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Acute but not chronic Donepezil administration increases muscarinic receptor-mediated brain signaling involving arachidonic acid in unanesthetized rats

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Abstract

Donepezil, an acetylcholinesterase (AChE) inhibitor that is used to treat patients with Alzheimer's disease, is thought to act by increasing brain extracellular acetylcholine (ACh), and thus ACh binding to cholinergic receptors. Cholinergic muscarinic receptors may be coupled to cytosolic phospholipase A_2 ($cPLA_2$) activation and arachidonic acid (AA) release from synaptic membrane phospholipid, and this activation can be imaged in rodents as an AA incorporation coefficient k*, using quantitative autoradiography. To examine acute and chronic effects of Donepezil on the AA signal, k* for AA was measured with quantitative autoradiography in 81 brain regions of unanesthetized rats. Twenty min after a single oral dose (3.0 mg/kg) of Donepezil compared with saline, k* was increased significantly in 37 brain regions, whereas k* did not differ from control 7 h afterwards or following chronic (21 days) of Donepezil. Pretreatment with atropine prevented the 20-min increments in k* following Donepezil. Donepezil also increased the brain ACh concentration and reduced brain AChE activity, but did not change $cPLA_2$ activity, regardless of administration regimen. These results show that Donepezil acutely increases the brain AA signal that is mediated by ACh acting at muscarinic receptors, but that this signal is rapidly desensitized despite continued elevated brain ACh concentration. In contrast, the AA signal in response to arecoline was not altered following Donepezil.

Keywords

Donepezil; muscarinic receptors; acetylcholine; arachidonic acid; phospholipase A_2; acetylcholinesterase; anticholinesterase; Alzheimer disease; imaging; brain; desensitization

Introduction

Alzheimer's disease is an age-associated neurodegenerative disorder that affects an estimated 5.1 million Americans [1]. The postmortem brain from Alzheimer's disease patients shows several indices of reduced cholinergic function, including deficits in the enzyme responsible for the synthesis of acetylcholine (ACh), choline acetyltransferase, reduced ACh release, and loss of cholinergic perikarya in the nucleus basalis of Meynert [2,3]. These observations, together with an accepted role of ACh in learning and memory, have suggested that cholinergic dysfunction contributes to cognitive and behavioral deficits in Alzheimer's disease [4]. Thus, drugs that potentiate central cholinergic function have been approved for treating Alzheimer's

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disease, including the reversible acetylcholinesterase (AChE) inhibitor, Donepezil [(±)-2,3-dihydro-5,6-dimethoxy-2-[[1-(phenylmethyl)-4-piperidinyl]methyl]-1H-inden-1-one hydrochloride] [5]. In some randomized clinical trials, patients on Donepezil compared with placebo performed better on memory, language, orientation and attention [6-8]. However, two trials found no benefit of Donepezil [9,10], and progression of brain atrophy did not differ in patients with mild cognitive impairment on or off Donepezil [11,12], suggesting only symptomatic relief.

Donepezil (2.5 mg/kg, p.o.), when given to rats, inhibited whole brain AChE activity and increased the synaptic ACh concentration in the hippocampus [13]. Chronic daily Donepezil (14 to 21 days) increased brain ACh [14,15] and nicotinic receptor levels [16,17], and enhanced memory on behavioral tests [18]. It did not affect baseline synaptic transmission after one week [16], expression of cholinergic muscarinic M_3 receptor genes, or muscarinic receptor binding at baseline or after activation by carbachol [15,19].

Postsynaptic muscarinic $M_{1,3,5}$ receptors can be coupled *via* $G_{q/11}$ proteins to Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$), which when activated selectively releases the second messenger, arachidonic acid (AA, 20:4n-6), from synaptic membrane phospholipid [20-22]. The signaling process has been imaged using quantitative autoradiography in unanesthetized rodents administered the nonspecific muscarinic receptor agonist, arecoline, and could be prevented by pretreatment with muscarinic receptor antagonist, atropine. In those studies, radiolabeled AA was injected intravenously, and regional brain AA incorporation coefficients k* and incorporation rates J_{in} were measured using quantitative autoradiography [23-27]. These parameters represent brain AA metabolic consumption and are unaffected by changes in cerebral blood flow [28-31], and thus are ideal for flow-independent imaging of resting and activated brain AA metabolism. AA and its metabolites can influence many physiological processes, including membrane excitability, gene transcription, apoptosis, sleep, brain blood flow and behavior [32].

In this study, we tested the hypothesis that k* for AA would be elevated in rats given acute or chronic Donepezil, insofar as both regimens increase the brain extracellular ACh concentration (see above). The fatty acid method was used with quantitative autoradiography to image k* for AA in unanesthetized rats at 20 min or 7 h after receiving 3.0 mg/kg p.o. Donepezil, or after receiving 1.5 mg/kg p.o. Donepezil twice daily for 21 days. These doses and times are reported to inhibit AChE activity and to increase the ACh concentration in rat brain [13-15]. In the rat, the brain Donepezil concentration reaches a peak 30 min after a single oral dose, then declines rapidly [13,33]. Effects of 5.0 mg/kg arecoline and of atropine on k* for AA [23,25,27] also were measured, as were the cortical ACh concentration and AChE activity, and whole brain $cPLA_2$ activity.

Materials and Methods

Animals

Experiments were conducted following the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication No. 86-23) and were approved by the Animal Care and Use Committee of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development. Two- or 3-month-old male Fischer CDF (F-344)/CrlBR rats (Charles River Laboratories, Wilmington, MA) were acclimated for 1 week in an animal facility in which temperature, humidity and dark-light cycle were regulated. The rats had *ad libitum* access to water and food (Rodent NIH-31 auto 18-4 diet, Zeigler Bros, Gardners, PA). The diet contained (as percent of total fatty acids) 20.1% saturated, 22.5% monounsaturated, 47.9% linoleic, 5.1% α-linolenic, 0.02% AA, 2.0% eicosapentaenoic, and 2.3% docosahexaenoic acid [34].

Drug administration

For the chronic study, rats received 1.5 mg/kg p.o. Donepezil twice daily (7:30 A.M. and 3:30 P.M.) for 21 days (Donepezil HCl, 99% pure, Ivy Fine Chemicals, Cherry Hill, NJ), as done previously [14]. For the 20-min and 7-h acute Donepezil studies, rats received a single dose of 3.0 mg/kg p.o.. A pilot study showed that a single dose of 1.5 mg/kg, p.o. Donepezil did not significantly decrease total brain cholinesterase (ChE) activity after 7 h (36.10 ± 12.67 versus 40.83 ± 12.36 nmol/min/mg protein, n = 8, p = 0.4623). Donepezil was dissolved in saline (0.9% NaCl). Control groups received the same volume of saline under parallel conditions. Arecoline hydrobromide, methylatropine bromide and atropine were purchased from Sigma-Aldrich (St Louis, MO). The competitive muscarinic receptor antagonist, methylatropine bromide (4 mg/kg, s.c.), which does not enter the brain, was given 17 min before arecoline to block peripheral autonomic effects of arecoline [35]. Atropine (5 mg/kg s.c.), which enters the brain but does not affect k* [26], was given 17 min before administration of Donepezil.

Surgical procedures and tracer infusion

Three hours after receiving the single dose of Donepezil or 7 h after the last of the 21-day dose or after receiving saline, a rat was anesthetized with 2-3% halothane in O_2. Polyethylene catheters were inserted into the right femoral artery and vein, as described [25]. The rat was allowed to recover from anesthesia for 3 h in a sound-dampened temperature-controlled box, with its hindquarters loosely wrapped and taped to a wooden block. Arterial blood pressure and heart rate were measured with a blood pressure recorder (CyQ 103/302; Cybersense, Inc., Nicholasville, KY).

After the rat had recovered from anesthesia, it was given methylatropine, then arecoline or saline. Three min later, [1-^{14}C]AA (170 μCi/kg; 53 mCi/mmol, > 99% pure, Moravek Biochemicals, Brea, CA) in 2 ml of 5 mM HEPES buffer, pH 7.4, containing 50 mg/ml of bovine serum albumin essentially fatty acid free, was infused into the femoral vein for 5 min at a rate of 400 μl/min using an infusion pump (Harvard Apparatus Model 22, Natick, MA) [25]. For the 20-min study, saline, Donepezil (3.0 mg/kg, p.o.) or atropine was administered 20 min before [1-^{14}C]AA infusion. Twenty min after beginning tracer infusion, the rat was killed with Nembutal→ (80 mg/kg, i.v.). Its brain was removed within 30 s, frozen in 2-methylbutane maintained at -40°C with dry ice, and stored at -80°C.

Chemical analysis

Thirteen arterial blood samples were collected before, during and after [1-^{14}C]AA infusion and were centrifuged immediately (30 s at 18,000 g) to determine radioactivity of unesterified AA in the plasma. Total lipids were extracted from 30 μl of plasma with 3 ml chloroform:methanol (2:1, by vol) and 1.5 ml 0.1 M KCl, using the Folch procedure [36]. Radioactivity was determined in 100 μl of the organic phase by liquid scintillation counting. As reported, following [1-^{14}C]AA infusion, greater than 97% of plasma radioactivity is radiolabeled AA at 5 min, and brain phospholipids account for greater than 81% of brain lipid radioactivity over 2 h [37]. Concentrations of unlabeled unesterified fatty acids were determined in 100-150 μl of the arterial plasma. Total lipids were extracted [36] and were separated by thin layer chromatography on silica gel 60 plates using the solvent system heptane:diethylether:glacial acetic acid (60:40:3, by vol). Unesterified fatty acids were scraped from the plate and methylated with 1% H_2SO_4 in anhydrous methanol for 3 h at 70°C. Fatty acid methyl esters were then separated and quantified by gas chromatography using an internal standard heptadecanoic acid (17:0).

Quantitative autoradiography

Frozen brains were cut in 20-μm-thick coronal sections in a cryostat at -20°C. The sections were exposed together with [^{14}C]methylmethacrylate standards to Ektascan C/RA film (Eastman Kodak Company, Health Imaging Group, Rochester, NY) for 5 weeks. Radioactivity (nCi/g of brain) in 81 anatomically identified regions [38] was measured bilaterally six times by quantitative densitometry using the public domain NIH Image program 1.62 (http://rsb.info.nih.gov/nih-image/). Regional AA incorporation coefficients k* (ml/s/g brain) of AA were calculated as [28],

$$k^* = \frac{c^*_{\text{brain}}\,(20 \quad \text{min})}{\int_0^{20} c^*_{\text{plasma}}\,dt} \qquad \text{(Eq. 1)}$$

where c^*_{brain} (nCi/g brain) is brain radioactivity at 20 min after the onset of infusion as determined by densitometry, c^*_{plasma} (nCi/ml plasma) is the arterial plasma concentration of labeled unesterified AA as determined by scintillation counting, and t (min) is time after beginning [1-^{14}C]AA infusion. Integrals of plasma radioactivity (input function in denominator) were determined in each experiment by trapezoidal integration, and divided into c^*_{brain} to calculate k* for each experiment.

The regional rate of incorporation of unesterified AA from plasma into brain phospholipids, J_{in} (fmol/s/g), was calculated as,

$$J_{\text{in}} = k^* c_{\text{plasma}} \qquad \text{(Eq. 2)}$$

where c_{plasma} (nmol/ml) is the plasma concentration of unlabeled unesterified AA.

Brain cPLA$_2$ activity

After being given Donepezil or saline, a rat was anesthetized with Nembutal→ (50 mg/kg, i.p.) and decapitated. The brain was removed within 30 s, frozen in 2-methylbutane maintained at -40°C with dry ice, and stored at -80°C. Half-brains were homogenized using a Tenbroeck tissue grinder on ice (Kontes Glass Co., Vineland, NJ) in 3 vol of cold buffer containing 10 mM HEPES, pH 7.5, 1 mM EDTA, 0.34 M sucrose and protease inhibitor cocktail tablet (Complete, Roche, Mannheim, Germany). The homogenates were centrifuged at 100,000 g for 1 h at 4°C (Beckman L8-M Ultracentrifuge, Fullerton, CA). The supernatants corresponding to the cytosolic fractions were assayed for cPLA$_2$ activity, using a cPLA$_2$ assay kit and secretory PLA$_2$ and Ca^{2+}-independent PLA$_2$ inhibitors (Cayman, Ann Arbor, MI). The protein concentration of the cytosolic fraction was determined by the Bradford method [39].

Brain cholinesterase activity

Cerebral cortical samples weighing 65-90 mg were homogenized in 5 volumes of cold 0.1 M phosphate buffer (pH = 7.5) in a Dounce tissue grinder on ice (Kontes Glass Co., Vineland, NJ). Samples were centrifuged at 1,000 g for 10 min at 4°C, and 10 μl of the supernatant was used for the assay with a Quantichrom™ AChE assay kit (BioAssay Systems, Hayward, CA) and acetylthiocholine chloride as substrate. Since acetylthiocholine chloride is a good substrate for both AChE and butyrylcholinesterase, a selective AChE inhibitor, BW284C51 (Sigma-Aldrich), was added to a final concentration of 0.01 mM.

Acetylcholine concentration

In separate experiments, a rat was anesthetized with Nembutal→ (45 mg/kg, i.p.) and immediately subjected to head-focused microwave irradiation (5.5 kW, 90% power, 3.2 s; Cober Electronics, Stamford, CT) to inactivate ChE prior to ACh analysis [40]. Cortical samples of about 300-450 mg were homogenized in 2.4 volumes of choline assay buffer provided with the choline/ACh quantification kit (BioVision, Mountain View, CA) in a Dounce tissue grinder on ice. Samples were centrifuged at 2,000 g for 15 min at 4°C, and 50 μl of the supernatant was used for the assay.

Statistical analyses

Physiological parameters before and after drug administration were compared in the same animal using paired *t*-tests (GraphPad Software, San Diego, CA, www.graphpad.com). Enzyme activities and ACh and plasma unesterified fatty acid concentrations were compared using an unpaired *t*-test. Regional values of k* following 20-min Donepezil, atropine plus Donepezil, or saline were compared by a one-way analysis of variance (ANOVA) followed by Dunnett's test. Values of k*, J_{in} and the input function after 7-h or 21-day Donepezil, followed by saline or arecoline, were compared by a two-way ANOVA. Where interactions between Donepezil and arecoline were statistically significant, probabilities of main effects are not reported because they cannot be interpreted clearly [41]. Instead, a Bonferroni post hoc test with correction for three comparisons was performed to compare arecoline and saline responses between Donepezil- and saline-treated rats. Other comparisons were not considered relevant. Data are reported as mean ± SD, with statistical significance taken as $p \leq 0.05$.

Results

Physiology and arterial plasma radioactivity

There was no significant effect 20 min after acute (single-dose) Donepezil compared to saline on mean rectal temperature, arterial blood pressure, pH, pCO_2 or pO_2, whereas heart rate was increased significantly by 11% ($p = 0.03$). The latter change could be blocked by atropine. Donepezil produced tremors and repetitive head movements at 20 min, as reported [42], and these behaviors could be prevented by atropine pretreatment. We did not quantify them.

Acute Donepezil at 7 h, or 21 days of daily Donepezil, did not significantly change mean body weight, arterial blood pressure, heart rate or rectal temperature compared with saline. Arecoline significantly increased mean heart rate in all treatment groups, a change that has been ascribed to its effect on brain ACh [43].

Neither Donepezil after 20 min or 7 h, nor 21 days of Donepezil treatment, nor acute arecoline, modified the time-course of arterial plasma [1-^{14}C]AA radioactivity, the input function for calculating k* in Eq. 1 (data not shown).

Plasma concentrations of unesterified fatty acids

The mean arterial plasma concentration of each of 8 measured unesterified fatty acids, including AA, was not significantly affected by 21 days of Donepezil compared with saline (Table 1). Arecoline's effects were not measured, because no effects were found in a comparable prior study [24].

Regional brain AA incorporation coefficients, k*

Figure 1 presents representative coronal autoradiographs from brains of rats at 20 min and 7 h after acute Donepezil or saline, or after 21 days of repeated administration. The effects of pretreatment with atropine at 20 min also are shown. k* for AA was increased compared with

saline at 20 min but not at 7 h after a single dose or after 21 days of Donepezil, and atropine blocked the 20 min effects on k*.

Mean values of k* for AA, determined in each of 81 brain regions, were subjected to a one- or two-way ANOVA for each of the three treatment paradigms. Twenty min after the single Donepezil dose compared with saline, k* was elevated significantly by 22-42% in 37 of 81 brain regions (Table 2). Affected were prefrontal cortex layers I and IV (22-23%), frontal (10) and (8) layers I and IV (26-44%), pyriform (33%), anterior cingulated (33%), motor layers I to VI (28-35%), somatosensory layers I to VI (29-37%), auditory layers IV and VI (38-41%) cortex, preoptic area (30%), caudate putamen (28-33%), medial septal nucleus (26%), medial and lateral habenular nuclei (34-41%), dorsal lateral and medial geniculate nuclei (22-42%), paratenial (26%), anteroventral (28%), anteromedial (30%) and paraventricular (38%) thalamus nuclei, lateral (38%) and anterior (39%) hypothalamus area and interpeduncular nucleus (30%). Atropine pretreatment prevented all significant elevations.

Seven h after the single Donepezil dose, none of the 81 regions had a statistically significant Donepezil × arecoline interaction with regard to k* for AA, whereas Donepezil had a main effect in 9 regions (data not shown). Arecoline had a significant main effect on k* in 49 regions, elevating k* for AA by 12-69%. Affected were prefrontal layer IV, frontal, anterior cingulated, motor, somatosensory, auditory and visual cortex, olfactory tubercle, caudate putamen, habenular nuclei, dorsal lateral geniculate nucleus, thalamus (6 of 8 regions), hypothalamus (2 of 7 regions), mesencephalon (6 of 6 regions) and rhombencephalon (3 of 3 regions).

None of the 81 regions had a statistically significant Donepezil × arecoline interaction or a Donepezil main effect with regard to k* after 21 days of daily Donepezil (Table 3). Arecoline had a significant main effect on k* for AA in 32 regions, increasing k* by 15-46%. Many of these regions were the same as at 7 h after single dose of Donepezil. k* responses to arecoline did not differ significantly between animals treated chronically with Donepezil compared with saline.

Regional incorporation rates of unesterified plasma AA into brain

Because mean plasma unesterified AA concentrations did not differ significantly between animals given Donepezil or saline daily for 21 days (Table 1), baseline differences and percent changes in J_{in} for AA corresponded to the differences and percent changes in respective values of k* (Table 3). In chronic saline-treated rats, baseline J_{in} ranged from 11.5 fmol/s/g in the internal capsule to 23.6 fmol/s/g in the inferior colliculus. In the 21-day Donepezil-treated rats, baseline J_{in} ranged from 11.3 fmol/s/g in white matter of the cerebellum to 24.6 fmol/s/g in the interpeduncular nucleus.

Cortical cholinesterase activity

Mean cortical ChE activity was decreased significantly by 50% (n = 4), 55% (n = 4) and 68% (n = 8) at 20 min and 7 h after the single Donepezil dose, and after 21 days of repeated Donepezil, respectively (Fig 2A). This activity largely represented AChE activity because it was reduced by 90% in homogenates from saline- or Donepezil-treated rats following incubation with the selective AChE inhibitor, BW284C51 (Fig. 2B), consistent with the literature [44].

Cortical acetylcholine concentration

Twenty min and 7 h after a single Donepezil dose and after 21 days of repeated Donepezil, compared to saline, the cortical ACh concentration was increased by 141% (n = 4), 214% (n = 4) and 259% (n = 6), respectively (Fig 2C).

Brain $cPLA_2$ activity

Whole brain $cPLA_2$ activity was not significantly changed at 7 h after a single dose of Donepezil (n = 4) or after 21 days of daily drug (n = 8) (Fig. 2D). Since a calcium chelator is used to determine $cPLA_2$ activity *in vitro* (see Methods), we did not measure $cPLA_2$ activity following arecoline because we could not reproduce increments in intracellular Ca^{2+} likely caused by the drug [20].

Discussion

At 20 min following a single (acute) dose of 3.0 mg/kg p.o. Donepezil compared with saline, k* for AA was increased significantly in 37 of 81 brain regions in unanesthetized rats; these increases could be prevented by pretreatment with atropine. There were no relevant significant effects on k* at 7 h after the single dose or after 21 days of twice daily 1.5 mg/kg p.o. Donepezil. Arecoline-induced elevations in k* or J_{in} in saline-treated rats were unaffected at 7 h after the single dose or after 21 days of daily Donepezil. Each of three Donepezil regimens significantly inhibited cortical AChE activity and elevated the cortical ACh concentration, without changing global brain $cPLA_2$ activity.

The increments in k* at 20 min after the single dose of Donepezil likely arose from muscarinic receptor activation by an elevated brain ACh concentration, as they could be blocked by atropine and occurred in anterior brain regions also stimulated by arecoline (arecoline effects were blocked by atropine) and having high densities of muscarinic receptors [23,26,27]. The lack of significant changes in k* at 7 h after the single dose and after chronic Donepezil, despite an elevated brain ACh concentration at these times, suggests desensitization of the $cPLA_2$-coupled muscarinic receptors and the AA response to increased synaptic ACh [20-22,45,46]. This desensitization likely was unrelated to altered muscarinic receptor number [15] and was distinct from the lack of desensitization of the signal to arecoline.

One possible explanation for desensitization of the Donepezil but not the arecoline effect on k* is that the tonically elevated synaptic concentration of ACh, a full agonist in neuronal cells [47], caused internalization of surface muscarinic receptors into a lipophilic environment [48]. Arecoline, a lipophilic muscarinic agonist like [^{3}H]quinuclidinyl benzilate (QNB) [49], would have access to both non-internalized and internalized muscarinic receptors, each of which remained coupled to $cPLA_2$. Indeed, internalization of muscarinic receptors without a change in receptor affinity was reported with acute and chronic AChE inhibitors in rodents by using as a ligand [^{3}H]N-methylscopolamine (NMS), which detects only cell surface receptors [48,50,51]. Internalization did not result in desensitization of the phosphoinositide-turnover response in neuronal cell lines in response to muscarinic agonists [46,47,49,52]. Internalization following Donepezil could be tested by measuring [^{3}H]NMS binding.

The elevations in the brain ACh concentration and in AChE inhibition at 20 min and 7 h following 3 mg/kg p.o. Donepezil are consistent with prior observations [13]. Additionally, the data obtained 7 h after the 21-day Donepezil regimen agree with a report that AChE remained inhibited for about 12 h after a single dose of Donepezil [13]. The 60% AChE inhibition after 21 days is comparable to the percent inhibition in cerebrospinal fluid of Alzheimer's disease patients treated with Donepezil [53].

Donepezil 2.0 mg/kg i.p. was reported to increase rat brain levels of dopamine, serotonin and norepinephrine in rats and to potentiate N-methyl-D-aspartic acid (NMDA) systems *in vitro* [54,55]. Although these changes might activate $cPLA_2$-coupled neuroreceptors so as to increase k* for AA [56-58], since the k* responses to Donepezil at 20 min were blocked by atropine, they likely were mediated entirely by cholinergic muscarinic receptors. Nicotinic receptors, which are largely presynaptic, also can be activated by Donepezil [59], but in

separate studies nicotine inhibited neuronal PLA_2 [60] and transiently reduced k* for AA in unanesthetized rats [61].

Chronically administered AChE inhibitors did not desensitize muscarinic receptor function in a number of studies. Fourteen days of Donepezil (10 mg/kg twice daily) did not desensitize muscarinic receptor-coupled G proteins in rat brain or spinal cord [19]. Chronic tacrine, metrifonate or diisopropylfluorophosphate did not alter muscarinic receptor densities, or their phosphoinositide responses at baseline or after muscarinic agonist administration in rodents [62-64]. Prolonged exposure of human neuroblastoma cells to galantamine or rivastigmine also did not affect muscarinic receptor-evoked increases in intracellular Ca^{2+} [65]. On the other hand, a life-long genetic deletion of AChE [66] or galantamine administration to transgenic mice that overexpressed AChE [67] downregulated M_1 receptors and reduced responses to cholinergic drugs.

The absence of a significant change in brain $cPLA_2$ activity at 7 h after a single dose of Donepezil or after 21 days of daily administration is consistent with the lack of a significant Donepezil effect on k* for AA at these times. In contrast, elevated baseline values of k* for AA were accompanied by increased $cPLA_2$ activity in rats treated for 3 weeks with fluoxetine, which increases the synaptic serotonin concentration to activate 5-HT2A/2C receptors coupled to $cPLA_2$ [68,69], and in rats chronically administered NMDA to activate NMDA receptors coupled to $cPLA_2$ [70,71].

Arecoline-induced increments in k* for AA in saline-pretreated rats were reported in frontal cortical regions having high densities of M1,3,5 receptors and high AChE activity [23,25,27, 72]. Arecoline may activate nicotinic as well as muscarinic receptors [73], but since its effects on k* were prevented by pre-treatment with atropine [26], a nicotine receptor contribution to arecoline-induced increments in k* is unlikely.

In some randomized trials, Alzheimer's disease patients on Donepezil compared with placebo performed better on memory, language, orientation and attention [6-8], but in others only a "small" benefit [74] or no effect [9,10] was noted. Furthermore, progression of brain atrophy, a marker of structural disease [75], was not slowed by Donepezil [11,12]. Thus, despite the hypothesis that Donepezil would ameliorate cognitive changes due to cholinergic deficits in Alzheimer's disease (see Introduction), it is not certain that the drug actually slows underlying disease progression. Our data suggest that a lack of effect of chronic Donepezil may be related to desensitization of ACh-induced muscarinic receptor-initiated signaling involving AA. Reduced functional G protein coupling of the M_1 muscarinic receptor subtype also has been suggested [76,77]. On the other hand, Donepezil may be neuroprotective in Alzheimer's disease patients independently of its effect on muscarinic transmission [78,79]. The results of this study put into question the mechanism of action of chronically administered Donepezil and other AChE inhibitors in patients with Alzheimer's disease.

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Abbreviations

AA, arachidonic acid; ACh, acetylcholine; AChE, acetylcholinesterase; ChE, cholinesterase; $cPLA_2$, cytosolic PLA_2; NMDA, N-methyl-D-aspartate.

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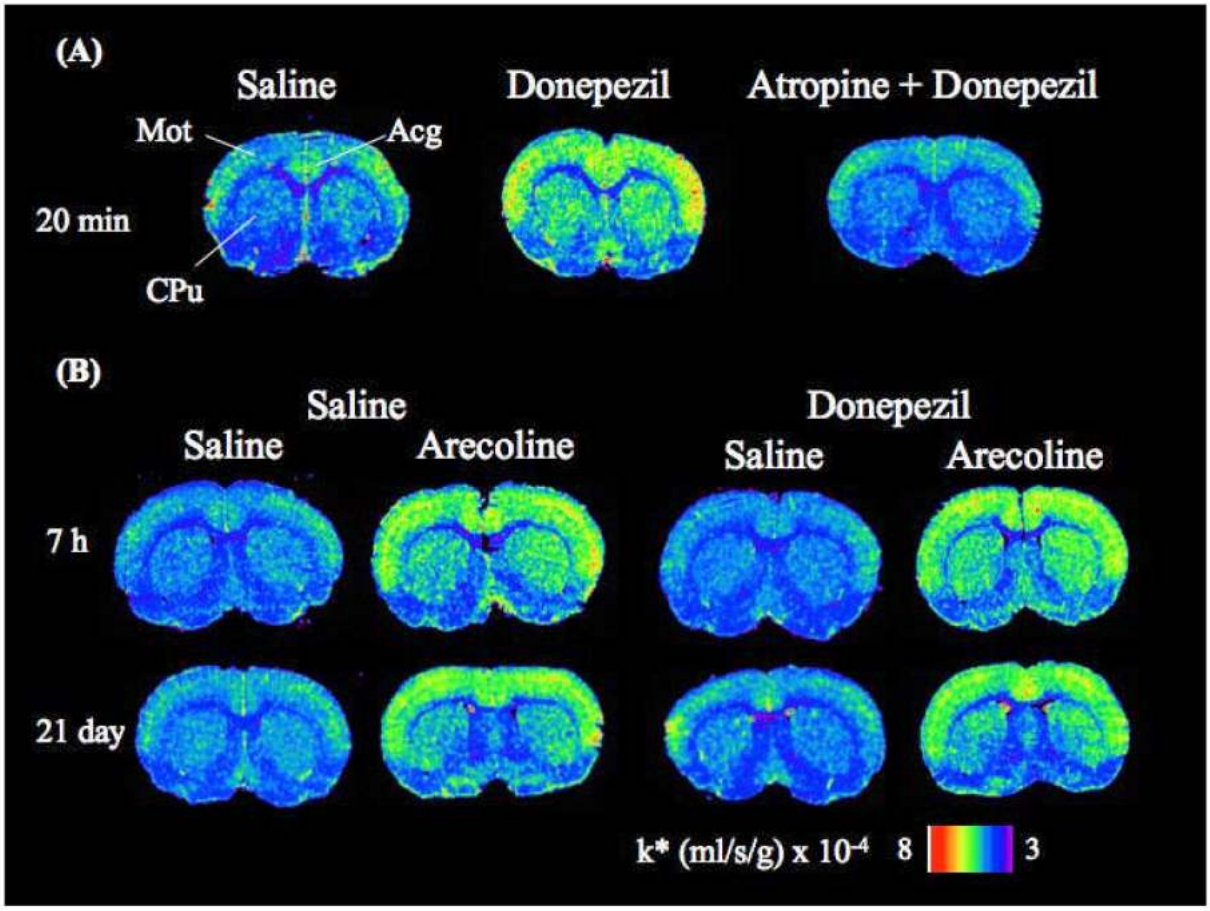

Figure 1.
Coronal autoradiographs of effects of Donepezil, atropine and arecoline on k* for AA in rat brain. (A) Effect of atropine (5.0 mg/kg, s.c.) on 20-min Donepezil's stimulation of k* for AA. (B) Effect of 7-h (3.0 mg/kg, p.o.) and 21-day Donepezil (1.5 mg/kg, twice daily, p.o.) and arecoline (5 mg/kg, i.p.) on k* for AA. Values of k* are color-coded. Abbreviations: Acg, anterior cingulate cortex; CPu, caudate putamen; Mot, motor cortex.

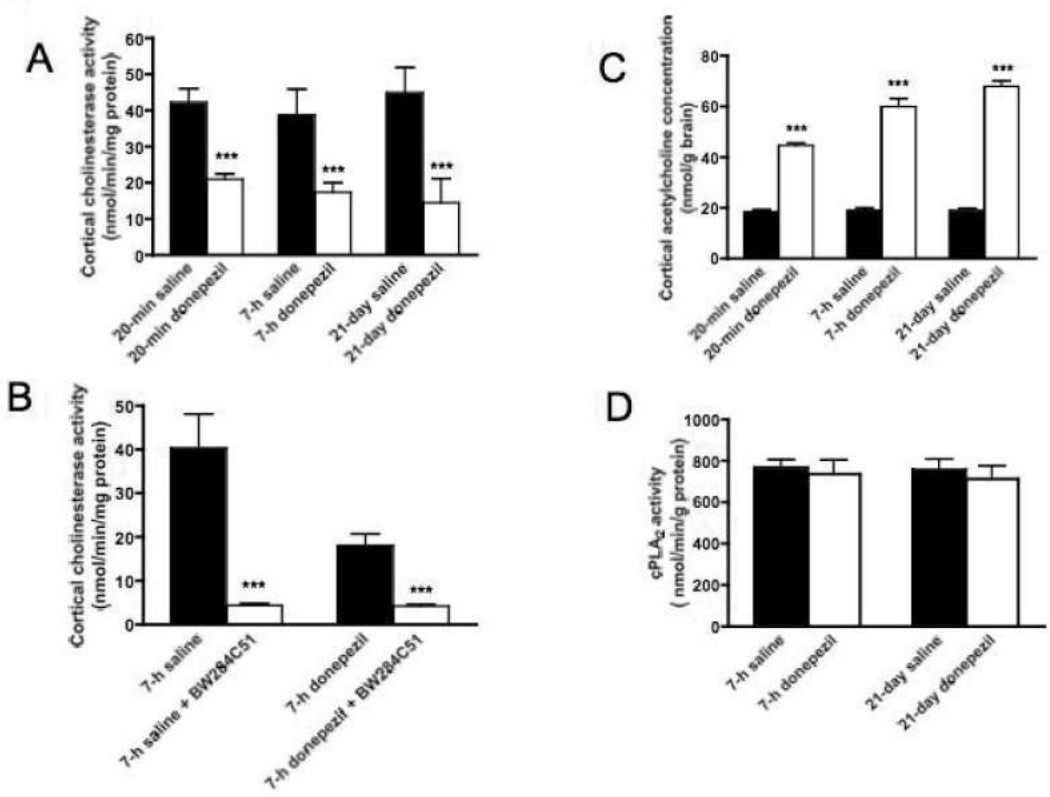

Figure 2.

Effects of Donepezil on cortical cholinesterase activity, cortical acetylcholine concentration and $cPLA_2$ activity in rat brain. Each bar represents mean ± SD. ***$p < 0.0001$ compared to saline (t-test). (A) Effects of 20-min, 7-h and 21-day Donepezil administration on cortical cholinesterase activity. (B) Effects of BW284C51 on cortical cholinesterase activity in vitro. (C) Effects of 20-min, 7-h and 21-day Donepezil administration on cortical acetylcholine concentration. (D) Effects of 7-h and 21-day Donepezil administration on $cPLA_2$ activity.

Table 1

Plasma concentrations of unesterified fatty acids after 21 days of Donepezil

Fatty Acid	Plasma unesterified fatty acid (nmol/ml)	
	Saline	Donepezil
Palmitic (16:0)	311.1 ± 100.7	265.6 ± 76.1
Palmitoleic (16:1n-7)	39.8 ± 16.1	31.3 ± 10.1
Stearic (18:0)	79.9 ± 19.3	70.4 ± 15.3
Oleic (18:1n-9)	229.7 ± 82.0	193.8 ± 51.3
Linoleic (18:2n-6)	310.9 ± 127.7	255.3 ± 82.5
α-Linolenic (18:3n-3)	21.7 ± 13.2	14.9 ± 4.9
Arachidonic (20:4n-6)	34.2 ± 7.5	33.9 ± 6.7
Docosahexaenoic (22:6n-3)	31.3 ± 10.3	27.6 ± 8.4

Values are mean ± SD (n = 12), measured from arterial plasma collected before [1-^{14}C]AA infusion.

Table 2

Mean values of arachidonic acid incorporation coefficients k* in rats, at baseline (in response to saline), in response to 20-min Donepezil and, following atropine + Donepezil

Brain Region	Saline	Donepezil	Atropine + Donepezil
Prefrontal cortex layer I	3.40 ± 0.42	4.20 ± 0.42*	3.38 ± 0.60
Prefrontal cortex layer IV	3.61 ± 0.51	4.41 ± 0.50*	3.68 ± 0.58
Primary olfactory cortex	3.63 ± 0.76	4.43 ± 0.52	3.63 ± 0.94
Frontal cortex (10)			
Layer I	3.44 ± 0.58	4.44 ± 0.39*	3.57 ± 0.66
Layer IV	4.02 ± 0.80	5.06 ± 0.52*	3.99 ± 0.75
Frontal cortex (8)			
Layer I	3.78 ± 0.75	5.01 ± 0.96*	3.90 ± 0.70
Layer IV	4.07 ± 0.78	5.87 ± 1.17*	4.49 ± 1.01
Pyriform cortex	2.60 ± 0.41	3.47 ± 0.43*	2.85 ± 0.66
Anterior cingulate cortex	4.03 ± 0.99	5.36 ± 0.57*	4.29 ± 0.88
Motor cortex			
Layer I	3.17 ± 0.41	4.27 ± 0.78*	3.59 ± 0.64
Layer II — III	3.28 ± 0.46	4.25 ± 0.69*	3.52 ± 0.70
Layer IV	3.83 ± 0.80	4.96 ± 0.50*	4.03 ± 0.97
Layer V	2.98 ± 0.58	3.82 ± 0.32*	3.10 ± 0.60
Layer VI	2.73 ± 0.44	3.65 ± 0.36*	2.76 ± 0.40
Somatosensory cortex			
Layer I	3.46 ± 0.59	4.46 ± 0.65*	3.54 ± 0.66
Layer II—III	3.47 ± 0.70	4.56 ± 0.44*	3.70 ± 0.75
Layer IV	3.95 ± 0.85	5.41 ± 0.76*	4.35 ± 0.96
Layer V	3.41 ± 0.45	4.54 ± 0.74*	3.84 ± 0.71
Layer VI	3.35 ± 0.57	4.44 ± 0.87*	3.65 ± 0.61
Auditory cortex			
Layer I	4.13 ± 1.55	4.66 ± 1.51	3.61 ± 0.48
Layer IV	4.16 ± 1.31	5.87 ± 1.03*	4.46 ± 0.65
Layer VI	3.31 ± 0.66	4.56 ± 1.05*	3.85 ± 0.54
Visual cortex			
Layer I	3.40 ± 0.99	3.65 ± 1.06	3.32 ± 0.61
Layer IV	4.04 ± 1.13	4.36 ± 1.18	3.69 ± 0.52
Layer VI	3.48 ± 1.00	4.09 ± 1.12	3.28 ± 0.57
Preoptic area (LPO/MPO)	2.62 ± 0.32	3.40 ± 0.70*	2.96 ± 0.53
Suprachiasmatic nu	2.60 ± 0.55	3.46 ± 0.74	2.96 ± 0.56
Globus pallidus	2.55 ± 0.42	3.35 ± 0.76	2.89 ± 0.54
Bed nu stria terminalis	3.03 ± 0.36	3.66 ± 0.76	2.98 ± 0.77
Olfactory tubercle	3.71 ± 0.90	4.07 ± 0.76	3.57 ± 0.51
Diagonal band Dorsal	3.11 ± 0.57	3.81 ± 0.79	3.52 ± 0.70
Ventral	3.22 ± 0.49	3.97 ± 0.71	3.26 ± 0.87
Amygdala basolat/med	3.25 ± 0.49	3.96 ± 0.67	3.09 ± 0.49
Hippocampus			
CA1	2.86 ± 0.42	3.24 ± 0.52	2.60 ± 0.61
CA2	3.16 ± 0.58	3.30 ± 0.65	2.87 ± 0.39
CA3	2.96 ± 0.43	3.24 ± 0.65	2.75 ± 0.42
Dentate gyrus	3.02 ± 0.33	3.51 ± 0.79	3.04 ± 0.58
SLM	3.78 ± 1.10	4.17 ± 1.13	3.81 ± 1.06
Accumbens nucleus	3.77 ± 0.81	4.36 ± 0.81	3.74 ± 0.69
Caudate putamen			
Dorsal	3.52 ± 0.54	4.50 ± 0.47*	3.56 ± 0.57
Ventral	3.50 ± 0.57	4.65 ± 0.39*	3.63 ± 0.58
Lateral	3.59 ± 0.65	4.65 ± 0.43*	3.57 ± 0.62
Medial	3.46 ± 0.55	4.57 ± 0.37*	3.56 ± 0.63
Septal nucleus lateral	2.91 ± 0.35	3.60 ± 0.78	2.82 ± 0.64
medial	3.56 ± 0.79	4.49 ± 0.37*	3.58 ± 0.70
Habenular nu lateral	4.33 ± 0.76	5.82 ± 0.41*	5.04 ± 1.30
medial	4.64 ± 1.05	6.54 ± 0.66**	5.02 ± 1.41
Dorsal lateral geniculate nu	3.67 ± 1.05	5.20 ± 1.19*	4.10 ± 0.97
Medial Geniculate nu	3.84 ± 0.46	4.70 ± 0.57*	4.30 ± 0.44
Thalamus			
Ventroposterior nu lat	4.12 ± 1.02	3.90 ± 1.13	3.37 ± 0.47
Ventroposterior nu med	3.72 ± 0.87	3.95 ± 1.08	3.29 ± 0.45
Paratenial nu	3.21 ± 0.44	4.05 ± 0.69*	3.37 ± 0.52
Anteroventral nu	4.78 ± 1.15	6.12 ± 0.92*	5.54 ± 0.58
Anteromedial nu	3.56 ± 0.57	4.63 ± 0.47*	3.75 ± 0.73
Reticular nu	3.78 ± 0.68	4.55 ± 0.81	4.25 ± 0.84
Paraventricular nu	3.29 ± 0.35	4.53 ± 0.59**	3.42 ± 0.60

Brain Region	Saline	Donepezil	Atropine + Donepezil
Parafascicular nu	3.86 ± 1.20	3.86 ± 1.32	3.62 ± 1.49
Subthalamic nucleus	3.82 ± 1.05	3.91 ± 1.22	4.35 ± 0.53
Hypothalamus			
Supraoptic nu	3.01 ± 0.60	3.56 ± 0.62	2.75 ± 0.40
Lateral	2.74 ± 0.40	3.78 ± 1.00*	3.02 ± 0.55
Anterior	2.79 ± 0.31	3.88 ± 0.87*	2.86 ± 0.42
Periventricular	2.30 ± 0.37	2.99 ± 0.88	2.26 ± 0.29
Arcuate	2.74 ± 0.32	3.35 ± 0.75	2.90 ± 0.51
Ventromedial	2.85 ± 0.22	3.44 ± 0.57	3.04 ± 0.52
Posterior	3.70 ± 0.82	3.73 ± 1.05	3.57 ± 0.63
Mammillary nucleus	3.37 ± 1.19	3.11 ± 0.81	2.84 ± 0.50
Interpeduncular nu	5.01 ± 1.23	6.52 ± 0.56*	5.62 ± 1.20
Substantia nigra	3.43 ± 1.06	3.37 ± 1.12	3.05 ± 0.39
Pretectal area	3.98 ± 1.31	4.40 ± 1.15	3.60 ± 0.66
Superior colliculus	3.73 ± 0.97	3.93 ± 1.02	3.36 ± 0.56
Deep layers	3.69 ± 0.85	4.47 ± 1.17	3.67 ± 0.35
Inferior colliculus	5.52 ± 1.50	6.18 ± 1.99	6.11 ± 1.45
Flocculus	4.01 ± 1.02	4.08 ± 1.00	4.42 ± 1.03
Cerebellar gray matter	3.70 ± 0.86	3.97 ± 1.12	3.60 ± 0.57
Molecular layer cerebellar gray matter	4.63 ± 1.08	5.46 ± 1.76	5.27 ± 1.15
White matter			
Corpus callosum	2.44 ± 0.43	2.21 ± 0.54	2.80 ± 0.42
Zona incerta	2.84 ± 0.47	3.04 ± 1.19	2.89 ± 0.45
Internal capsule	2.07 ± 0.52	2.63 ± 0.69	2.33 ± 0.42
Cerebellar white matter	2.10 ± 1.07	2.58 ± 0.62	2.46 ± 0.45
Non-blood-brain barrier regions			
Subfornical organ	4.09 ± 0.61	4.47 ± 0.74	4.05 ± 0.78
Median eminence	3.07 ± 0.46	3.26 ± 0.54	3.05 ± 0.52
Choroid plexus	16.89 ± 2.85	15.66 ± 3.18	17.32 ± 3.57

Abbreviations: nu, nucleus; lat, lateral; med, medial; SLM, stratum lacunosum-molecular of the hippocampus $k^* = (ml/s/g) \times 10^{-4}$

Donepezil administration: 3.0 mg/kg p.o. 20 min

Atropine plus Donepezil administration: 5.0 mg/kg s.c., 17 min following by Donepezil 3.0 mg/kg, p.o. 20 min

Each value is a mean ± S.D. (n = 6).

* $p < 0.05$

** $p < 0.01$; compared with the saline group (one-way ANOVA Dunnett tests).

Table 3

Effect of 21-day Donepezil or saline pretreatment on saline- or arecoline-induced k* responses for AA in specific brain regions.

Brain region	Saline		Donepezil		Saline × Donepezil interaction *p*-value	Donepezil effect *p*-value	Arecoline effect *p*-value
	Saline (n = 13)	Arecoline (n = 11)	Saline (n = 10)	Arecoline (n = 10)			
Prefrontal cortex layer I	4.11 ± 1.69	5.06 ± 1.38	4.88 ± 1.63	5.18 ± 2.11	NS	NS	NS
Prefrontal cortex layer IV	4.93 ± 1.60	5.86 ± 1.62	5.48 ± 1.93	6.70 ± 1.86	NS	NS	0.0487
Primary olfactory cortex	4.79 ± 1.77	5.25 ± 1.44	5.46 ± 2.00	5.55 ± 1.90	NS	NS	NS
Frontal cortex (10)							
Layer I	4.58 ± 1.52	5.12 ± 1.34	5.21 ± 1.90	5.41 ± 1.85	NS	NS	NS
Layer IV	5.03 ± 1.66	6.36 ± 1.62	5.73 ± 2.06	7.23 ± 2.06	NS	NS	0.0153
Frontal cortex (8)							
Layer I	4.67 ± 1.52	5.65 ± 1.60	5.23 ± 1.80	5.85 ± 1.92	NS	NS	NS
Layer IV	5.61 ± 1.89	7.25 ± 1.95	6.09 ± 2.29	7.82 ± 2.15	NS	NS	0.0102
Pyriform cortex	3.94 ± 1.34	4.81 ± 1.17	3.81 ± 2.37	5.28 ± 1.83	NS	NS	0.0289
Anterior cingulate cortex	5.37 ± 1.83	7.21 ± 1.61	6.16 ± 2.20	7.99 ± 2.04	NS	NS	0.0030
Motor cortex							
Layer I	4.44 ± 1.56	5.53 ± 1.49	5.12 ± 1.78	5.74 ± 1.92	NS	NS	NS
Layer II - III	4.71 ± 1.50	6.23 ± 1.80	5.47 ± 2.00	6.87 ± 2.08	NS	NS	0.0121
Layer IV	5.49 ± 1.76	8.02 ± 2.27	6.13 ± 2.34	8.81 ± 2.30	NS	NS	0.0003
Layer V	4.92 ± 1.51	6.51 ± 1.71	5.60 ± 1.92	7.27 ± 2.01	NS	NS	0.0043
Layer VI	5.10 ± 1.54	6.60 ± 1.78	5.78 ± 2.06	7.57 ± 2.15	NS	NS	0.0060
Somatosensory cortex							
Layer I	4.67 ± 1.42	5.51 ± 1.54	5.44 ± 1.97	6.05 ± 2.09	NS	NS	NS
Layer II - III	4.94 ± 1.59	6.07 ± 1.64	5.66 ± 2.09	6.67 ± 1.09	NS	NS	0.0356
Layer IV	5.69 ± 1.81	7.39 ± 1.87	6.29 ± 2.27	8.19 ± 2.29	NS	NS	0.0061
Layer V	4.99 ± 1.58	6.12 ± 1.67	5.62 ± 2.02	7.55 ± 1.92	NS	NS	0.0156
Layer VI	5.11 ± 1.60	6.52 ± 1.80	5.81 ± 2.00	7.33 ± 2.05	NS	NS	0.0126
Auditory cortex							
Layer I	4.49 ± 1.45	5.18 ± 1.70	4.74 ± 1.06	5.88 ± 1.25	NS	NS	0.0371
Layer IV	5.13 ± 1.79	6.37 ± 2.01	5.44 ± 1.54	7.08 ± 1.77	NS	NS	0.0115
Layer VI	4.59 ± 1.47	5.95 ± 1.72	4.56 ± 1.15	5.41 ± 1.30	NS	NS	0.0152
Visual cortex							
Layer I	4.45 ± 1.32	5.19 ± 1.63	4.43 ± 0.73	5.78 ± 0.82	NS	NS	0.0100
Layer IV	5.05 ± 1.81	6.63 ± 2.01	5.18 ± 1.12	7.38 ± 1.40	NS	NS	0.0005
Layer VI	4.82 ± 1.63	5.97 ± 1.95	4.90 ± 0.96	6.82 ± 1.55	NS	NS	0.0027
Preoptic area (LPO/MPO)	4.46 ± 1.28	4.38 ± 1.91	5.15 ± 1.74	4.86 ± 1.84	NS	NS	NS
Suprachiasmatic nu	4.54 ± 1.28	4.23 ± 1.79	5.12 ± 1.60	4.89 ± 1.76	NS	NS	NS
Globus pallidus	4.18 ± 1.54	4.74 ± 1.24	4.97 ± 1.60	5.03 ± 1.86	NS	NS	NS
Bed nu stria terminalis	3.95 ± 1.49	4.29 ± 1.01	4.87 ± 1.44	4.74 ± 1.80	NS	NS	NS
Olfactory tubercle	4.79 ± 1.64	5.49 ± 1.33	4.49 ± 2.12	6.85 ± 1.92	NS	NS	0.0065
Diagonal band dorsal	5.04 ± 1.50	5.14 ± 2.32	5.73 ± 1.92	5.88 ± 2.08	NS	NS	NS
ventral	4.97 ± 1.62	5.45 ± 1.54	5.66 ± 1.96	5.87 ± 2.03	NS	NS	NS
Amygdala basolateral/medial	4.53 ± 1.35	4.95 ± 1.64	4.91 ± 1.52	5.54 ± 1.81	NS	NS	NS
Hippocampus							
CA1	4.12 ± 1.04	4.69 ± 1.56	4.05 ± 1.52	5.14 ± 1.69	NS	NS	NS
CA2	4.29 ± 1.19	4.88 ± 1.66	4.32 ± 1.58	5.27 ± 1.76	NS	NS	NS
CA3	4.26 ± 1.22	4.70 ± 1.58	4.18 ± 1.40	5.10 ± 1.69	NS	NS	NS
Dentate gyrus	4.53 ± 1.47	5.27 ± 1.78	4.66 ± 1.48	5.71 ± 1.93	NS	NS	NS
SLM	4.94 ± 1.73	6.03 ± 1.86	5.57 ± 1.75	6.51 ± 1.79	NS	NS	NS
Accumbens nucleus	4.93 ± 1.55	5.94 ± 1.67	5.52 ± 1.94	6.33 ± 2.02	NS	NS	NS
Caudate putamen							
dorsal	4.73 ± 1.61	5.16 ± 1.29	5.38 ± 1.84	5.90 ± 1.82	NS	NS	NS
ventral	4.89 ± 1.68	5.26 ± 1.53	5.41 ± 1.91	5.66 ± 1.97	NS	NS	NS
lateral	4.79 ± 1.64	5.56 ± 1.47	5.44 ± 1.91	6.18 ± 1.89	NS	NS	NS

J Alzheimers Dis. Author manuscript; available in PMC 2009 December 8.

Brain region	Saline		Donepezil		Saline × Donepezil interaction *p*-value	Donepezil effect *p*-value	Arecoline effect *p*-value
	Saline (n = 13)	Arecoline (n = 11)	Saline (n = 10)	Arecoline (n = 10)			
medial	4.86 ± 1.70	5.18 ± 1.37	5.42 ± 1.85	5.82 ± 2.01	NS	NS	NS
Septal nu lateral	4.11 ± 1.37	4.43 ± 1.00	4.80 ± 1.49	4.79 ± 1.79	NS	NS	NS
Septal nu medial	5.06 ± 1.66	5.44 ± 1.51	5.53 ± 1.97	5.88 ± 1.86	NS	NS	NS
Habenular nu lateral	6.05 ± 2.23	7.48 ± 1.95	6.85 ± 1.87	8.38 ± 2.18	NS	NS	0.0295
Habenular nu medial	5.38 ± 1.90	7.32 ± 1.90	6.24 ± 2.03	8.11 ± 2.03	NS	NS	0.0311
Lateral geniculate nu dorsal	5.43 ± 1.94	6.29 ± 2.02	5.78 ± 1.75	6.74 ± 1.85	NS	NS	NS
Medial geniculate nu	5.28 ± 1.97	6.54 ± 2.04	5.66 ± 1.24	6.86 ± 1.96	NS	NS	0.0340
Thalamus							
Ventroposterior lateral nu	5.04 ± 1.85	5.99 ± 1.83	5.67 ± 1.74	6.54 ± 1.70	NS	NS	NS
Ventroposterior medial nu	5.08 ± 1.84	6.17 ± 1.90	5.77 ± 1.65	6.64 ± 1.64	NS	NS	NS
Paratenial nu	4.83 ± 1.44	5.09 ± 1.34	5.43 ± 1.90	5.62 ± 1.86	NS	NS	NS
Anteroventral nu	6.13 ± 2.25	7.92 ± 1.84	7.20 ± 2.67	8.99 ± 2.24	NS	NS	0.0125
Anteromedial nu	5.11 ± 1.69	5.45 ± 1.58	5.66 ± 1.92	6.17 ± 1.85	NS	NS	NS
Reticular nu	5.21 ± 1.67	5.87 ± 1.66	5.85 ± 2.17	6.81 ± 2.01	NS	NS	NS
Paraventricular nu	4.80 ± 1.54	5.08 ± 1.27	5.49 ± 2.03	5.60 ± 1.80	NS	NS	NS
Parafascicular nu	4.92 ± 1.19	6.20 ± 1.02	5.60 ± 1.58	6.46 ± 1.66	NS	NS	0.0136
Subthalamic nu	5.08 ± 1.78	6.20 ± 1.96	5.93 ± 1.95	6.90 ± 1.67	NS	NS	NS
Hypothalamus							
Supraoptic nu	4.44 ± 1.39	4.77 ± 1.09	5.36 ± 1.90	5.59 ± 2.25	NS	NS	NS
Lateral	4.10 ± 1.44	4.50 ± 1.04	4.97 ± 1.56	4.91 ± 1.78	NS	NS	NS
Anterior	4.19 ± 1.29	4.43 ± 0.92	5.03 ± 1.62	4.99 ± 1.78	NS	NS	NS
Periventricular	4.28 ± 1.15	4.32 ± 0.90	5.07 ± 1.61	4.96 ± 1.67	NS	NS	NS
Arcuate	4.40 ± 1.21	4.52 ± 1.44	5.03 ± 1.35	5.23 ± 1.64	NS	NS	NS
Ventromedial	4.45 ± 1.31	4.53 ± 1.42	4.75 ± 1.42	5.18 ± 1.68	NS	NS	NS
Posterior	4.68 ± 1.55	5.13 ± 1.72	4.96 ± 1.49	5.68 ± 1.84	NS	NS	NS
Mammillary nu	4.41 ± 1.23	4.73 ± 1.59	4.66 ± 1.15	5.32 ± 1.36	NS	NS	NS
Interpeduncular nu	6.65 ± 2.01	8.33 ± 2.17	7.27 ± 2.21	8.78 ± 1.34	NS	NS	0.0110
Substantia nigra	4.55 ± 1.42	4.85 ± 1.41	4.42 ± 1.20	5.54 ± 1.27	NS	NS	NS
Pretectal area	5.13 ± 1.76	6.21 ± 1.81	5.45 ± 1.25	6.73 ± 1.55	NS	NS	0.0214
Grey layer superior colliculus	5.10 ± 1.84	6.85 ± 1.83	5.75 ± 1.77	6.17 ± 1.50	NS	NS	0.0476
Superior colliculus	5.30 ± 1.89	6.19 ± 1.85	5.50 ± 1.23	7.03 ± 1.63	NS	NS	0.0234
Inferior colliculus	6.90 ± 2.76	8.72 ± 1.80	7.23 ± 1.48	9.71 ± 1.67	NS	NS	0.0013
Flocculus	5.49 ± 1.83	6.49 ± 2.11	5.74 ± 1.47	7.07 ± 1.68	NS	NS	0.0407
Cerebellar gray matter	4.98 ± 1.87	5.65 ± 1.75	4.98 ± 1.00	6.22 ± 1.61	NS	NS	NS
Molecular layer cerebellar gray	5.97 ± 2.42	7.63 ± 2.18	6.42 ± 1.64	8.53 ± 2.22	NS	NS	0.0064
White matter							
Corpus callosum	3.56 ± 1.33	3.96 ± 0.74	4.34 ± 1.19	3.96 ± 0.74	NS	NS	NS
Zona incerta	4.61 ± 1.53	5.14 ± 1.76	4.82 ± 1.38	5.52 ± 1.43	NS	NS	NS
Internal capsule	3.37 ± 1.25	3.86 ± 0.70	4.27 ± 1.16	3.86 ± 0.70	NS	NS	NS
Cerebellar white matter	3.81 ± 0.78	3.81 ± 1.16	3.32 ± 1.19	4.41 ± 0.96	NS	NS	NS
Non-blood-brain barrier regions							
Subfornical organ	4.34 ± 1.37	4.45 ± 1.04	4.94 ± 1.49	4.45 ± 1.04	NS	NS	NS
Median eminence	4.49 ± 1.28	4.64 ± 1.53	5.12 ± 1.35	5.22 ± 1.35	NS	NS	NS

Abbreviations: nu, nucleus; SLM, stratum lacunosum-molecular of the hippocampus

Each k* value is a mean ± S.D.

NS: not significant.

k* = (ml/s/g) × 10^{-4}.

Rats were pre-treated with saline or Donepezil (1.5 mg/kg, twice daily, p.o.) for 21 days and injected with arecoline (5.0 mg/kg, i.p.) or saline. Each bar represents the mean ± SD . Statistics were calculated by 2-way ANOVA.