The image of the cells in the slice was displayed on a video monitor, and glass pipettes used for electrophysiological recordings were visually advanced through the slice to the surface of the cell from which recordings were made. Extracellular recordings were performed with glass micropipettes filled with 2 M NaCl (5-10 MΩ). Whole-cell patch-clamp recordings were performed using previously described methods (Alreja and Liu, 1996). In brief, low resistance (2.5-3.5 MΩ) patch pipettes were filled with a solution containing (in mM): K methylsulfonate/KCl, 120; HEPES, 10; BAPTA K$_4$, 5; sucrose, 20; CaCl$_2$, 2.38; MgCl$_2$, 1; K$_2$ATP, 1 and GTP, 0.1 (pH - 7.32-7.35). All recordings were made using an Axoclamp-2B amplifier (Axon Instruments, CA). **Current-clamp recordings were made in the bridge mode** and the output signal was filtered at 10 KHz. The cells selected for study had spike amplitudes of 70-100 mV. Spike durations were measured at half-spike amplitude. In spontaneously firing cells these measurements were done at the resting potential and in quiescent cells firing was induced by injecting a small amount of depolarizing current. Voltage clamp recordings were performed using the continuous single electrode voltage clamp mode. The firing rate, current and voltage signals were amplified and continuously recorded on a chart recorder (Gould 2200).

**Combined Immunocytochemistry and in situ hybridization**

**Immunocytochemistry**
16 μm thick rat brain cryocut sections were fixed in 4% paraformaldehyde for 10 min followed by a 5 min 1% hydrogen peroxide treatment and 2 rinses of 5 min each in PBS. Sections were blocked with 2.5% BSA and 3000 U/ml heparin in PBS for 30 min to prevent non-specific binding of antibody. Slides were then rinsed 3 times in PBS. Sections were then incubated for 48 hr in an antibody solution (0.25% triton X, 1% BSA, 3000 U/ml heparin, 10 U/ml anti-RNase in PBS) containing a 1:1000 dilution of monoclonal antiparvalbumin antibody. Unbound antibody was removed by 3 PBS rinses and 1 rinse in PB. Subsequently, sections were incubated in biotinylated anti-mouse IgG at a 1:200 dilution for 1 h at room temperature. Unbound secondary antibody was removed by three 5 min rinses in PBS. Sections were incubated in preformed avidin and biotinylated horseradish peroxidase macromolecular complex (ABC reagent, Vector labs). Following this incubation, sections were rinsed 3 times in PB and then stained with the DAB staining kit (Vector Labs) according to the manufacturer's directions. Slides were air dried and processed as follows for in situ hybridization.

**In situ hybridization (ISH)**

ISH was performed as per standard protocols for cryocut sections. Briefly, sections were fixed for 10 min in cold 4% paraformaldehyde, followed by a 1 min rinse in PBS and another 1 min rinse in 100 mM triethanolamine (TEA) buffer and acetylated in 100 mM TEA buffer containing 0.25% acetic anhydride. After rinsing twice in 2x SSC buffer, sections were then dehydrated in an alcohol series. Antisense cDNA template for muscarinic receptor 3 (Bonner et al., 1987) with an upstream Sp6 polymerase site was used to generate 35 S radiolabeled riboprobes. Hybridization was overnight at 55°C. Following post-hybridization washes and RNAse A treatment, slides were air dried and exposed to film to determine success of ISH and duration of exposure to emulsion. Slides were then
dipped in nuclear track emulsion (NTB2, Kodak), dried for 4h and then stored in a dark, dry box. After 4 weeks sections were developed using Kodak D-19 developer (4 min at 16 °C) and fixed in Kodak Fixer. Slides were counter-stained in cresyl violet, dehydrated in an alcohol series, cleared in Histochoice (15 sec) and coverslipped with DPX mountant. Sections were observed using a Carl Zeiss Axioplan brightfield microscope and photographed with a digital camera (DVC).

Specificity of signal was ascertained by first exposing sections to autoradiographic film and only then dipped in emulsion. A cell was considered colocalizing paravalbumin protein and 

M₃ mRNA when emulsion grains overlaying a parvalbumin positive cell had a higher density (50%) than background (determined by counting emulsion grains in a brain region not showing specific signal on film). 4 fields were examined from each section, and 4 sections were used from 3 animals.

Materials

The various AChE inhibitors, Acetylcholine chloride, Muscarine chloride, atropine sulfate, (-)-scopolamine hydrobromide, methoctramine tetrahydrochloride as well as nicotinic receptor agonists and antagonists were obtained from Sigma-Aldrich, USA. Muscarinic toxin 7 (MT7) was obtained from Peptide Institute, Inc., Osaka, Japan. All drugs were diluted in ACSF from previously prepared stock solutions that were prepared in water and stored at -20°C. Rhodamine microspheres were obtained from Lumafluor Inc., Naples, FL, USA and Cy3-192IgG was custom synthesized by Advanced Targeting Systems, San Diego CA.
Results

AChE inhibitors excite MSDB neurons in vitro

The effects of AChE inhibitors on MSDB neurons were tested using both extracellular and intracellular recordings in rat brain slices. In order to exclude non-specific effects of AChE inhibitors, such as agonist actions that may involve activation of nicotinic receptors (Bloch and Stallcup, 1979; Shaw et al., 1985), a wide array of AChE inhibitors with different primary mechanisms of action were chosen. The AChE inhibitors tested, included the long-acting, clinically used physostigmine, tetrahydroaminoacridine (THA/tacrine) and galanthamine, as well as the irreversible cholinesterase inhibitor, diisopropylfluorophosphate (DFP). The effects of short-acting inhibitors such as neostigmine, ambenonium, edrophonium were also tested. Physostigmine and neostigmine are carbamates and act as metabolic inhibitors of acetylcholinesterase. Edrophonium is a quaternary compound that blocks the AChE enzyme competitively by binding to its active site, whereas, ambenonium is a rapidly reversible noncovalent inhibitor of acetylcholinesterase. Tacrine causes a long-lasting but reversible allosteric inhibition of AChE by binding to a hydrophobic region near the anionic $\alpha$ or $\beta$ sites on its surface and DFP is an irreversible phosphorylating inhibitor. In all, we tested the effect of 7 AChE inhibitors on 80 unidentified MSDB neurons.

The effects of multiple AChE inhibitors were tested on the same cell. As shown in figure 1, a qualitatively similar response was obtained in all cases, albeit, as expected, the magnitude and duration of the effect varied amongst different AChE inhibitors. In extracellular recordings, 77.3 % of the neurons tested were excited by exogenous ACh/muscarine (58/75), 17.3 % (13/75) were inhibited and 5.4 % (4/75) of neurons were not affected by ACh/muscarine. Of the 58 neurons excited by
ACh/musc, 49 (84.5%) responded to AChE inhibitors with a strong excitation, suggesting the presence of a cholinergic tone. A similar effect was obtained with all the AChE inhibitors (Fig. 1a-e). Out of the 13 neurons inhibited by ACh/muscarine, two responded to AChE inhibitors with an inhibition and the remaining neurons showed no response (not shown). The 4 neurons not affected by ACh/muscarine showed no response to AChE inhibitors. Cumulative concentration-response studies performed in 5 cells with the clinically most prescribed AChE inhibitor, tacrine revealed an EC$_{50}$ of 3.2 μM (Fig. 1d).

The effect of the short-acting AChE inhibitors on GABA-type MSDB neurons was also tested using intracellular voltage-clamp recordings. GABA-type neurons were identified by their electrophysiological characteristics and their excitatory response to muscarine (Wu et al., 2000). 85.7% of neurons tested (12/14) responded to AChE inhibitors with an excitation and both neostigmine and ambenonium mimicked the effect of muscarine and produced inward currents (95.4 ± 22.7 pA; n= 12). The magnitude of the AChE inhibitor response at the concentration tested, was significantly lower than the effect of a near-maximal concentration of muscarine (254.2 ± 47.9 pA; Fig. 1f,g).

AChE inhibitor-induced excitation of MSDB neurons involves muscarinic but not nicotinic receptors

Since ACh excites septohippocampal GABA-type neurons via both nicotinic and muscarinic receptors (Wu et al., 2000) we determined the contribution of these 2 receptor subtypes to the AChE-induced excitation. As shown in Fig. 1, the broad-spectrum muscarinic receptor antagonists, atropine or scopolamine significantly reduced basal firing (Alreja et al., 2000) and also blocked both the excitatory (n=19) and the inhibitory (n=3) effects of AChE inhibitors (see below).
The contribution of nicotinic receptors to the AChE inhibitor response was determined using several nicotinic receptor antagonists. Within the MSDB, nicotinic responses are blocked by the broad-spectrum nicotinic receptor antagonist, d-tubocurarine and by the \( \alpha_{4}\beta_{2} \)-subtype selective antagonist, dihydro-beta-erythroidine (DHBE), but not by the \( \alpha_{7} \)-selective antagonists, methyllycaconitine (MLA) and \( \alpha \)-bungarotoxin (\( \alpha \)-BT) (Fig. 2a, b). Note that, in contrast to the muscarinic receptor antagonists which reduce basal firing, nicotinic receptor antagonists did not produce any change in basal firing rates in the population of cells tested, suggesting that MSDB neurons are under a muscarinic but not a nicotinic excitatory tone under resting conditions. Similarly, nicotinic receptor antagonists also had no effect on the AChE inhibitor-induced excitation, suggesting that AChE inhibitor-induced excitation of MSDB neurons involves activation of muscarinic but not nicotinic receptors (Fig. 2c,d). These findings are consistent with the behavioral effects of systemic AChE inhibitors on septohippocampal functions that are blocked by muscarinic receptors (see Introduction).

**Effect of AChE inhibitors on identified septohippocampal cholinergic and non-cholinergic neurons.**

Having ascertained the presence of AChE inhibitor effects on MSDB neurons, we next conducted a systematic study to determine the effect of AChE inhibitors on MSDB neurons that project to the hippocampus, as it is these septohippocampal neurons which control the activity of the hippocampus and facilitate performance in hippocampus-associated learning and memory tasks. Based on our earlier studies in which we have shown that neurons that are excited by ACh/muscarine belong to the non-cholinergic subpopulations (presumably, GABAergic) and project to the hippocampus (Wu et al., 2000), we hypothesized that AChE inhibitor-excited neurons would belong
to the septohippocampal GABAergic neuronal population. Septohippocampal cholinergic neurons, on the other hand are either inhibited or not affected by muscarine and were found to be under a minimal muscarinic tone as tested using muscarinic receptor antagonists (Alreja et al., 2000).

Septohippocampal cholinergic neurons were identified in the living state using the fluorescent marker, Cy3-192IgG (Alreja et al., 2000; Wu et al., 2000) as previously described. This technique exploits the fact that septohippocampal cholinergic neurons, but not the GABAergic neurons of the MSDB, exclusively express the low-affinity nerve growth factor receptor, p75. Thus, this marker, which is comprised of an antibody against the p75 receptor (192IgG) conjugated to the Cy3 fluorochrome, is taken up only by cholinergic terminals and therefore exclusively labels only the cholinergic subpopulation. The specificity of this marker and its inert nature has been thoroughly confirmed by us (Alreja et al., 2000; Wu et al., 2000) and by others in an earlier study (Hartig et al., 1998).

Consistent with our earlier findings (Wu et al., 2000), septohippocampal cholinergic neurons, identified using Cy3-192IgG, were either inhibited or not affected by exogenous ACh/muscarine and lacked a depolarizing sag in response to hyperpolarizing pulses (Fig. 3a1-a3). We tested the effect of 2 short-acting AChE inhibitors, neostigmine and ambenonium, and the clinically used AChE inhibitor, tacrine on 29 septohippocampal cholinergic neurons that were inhibited by ACh/muscarine. AChE inhibitors mimicked the effect of ACh/muscarine in 24% of cholinergic neurons tested (7/29) (Fig. 3a, c-e) and had no effect on the remaining 76% of neurons. The mean magnitude of the response to AChE inhibitors was 8.6 ± 2.4 mV, whereas the response to muscarine was 11.6 ± 2.4 mV in magnitude. The inhibitory effects of AChE inhibitors were blocked by atropine (n=3), suggesting involvement of muscarinic receptors (Fig. 3a3). Thus, AChE inhibitors can modulate the activity of a small subpopulation of septohippocampal cholinergic neurons.
In contrast to the Cy3-192IgG-labeled neurons mentioned above, a vast majority of the unlabeled neurons (94%; 15/16) recorded from brain slices taken from rats injected biventricularly with Cy3-192IgG, responded to ACh/musc with an excitation and exhibited a depolarizing sag in response to hyperpolarizing pulses that was absent in the Cy3-192IgG-labeled neurons (Fig. 3b1-b3). It should be mentioned that biventricular injections of Cy3-192IgG label at least 90% of ChAT-positive neurons and the unlabeled population is comprised primarily of the non-cholinergic neurons that co-localize the calcium-binding protein, parvalbumin. Parvalbumin is uniquely expressed by the septohippocampal GABAergic neurons in this nucleus that exhibit a depolarizing sag in response to hyperpolarizing current steps (Morris et al., 1999; Wu et al., 2000). All 15 unlabeled neurons responded to AChE inhibitors with a profound excitation and atropine blocked the excitatory effects in the 3 neurons tested (Fig. 3b-e).

**AChE inhibitors excite septohippocampal GABAergic neurons via M₃ receptors**

Septohippocampal neurons were retrogradely labeled following injection of rhodamine beads into the hippocampus. Rhodamine beads label both the septohippocampal cholinergic and GABAergic neurons. However, if a rhodamine-labeled septohippocampal neuron responded to ACh/musc with an excitation and displayed the appropriate electrophysiological signature, which included the presence of a depolarizing sag (Morris et al., 1999), then it was classified as a septohippocampal GABAergic neuron as septohippocampal cholinergic neurons are not excited by muscarine (Wu et al., 2000). 5/5 septohippocampal GABA-type neurons identified using these criteria were excited by AChE inhibitors, with the short-acting inhibitor, neostigmine, producing a 215 ± 15% increase in firing rate (Fig. 4a-f). Thus, AChE inhibitors enhance the activity of septohippocampal GABAergic neurons in vitro.
Since the M₃ receptor mediates the excitatory effects of muscarine on septohippocampal GABAergic neurons, and M₃ receptor antagonists also mimic the effects of scopolamine on MSDB neurons (Liu et al., 1998; Alreja et al., 2000; Wu et al., 2000), we tested the effect of an M₃ receptor antagonist on AChE inhibitor induced activation. Low nanomolar concentrations of 4-DAMP mustard, an irreversible antagonist that selectively inactivates M₃ receptors but has no effect on M₁, M₂, M₄ and M₅ receptors (see Liu et al., 1998), produced statistically significant reductions in the excitatory response to exogenous ACh/muscarine and neostigmine in 7/8 neurons tested (Fig. 4c, g). Consistent with our previous findings, 4-DAMP mustard also reduced basal firing rates. In contrast, the M₂/M₄ receptor antagonist, methoctramine, and the highly selective, M₁ receptor toxin, MT-7 (Olianas et al., 2000) did not reduce the excitatory response to AChE inhibitors in the 3 neurons tested (Fig. 4d,e,g). Thus, the excitatory effects of AChE inhibitors in the MSDB are mediated primarily via M₃ muscarinic receptors (Fig. 4g).

We next used immunolabeling techniques in combination with in situ hybridization to examine if septohippocampal GABAergic neurons co-localize the message for the M₃ muscarinic receptor. It should be mentioned that presence of M₃ receptor message has already been reported in about 80% of septohippocampal neurons, however, the phenotype of the M₃ mRNA-expressing septohippocampal neurons was not determined (Rouse and Levey, 1996). Septohippocampal GABAergic neurons were identified by an immunopositive reaction to an antibody against the calcium-binding protein, parvalbumin, which is exclusively expressed by the septohippocampal GABAergic neurons in the MSDB (Freund, 1989) and has therefore become an established marker for this neuronal subpopulation. As shown in Fig. 4h, parvalbumin-containing MSDB neurons were found to express M₃ receptor mRNA, thus supporting the pharmacological findings.
Discussion

The chief findings of this study are - 1) acetylcholinesterase inhibitors activate a vast majority of septohippocampal GABAergic neurons and have an opposing effect on a small subpopulation of septohippocampal cholinergic neurons; 2) the excitatory effects of AChE inhibitors are mediated via activation of muscarinic but not nicotinic receptors. These effects of AChE inhibitors within the MSDB, via recruitment of the septohippocampal disinhibitory circuit, may contribute or even be critical in mediating the facilitatory effects of AChE inhibitors in learning and memory functions associated with the septohippocampal pathway.

**AChE inhibitors activate MSDB neurons via muscarinic but not nicotinic receptors**

The profound effects of low micromolar concentrations of AChE inhibitors observed in this study are in support of behavioral studies which suggest that the MSDB may be a key locus in mediating the effects of AChE inhibitors (see Introduction). These effects, presumably, result from an increase in the availability of locally released ACh as a result of reduced degradation of ACh following inhibition of the AChE enzyme.

A blockade of AChE inhibitor effects by muscarinic but not nicotinic receptor antagonists, suggests the presence of a muscarinic but not nicotinic tone within the MSDB slice preparation which is consistent with published literature showing that AChE inhibitor effects on learning and memory functions and on theta rhythm are blocked by scopolamine (see Introduction). The presence of a muscarine tone supports our recent findings wherein, bath-application of muscarinic receptor antagonists reduce basal firing rates of GABA-type MSDB neurons in brain slices devoid of extrinsic cholinergic inputs (Alreja et al., 2000). **The absence of a nicotinic component in the AChE inhibitor**
responses may reflect a rapid desensitization of nicotinic receptors due to the augmented concentrations of ACh with AChE inhibitors. A lack of effect of nicotinic receptor antagonists on AChE inhibitor responses also suggests that ACh release within the MSDB is not under the influence of nicotinic receptors.

Interestingly, the number of MSDB neurons found to be under the influence of an excitatory muscarinic tone using AChE inhibitors is much larger (94%) than we previously estimated using muscarinic receptor antagonists (54%). A likely explanation for this difference may be that under basal conditions, the amount of ACh available at some synapses may not be high enough to elicit a physiological response, possibly due to inadequate release and/or high levels of local cholinesterase activity. AChE inhibitors, by preventing degradation may allow enough ACh to build up so as to have a noticeable effect on basal cell firing of MSDB neurons. Alternately, the increased number of neurons responding to AChE inhibitors may reflect spillover of released ACh from the synaptic clefts, which in the absence of degradation, may diffuse long enough distances to activate receptors located outside the synaptic cleft or even in neighboring synapses. Both these factors may also contribute to the AChE inhibitor effects that were detected in 25% of septohippocampal cholinergic neurons in this study. Previously, using muscarinic receptor antagonists, an inhibitory muscarinic tone was detected only in 7% (1/14) of septohippocampal cholinergic neurons tested.

A third possibility is that some of the actions of AChE inhibitors may be attributable to non-cholinesterase activity, albeit muscarinic receptor dependent, as AChE inhibitor responses were blocked by muscarinic receptor antagonists in 100% of the neurons tested. This seems unlikely for the following reasons, 1) the effects of AChE inhibitors, but not of exogenous ACh/muscarine, on basal firing rates are significantly reduced following blockade of synaptic transmission, although AChE inhibitors continue to potentiate the effects of exogenous ACh; 2) similar results were obtained
with the 7 AChE inhibitors tested even though these AChE inhibitors inhibit AChE via different mechanisms. It is unlikely that all the AChE inhibitors tested shared a common non-specific mechanism; that of behaving as an agonist at the muscarinic M₃ receptor (see below). Moreover, non-specific actions of AChE inhibitors are mostly attributed to activation of nicotinic receptors. In the present study, nicotinic receptor antagonists did not reduce the effects of AChE inhibitors.

It should also be mentioned that the effects of one of the AChE inhibitors used in this study, THA, have previously been studied in guinea pig MSDB slices, albeit, at concentrations 100-200 fold higher than those used in the present study; effects were observed on potassium channels of several populations of electrophysiological characterized MSDB neurons. However, THA effects on basal properties of neurons as well as the question of whether they involved activation of muscarinic receptors were not addressed (Griffith and Sim, 1990).

AChE inhibitor effects, similar to those described in the present study have been reported only in the rat dorsal cochlear nucleus slices, wherein, comparable concentrations of AChE inhibitors induced atropine-sensitive burst firing in a synaptically dependent manner (Chen et al., 1998).

**AChE inhibitors increase impulse flow in the septohippocampal GABAergic pathway**

The finding that increased impulse flow in the septohippocampal GABAergic pathway but not in the septohippocampal cholinergic pathway may underlie the mnemonic effects of AChE inhibitors further supports the emerging role of the septohippocampal GABAergic pathway in learning and memory. Thus, accumulating data from *in vivo* microdialysis studies, theta rhythm studies, behavioral studies as well as *in vitro*, cellular studies, indicates that contrary to current wisdom, manipulations that improve learning and memory and/or facilitate the hippocampal theta rhythm are often associated with a decrease in hippocampal ACh release and an increase in septohippocampal GABA release (see
Alreja et al., 2000; Wu et al., 2000). Septohippocampal GABAergic neurons exclusively innervate the GABAergic interneurons of the hippocampus, which, in turn, contact 500-1200 pyramidal neurons, connectivity, that results in a powerful disinhibition of hippocampal pyramidal cells following stimulation of septohippocampal GABAergic afferents (see Toth et al., 1997). Thus, while scopolamine disrupts ongoing impulse flow in the septohippocampal GABAergic pathway, AChE inhibitors, like muscarinic agonists recruit the septohippocampal disinhibitory pathway. This may be a key mechanism by which AChE inhibitors work in the brain as lesions of the MSDB can prevent both the memory-enhancing effects of AChE inhibitors (Teitelbaum et al., 1975; Olpe et al., 1987; Ennaceur, 1998), as well as the facilitatory effects of AChE inhibitors on the hippocampal theta rhythm (Ikonen and Tanila, 2001).

**AChE inhibitors decrease impulse flow in the septohippocampal cholinergic pathway**

The finding that AChE inhibitors actually decrease the activity of a small subpopulation of septohippocampal cholinergic neurons is also significant and is supported by the finding that in the presence of the AChE inhibitor, physostigmine, uptake of tritiated choline is reduced in the MSDB (Disko et al., 1998). This effect would therefore be counter-productive from the therapeutic angle as it would further reduce the release of ACh, although within the hippocampus this reduction could be countered by reduced degradation of available ACh. Nevertheless, a blockade of the inhibitory effects of AChE inhibitors on septohippocampal cholinergic neurons may be desirable especially as the neurodegenerative process escalates and available ACh continues to dwindle. The inhibitory effects of AChE inhibitors on septohippocampal cholinergic neurons could be due to a direct inhibitory effect of local ACh via the inhibitory muscarinic M2/M4 receptor subtypes localized on a subset of septohippocampal cholinergic neurons (Vilaro et al., 1992; Van der Zee and Luiten, 1994; Levey et
al., 1995; Rouse and Levey, 1996) or indirect due to an increase in local GABA release following AChE-inhibitor-induced excitation of the septal GABAergic neurons.

The results of this study also show that the slice preparation of the MSDB may be useful in evaluating drugs that may be effective for the treatment of cognitive deficits associated with neurodegenerative disorders and hopefully understand the cellular mechanisms that may limit the effectiveness of treatments over a prolonged period. For example, the MSDB slice preparation can in the future be used to test if prolonged treatment with AChE inhibitors or acute stress reduce AChE inhibitor effects in septohippocampal neurons, as has been suggested to occur in the cortex via a downregulation of the ACh-synthesizing enzyme and an upregulation of AChEs (Soreq and Seidman, 2001). In conclusion, it is hoped that the results of this study and future studies will help design better therapies for treatment of cognitive deficits that occur in various neurodegenerative disorders.
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References


Figure Legends

Fig. 1. Acetylcholinesterase inhibitors excite MSDB neurons in vitro via activation of muscarinic receptors. a, Extracellular recording from an MSDB neuron shows that an ACh-excited MSDB neuron responds to the short-acting AChE inhibitor, ambenonium (amb), and to the long-acting AChE inhibitor, tacrine with a profound increase in firing rate. Scopolamine, a muscarinic receptor antagonist, blocked tacrine-induced excitation and also lowered the basal firing rate by 15%. Scopolamine also prevented the effect of a second application of ambenonium and reduced the response to exogenous ACh, presumably due to blockade of the muscarinic component. b, Shows the excitatory response of a second MSDB neuron to the short-acting AChE inhibitors, neostigmine (NS), edrophonium (ED), ambenonium and the longer-acting, physostigmine (PS). Atropine, also a muscarinic receptor antagonist, blocked the response to physostigmine and produced a 78% reduction in the basal firing rate, consistent with our earlier findings (Alreja et al., 2000). Subsequent applications of various AChE inhibitors had no effect. c, shows that the AChE inhibitors, ambenonium and galanthamine, caused a marked increase in basal firing, an effect that was reversed by atropine. Atropine blocked basal firing, but note that the cell still responded to exogenous glutamate with an excitation. d, shows the concentration-response curve for tacrine plotted from data obtained in 5 cells. An EC$_{50}$ of 3.2 M was obtained. e, Summarizes the magnitude of the excitatory effect of various AChE inhibitors on basal firing rate of MSDB neurons that responded to ACh with an increase in firing rate (*p<.05, **p<.01, ***p<.001; Student’s paired t-test). Thus, MSDB neurons are under a constant muscarinic tone, presumably due to the presence of locally released ACh. f, Intracellular voltage clamp recording from an MSDB neuron (holding potential: -65 mV) shows that both muscarine and neostigmine produced an inward current. g, Bar chart summarizes the magnitude of the inward currents in response to the 2 drugs. Both drugs were tested in the same neurons.
Fig. 2, Nicotinic receptors do not contribute to the excitatory effects of AChE inhibitors on MSDB neurons. a, show the excitatory response to nicotine in an MSDB neuron. The nicotine excitation is blocked by d-TC, which blocks all nicotinic receptor subtypes, and also by DHBE, which selectively blocks $\alpha_4\beta_2$-subtype nicotinic receptors. $\alpha$-BGT and MLA, which block responses to $\alpha_7$ nicotinic receptors, had no effect on the nicotinic response in MSDB neurons. Note that in contrast to the muscarinic receptor antagonist, atropine, none of the nicotinic receptor antagonists reduced basal firing, suggesting that basal firing rates of MSDB neurons are under a muscarinic but not a nicotinic tone. b, bar chart summarizes the effect of nicotinic receptor antagonists on basal firing rates of MSDB neurons. c, Shows the excitatory effect of nicotine, muscarine and neostigmine on a MSDB neuron. Middle trace shows that while the $\alpha_4\beta_2$-subtype nicotinic receptor antagonist blocked the nicotine response, it had no effect on the muscarine or the neostigmine response. In contrast, the muscarinic receptor antagonist, atropine blocked both the muscarinic and the neostigmine response. d, Bar chart summarizes the data and shows that the excitatory effects of AChE inhibitors are mediated via muscarinic but not nicotinic receptors in MSDB neurons.

Fig. 3, Opposing effects of AChE inhibitors on identified septohippocampal cholinergic neurons and non-cholinergic neurons of the MSDB. a1, a2, Whole-cell current-clamp recordings showing the electrophysiological signature of a septohippocampal cholinergic neuron identified in the living state (right) following injection of the retrograde tracer, Cy3-192IgG, which selectively labels the p75-receptor expressing cholinergic neurons (see methods). a3, shows the hyperpolarizing effect of exogenous muscarine and the AChE inhibitor ambenonium on a Cy3-192IgG-labeled septohippocampal cholinergic neuron, both responses were blocked by the muscarinic receptor.
antagonist, atropine. a4, shows the inhibitory response of a second septohippocampal cholinergic neuron to muscarine and the AChE inhibitor, ambenonium (Amb). Note that blocked of synaptic transmission using low Ca\(^{2+}\), high Mg\(^{2+}\)-containing ACSF, blocked the response to ambenonium, but not to muscarine. b1, Electrophysiological signature of an unlabeled neuron (presumably, GABAergic), taken from a Cy3-192IgG-injected rat. Note that the electrophysiological characteristics of this cell differ from those of the labeled cell shown in a1 above and include the presence of a depolarizing sag in response to the hyperpolarizing pulses. b2, An IR-DIC image shows several MSDB neurons surrounding one larger neuron. Note in the fluorescent image on the right, that only one neuron was labeled, remaining neurons (arrows), were unlabeled and most likely GABAergic. b3, shows the response of unlabeled neurons (taken from rats injected biventricularly with Cy3-192IgG) to exogenous ACh and the AChE inhibitor, neostigmime. Note that neostigmine increased the basal firing rate as well as the response to exogenous ACh. c, Bar chart summarizes the number of neurons that responded to AChE inhibitors in the 2 neuronal subpopulations. Note that while only 24% of Cy3-192IgG-labeled septohippocampal neurons responded to AChE inhibitors, 94% of the un-labeled neurons (presumably GABAergic) responded to AChE inhibitors. d, e, Bar charts compare the magnitude of the response to AChE inhibitors and muscarine in the 2 neuronal subpopulations.

**Fig. 4, AChE inhibitors excite identified septohippocampal GABAergic neurons via the M3 muscarinic receptor.** a, coronal section of the rat hippocampus showing the injection site where the retrograde tracer, rhodamine beads were injected (arrow); b, shows a rhodamine-labeled septohippocamal neuron in an MSDB slice preparation; c, recording from a retrogradely-labeled rhodamine-containing septohippocampal neuron identified to be GABAergic on the basis of its excitatory response to muscarine and electrophysiological characteristics (see text). Note that both
ACh and the AChE inhibitor, neostigmine, activated the cell. Bath application of 4-DAMP mustard (200 nM, 20 min) reduced basal firing, presumably due to blockade of an M3-receptor-mediated muscarinic tone (Alreja et al., 2000) and also blocked the response to neostigmine and to exogenous ACh. 

d, e, Show that the M2/M4 receptor antagonist, methoctramine and the M1 receptor antagonist, MT-7, did not block the excitatory response to neostigmine. 

f, Bar chart summarizes the magnitude of the excitatory response to muscarine and the AChE inhibitor, neostigmine, on septohippocampal GABAergic-type neurons. Note that neostigmine produced a 215 ± 15% increase in basal firing. 

g, Summarizes the effects of M1, M2/M4 and M3 receptor antagonists on the effect of AChE inhibitors (n=3-7). 

h, Shows co-localization of M3 receptor mRNA with the calcium-binding protein, parvalbumin, in an MSDB neuron (solid arrow). The neuron on the right (open arrow) did not co-localize the receptor message. Parvalbumin-ir is observed as a brown immunoperoxidase precipitate and M3 receptor mRNA as emulsion grains. Parvalbumin is uniquely expressed by the septohippocampal GABAergic neurons of the MSDB (see text).
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Cy3-192IgG labeled neurons

a1

a2

Cy3-192IgG

a3

Musc 10
Amb 1

-15 mV

-1.65

-10 mM

2 min

unlabeled neurons

b1

b2

IR-DIC

Cy3-192IgG

b3

Spikes/10s

120

ACh 1 mM

0

NS ACh

1 mM

2 min

c

d

e

AChE-sensitive neurons (%)

n=29

n=84

n=4

n=5

n=19

n=19

Hyperpolarization (mV)

Musc AChE

Musc AChE

% increase in firing rate

Cy3-192IgG labeled neurons

unlabeled neurons