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Effect of donepezil hydrochloride (E2020) on basal concentration of extracellular acetylcholine in the hippocampus of rats

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Abstract

The effects of oral administration of the centrally acting acetylcholinesterase (AChE) inhibitors, donepezil hydrochloride (donepezil: E2020: (±)-2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one monohydrochloride), tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) and ENA-713 (rivastigmine: (*S*)-*N*-ethyl-3-[(1-dimethyl-amino)ethyl]-*N*-methyl-phenylcarbamate hydrogentartrate), which have been developed for the treatment of Alzheimer's disease, on the extracellular acetylcholine concentration in the hippocampus of rats were evaluated by using a microdialysis technique without adding cholinesterase inhibitor to the perfusion solution. We also compared the inhibition of brain AChE and the brain concentrations of these drugs. Donepezil at 2.5 mg/kg and tacrine at 5 mg/kg showed significant effects for more than 6 h. At these doses, the maximum increases were observed at about 1.5 h after administration of donepezil, and at about 2 h with tacrine, and were 499% and 422% of the pre-level, respectively. ENA-713 produced significant effects at doses of 0.625, 1.25 and 2.5 mg/kg, which lasted for about 1, 2 and 4 h, respectively. The maximum increases produced by these doses at about 0.5 h after administration were 190, 346 and 458% of the pre-level, respectively. The time courses of brain AChE inhibition with donepezil at 2.5 mg/kg, tacrine at 10 mg/kg and ENA-713 at 2.5 mg/kg were mirror images of the extracellular acetylcholine-increasing action at the same doses. The time courses of the brain concentrations of drugs after oral administration of donepezil at 2.5 mg/kg and tacrine at 10 mg/kg were consistent with those of brain AChE inhibition at the same doses, and there was a linear relation between these parameters. Brain concentration of ENA-713 at 2.5 mg/kg was below the limit of quantification at all time points measured. These results suggest that oral administration of donepezil, tacrine and ENA-713 increases acetylcholine concentration in the synaptic cleft of the hippocampus mostly through AChE inhibition, and that donepezil has a more potent activity than tacrine and a longer-lasting effect than ENA-713 on the central cholinergic system.

Keywords: Donepezil hydrochloride; E2020; Acetylcholinesterase inhibitor; Acetylcholine; Microdialysis; Tacrine; ENA-713

1. Introduction

Alzheimer's disease is a progressive neurodegenerative disease characterized by deficits in memory and cognitive function. One of the most pronounced changes in the brain of Alzheimer's disease patients occurs in the cholinergic systems. Cholinesterase inhibitors are the only class of drugs currently approved for the treatment of Alzheimer's disease.

We have developed a novel, piperidine-based, acetylcholinesterase (AChE) inhibitor, donepezil hydrochloride (donepezil: E2020: (±)-2-[(1-benzylpiperidin-4-yl)-methyl]-5,6-dimethoxy-indan-1-one monohydrochloride), for the treatment of Alzheimer's disease (Sugimoto et al., 1995). Donepezil was approved by the U.S. Food and Drug Administration (FDA) in 1996, and is now being prescribed world-wide. A large-scale multicenter, double-blind clinical study has demonstrated that donepezil is a well-tolerated drug that improves cognitive performance and global function in patients with mild to moderate Alzheimer's disease (Rogers and Friedhoff, 1998; Rogers et al., 1996, 1998a,b). Pharmacological studies in vitro have revealed that donepezil is a reversible and non-competitive cholinesterase inhibitor, and is a far more selective inhibitor of AChE, relative to butyrylcholinesterase, than is tacrine or physostigmine. It produces marked and long-lasting inhibition of brain AChE and increases the brain content of acetylcholine in vivo. Moreover, donepezil significantly ameliorates performance deficits in several

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learning and memory tasks including eight-arm radial maze impairments after scopolamine, and passive avoidance deficits in nucleus basalis magnocellularis-lesioned rats (Ogura et al., 1988; Yamanishi et al., 1988; Rogers et al., 1991). Other cholinesterase inhibitors available for the treatment of Alzheimer's disease are tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) and ENA-713 (rivastigmine: (*S*)-*N*-ethyl-3-[(1-dimethyl-amino)ethyl]-*N*-methyl-phenylcarbamate hydrogentartrate). Tacrine is the first drug approved by the U.S. FDA for the treatment of Alzheimer's disease, although it has adverse effects related to its actions on the peripheral nervous system and to hepatic toxicity (Beermann, 1993). ENA-713 is a pseudo-irreversible, carbamate-based cholinesterase inhibitor (Weinstock et al., 1994; Anand and Gharabawi, 1996) and is currently available in some European countries.

Since the extracellular acetylcholine concentration measured by the intracerebral microdialysis technique reflects the acetylcholine concentration in the synaptic cleft, it is a useful parameter with which to compare the potential efficacy of various cholinesterase inhibitors in the central cholinergic system. The effects of donepezil, tacrine and ENA-713 on extracellular acetylcholine concentration have been studied by microdialysis. However, in those studies, the drug effects were evaluated after intraperitoneal or subcutaneous injection (Kawashima et al., 1994; Giacobini et al., 1996), or in the presence of a second cholinesterase inhibitor, in the perfusion solution in order to increase extracellular acetylcholine to a detectable level (Ohara et al., 1997). In the present study, we compared the effects of orally administered donepezil, tacrine and ENA-713 on the basal concentration of extracellular acetylcholine in the hippocampus of rats under the same experimental conditions. Moreover, in order to validate the microdialysis data, we measured the inhibition of brain AChE and the brain concentrations of these drugs.

2. Materials and methods

2.1. Subjects

Male Wistar rats (Charles River Japan, Kanagawa, Japan; 7 weeks of age, 210–290 g) were housed at a room temperature of 23 ± 1°C and relative humidity of 55 ± 10%, under a 12-h light/dark cycle (start at 0700 h) for at least 1 week before experiments. The animals were given free access to food and water. All experiments were approved by the Animal Care and Use Committee of Eisai.

2.2. Drugs

Donepezil hydrochloride (E2020: (±)-2-[(1-benzyl-piperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one monohydrochloride) was supplied by Eisai Chemicals, (Ibaraki, Japan), and tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) by Sigma (St. Louis, MO, USA). ENA-713 (rivastigmine: (*S*)-*N*-ethyl-3-[(1-dimethyl-amino)ethyl]-*N*-methyl-phenylcarbamate hydrogentartrate) was synthesized by Eisai (Ibaraki, Japan). All other chemicals were commercial products of reagent grade. Donepezil, tacrine, and ENA-713 were dissolved in distilled water and administered by gavage.

2.3. Implantation of microdialysis probe

Rats were anesthetized with pentobarbital Na (50 mg/kg, i.p.) and placed in a stereotaxic frame (Narishige, Tokyo, Japan). The skull was exposed and a hole was drilled for implantation of a microdialysis probe. In order to increase the recovery of acetylcholine, a probe with a long membrane (5.0 mm membrane length, 0.5 mm diameter; I-4-05 Eicom, Kyoto, Japan) was used. The probe was implanted into the right lateral hippocampus at 4.7 mm lateral and 5.3 mm posterior to the bregma and to a depth of 8.5 mm from the brain surface according to the atlas of Paxinos and Watson (1986). The probe was fixed with dental cement. After surgery, the rats were housed in their home cage. Placement of the probe within the hippocampus was confirmed by visual inspection of the probe track at the end of the experiment.

2.4. Microdialysis

One day after surgery, the microdialysis probe was perfused with Ringer's solution (147 mM NaCl, 4.7 mM KCl, 0.6 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5.0 mM HEPES) at a flow rate of 1.5 μl/min. The perfusate was discarded during the first 1 h of perfusion and then collected at 20-min intervals into microtubes containing 5 μl of 0.1 M phosphate buffer (pH 3.5). Donepezil, tacrine or ENA-713 was orally administered after three fractions had been collected. The perfusate was collected until 6 h after administration.

2.5. Acetylcholine assay

Acetylcholine concentration in dialysis samples was measured by high-performance liquid chromatography (HPLC) with electrochemical detection. As an internal standard, 500 fmol of isopropylhomocholine was added to the sampling tubes, and the mixture was subjected to HPLC. The HPLC system (DSA-300, Eicom) consisted of a degasser, an HPLC pump system, an autosampler, a column temperature controller, and an electrochemical detector with a platinum electrode (5 mm ϕ). A guard column and an enzyme reactor (3.0 mm $\phi \times 4.0$ mm; AC-Enzympak, Eicom) were placed before and after the analytical column (2.0 mm $\phi \times 160$ mm; Eicompak AC-GEL, Eicom), respectively. Following its separation from the analytical column, acetylcholine was converted to hydrogen peroxide inside the enzyme reactor. The analytical and enzyme columns and the electrode were kept at 30°C in a column temperature controller. The mobile phase was

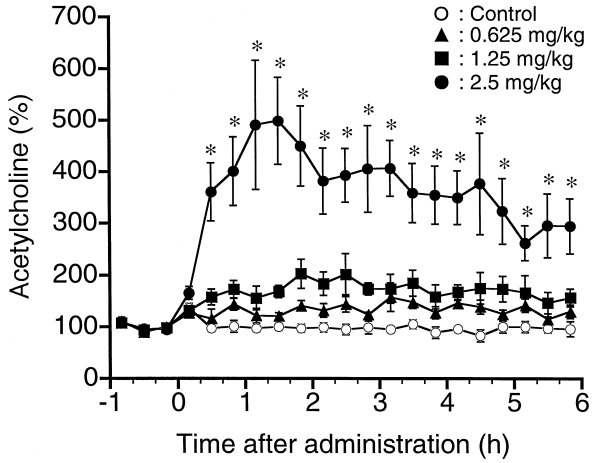

Fig. 1. Effect of oral administration of donepezil on the basal concentration of extracellular acetylcholine in the hippocampus of rats. Data are expressed as percentages of the pre-levels (average of three samples prior to administration = 100%). Values are means ± S.E.M. $n = 6$. Pre-levels were: control: 102.4 ± 18.77; 0.625 mg/kg: 78.3 ± 6.98; 1.25 mg/kg: 92.4 ± 15.20; 2.5 mg/kg: 72.4 ± 6.89 fmol/tube. $^{*}P < 0.05$ vs. control (Dunnett's multiple comparison test).

0.1 M phosphate buffer (pH 8.3) containing 200 mg/l sodium 1-decanesulfonate, 65 mg/l tetramethylammonium chloride and 50 mg/l EDTA · 2Na. The flow rate of the mobile phase was 0.15 ml/min. Peaks were recorded on a Powerchrom integrator (Eicom). Acetylcholine in the dialysate sample was quantified by the internal standard method. The data are expressed as percentages of the pre-level (average of three samples prior to administration).

2.6. Measurement of AChE activity

Rats were decapitated at 0.5, 1, 2, 4, 8 and 12 h after receiving donepezil (2.5 mg/kg), tacrine (10 mg/kg) or ENA-713 (2.5 mg/kg) by oral administration. Control animals received no treatment. The whole brain, excluding cerebellum and olfactory bulb, was removed and the two hemispheres were split for assays of AChE inhibition and brain concentrations of the drugs.

AChE activity was measured using the radiometric method of Thomsen et al. (1989) as modified by Sherman (1991). [^{3}H]acetylcholine iodide (New England Nuclear, Boston, MA, USA) was used as a substrate. In order to minimize the dilution effect which is seen in ex vivo assays of the inhibition by reversible cholinesterase inhibitors, the brain hemisphere was homogenized in four volumes of buffer, and finally 10 mg of brain tissue was diluted in 100 μl of assay solution. Data are presented as percentages of the intact level.

2.7. Measurement of brain concentrations of cholinesterase inhibitors

Brain concentrations of donepezil, tacrine and ENA-713 were measured by LC-MS/MS. ESI-MS/MS was carried out on a Finnigan MAT TSQ7000 (Finnigan MAT Instrument, Tokyo, Japan) mass spectrometer equipped with a liquid chromatography system. The spectrometer was set to admit the protonated molecules $[M + H]^{+}$ at m/z 380 (donepezil), m/z 199 (tacrine), m/z 251 (ENA-713) and m/z 394 (internal standard: (*R*,*S*)-1-benzyl-4-[2-[(5,6-dimethoxy-1-indanon)-2-yl]-ethyl]piperidine hydrochloride), with monitoring of the product ions at m/z 91 (donepezil), m/z 144 (tacrine), m/z 206 (ENA-713), and m/z 91 (internal standard). The limits of quantification for the three cholinesterase inhibitors were set at 0.01 nmol/g.

2.8. Statistical analysis

The data are expressed as means ± S.E.M. Microdialysis data were analyzed using a repeated measures analysis of variance with dose and fraction as factors. If a significant dose × fraction interaction existed, post-hoc analysis using Dunnett's multiple comparison test was applied between doses for individual fractions. Cholinesterase inhibition data were analyzed with Dunnett's multiple comparison test. A P value of less than 0.05 was considered significant. Statistical analysis was conducted using the software package SAS ver. 6.12® (SAS Institute Japan, Tokyo, Japan), available on the statistical analysis support system.

3. Results

3.1. Effects of cholinesterase inhibitors on hippocampal extracellular acetylcholine concentration

We examined the effects of donepezil, tacrine and ENA-713 on the extracellular acetylcholine concentration

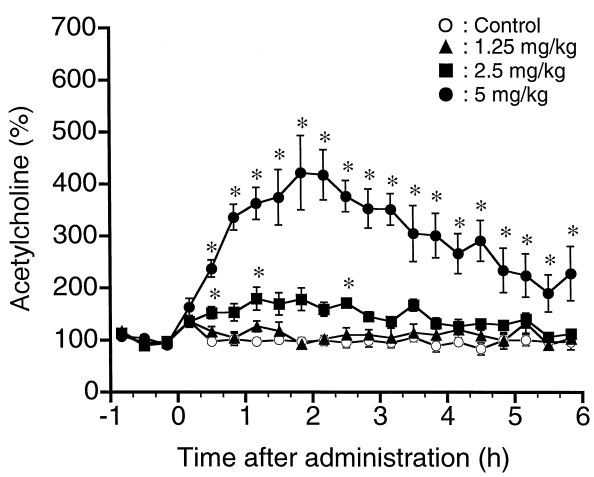

Fig. 2. Effect of oral administration of tacrine on the basal concentration of extracellular acetylcholine in the hippocampus of rats. Data are expressed as percentages of the pre-levels (average of three samples prior to administration = 100%). Values are means ± S.E.M. $n = 6$. Pre-levels were: control: 102.4 ± 18.77; 1.25 mg/kg: 91.8 ± 12.65; 2.5 mg/kg: 83.2 ± 8.08; 5 mg/kg: 70.0 ± 13.95 fmol/tube. $^{*}P < 0.05$ vs. control (Dunnett's multiple comparison test).

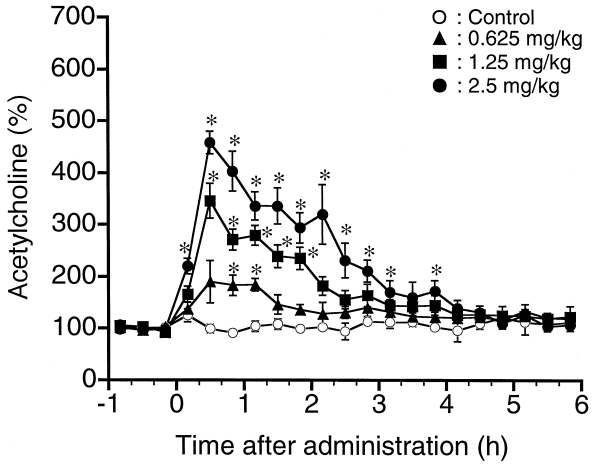

Fig. 3. Effect of oral administration of ENA-713 on the basal concentration of extracellular acetylcholine in the hippocampus of rats. Data are expressed as percentages of the pre-levels (average of three samples prior to administration = 100%). Values are means ± S.E.M. $n = 6$. Pre-levels were: control: 136.2 ± 19.07; 0.625 mg/kg: 98.6 ± 12.35; 1.25 mg/kg: 110.4 ± 6.26; 2.5 mg/kg: 120.6 ± 8.45 fmol/tube. $^{*}P < 0.05$ vs. control (Dunnett's multiple comparison test).

in the hippocampus of rats. These cholinesterase inhibitors produced dose-dependent increases in the extracellular acetylcholine concentration (Fig. 1–3). Donepezil had a significant effect at a dose of 2.5 mg/kg. The maximum increase produced by this dose was seen at about 1.5 h, when acetylcholine reached 499% of the pre-treatment level. The duration of the acetylcholine increase produced by the drug at this dose was more than 6 h (Fig. 1). The increase in the extracellular acetylcholine concentration produced by tacrine at 5 mg/kg was maximum at 2 h, when the level was 422% of the pre-treatment level. The acetylcholine-increasing action at this dose continued for more than 6 h (Fig. 2). ENA-713 at 0.625 mg/kg had a significant but transient effect on the extracellular acetylcholine concentration, while at 1.25 and 2.5 mg/kg, it showed significant and profound effects. The maximum increases were observed at 0.5 h after administration, and at 1.25 and 2.5 mg/kg, the levels of acetylcholine reached 346% and 458% of pre-treatment levels, respectively. The duration of the acetylcholine increase produced by the drug increased in a dose-dependent manner, and was about 4 h at the dose of 2.5 mg/kg (Fig. 3).

3.2. Effects of cholinesterase inhibitors on rat brain AChE activity

Fig. 4 shows the effects of donepezil (2.5 mg/kg), tacrine (10 mg/kg) and ENA-713 (2.5 mg/kg) on rat brain AChE activity ex vivo. The effects of these drugs, at the same doses, on the hippocampal extracellular acetylcholine concentration are shown in the same figure for reference. The data for donepezil and ENA-713 are the same as those in Figs. 1 and 3, respectively. Donepezil produced maximal inhibition at 1 h after administration, when AChE activity was 46% of the intact level. AChE activity gradually recovered thereafter, and reached 81% of the intact level at 12 h after administration. The maximal

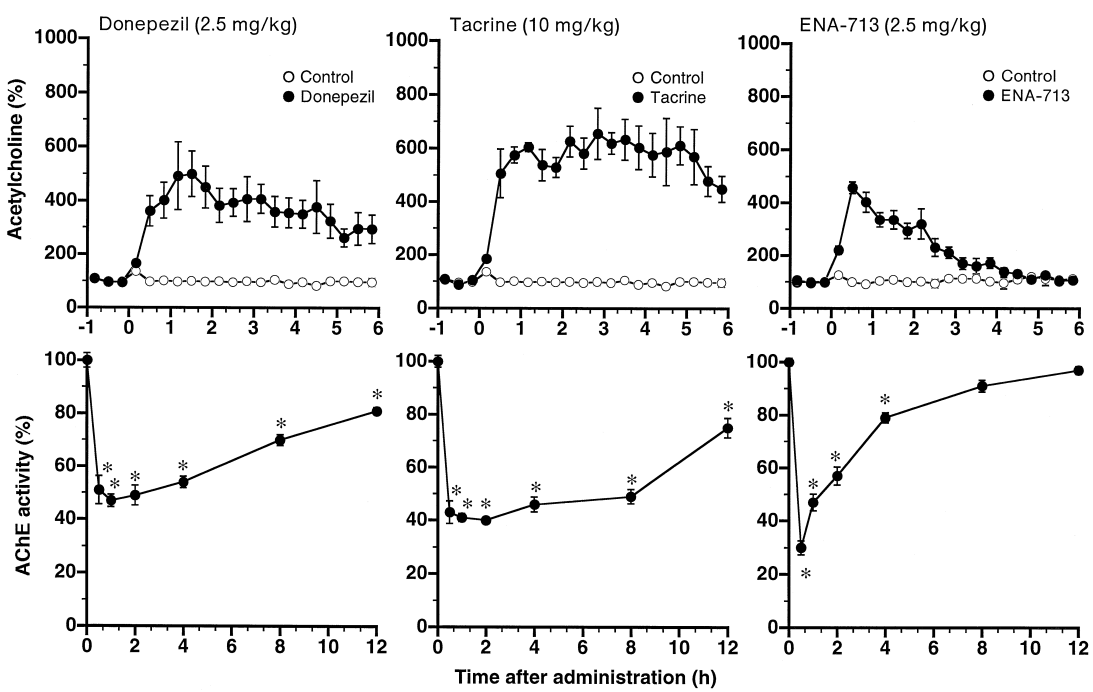

Fig. 4. Comparison of the hippocampal extracellular acetylcholine elevation and brain AChE inhibition induced by donepezil, tacrine and ENA-713. Extracellular acetylcholine data are expressed as percentages of the pre-level (average of three samples prior to administration = 100%). Values are means ± S.E.M. $n = 6$. The data for donepezil and ENA-713 are from Figs. 1 and 3, respectively. AChE activity is expressed as a percentage of the intact level shown at 0 h (100%). Values are means ± S.E.M. $n = 5$. $^{*}P < 0.05$ vs. intact (Dunnett's multiple comparison test).

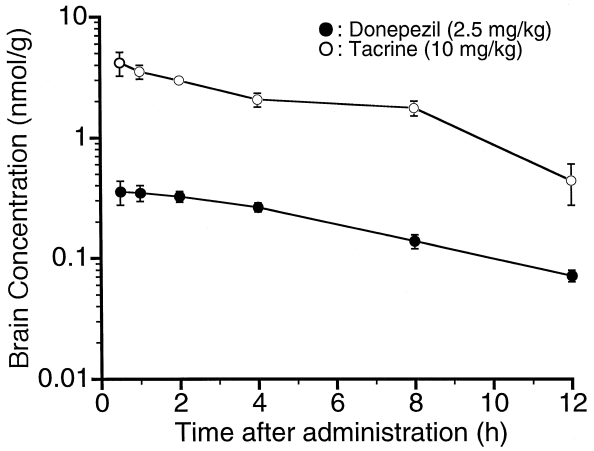

Fig. 5. Concentrations of donepezil and tacrine in the rat brain after oral administration. ENA-713 was not shown because the brain concentration of ENA-713 was below the quantification limit at all time points measured. Values are means ± S.E.M. $n = 5$.

inhibition of AChE by tacrine was observed at 1–2 h after administration, although AChE inhibition remained at a plateau for 0.5–8 h after administration (0.5 h, 43%; 1 h, 41%; 2 h, 41%; 4 h, 45%). The AChE activity was 74% of the intact level at 12 h after administration. The effect of ENA-713 on AChE activity was maximal at 0.5 h after administration, when the AChE activity was 30% of the intact level. The activity rapidly recovered thereafter. AChE activity was 78% of the intact level at 4 h after administration, and had recovered almost completely (97% of intact level) at 12 h. Acetylcholine elevation in the hippocampus was a mirror image of AChE inhibition.

3.3. Concentrations of cholinesterase inhibitors in the brain after oral administration

The concentrations of donepezil, tacrine and ENA-713 in the rat brain were measured after oral administration (Fig. 5). The result for ENA-713 at 2.5 mg/kg was not shown because the brain concentrations of ENA-713 were below the quantification limit at all time points measured. After oral administration of donepezil at 2.5 mg/kg, its concentration was maximum (0.355 nmol/g) at 0.5 h. Although elimination of donepezil from the brain was rapid, 0.072 nmol/g remained at 12 h after administration. The maximal concentration of tacrine after oral administration at 10 mg/kg was 4.185 nmol/g at 0.5 h. Elimination of tacrine was rapid, and the concentration at 12 h after administration was 0.441 nmol/g. There was a linear relation between AChE inhibition and the drug concentration in the brain after oral administration of these compounds (Fig. 6).

4. Discussion

The results in this study clearly demonstrate that donepezil has a more potent activity than tacrine and a longer-lasting effect than ENA-713 on the central cholinergic system, in terms of the basal concentration of extracellular acetylcholine in the hippocampus and the AChE activity in the brain of rats.

Oral administration of donepezil dose-dependently increased the basal concentration of extracellular acetylcholine in the hippocampus of rats. Previously, Kawashima et al. (1994) had examined the effect of intraperitoneal administration of donepezil (0.65 and 2 mg/kg) on the basal concentration of extracellular acetylcholine in the hippocampus of rats. Giacobini et al. (1996) also examined the effect of subcutaneous injection of donepezil (0.5 and 2 mg/kg) on the basal concentration of extracellular acetylcholine in the cerebral cortex of rats. In these studies, donepezil caused a marked and dose-dependent increase in extracellular acetylcholine concentration. Our data show that extracellular acetylcholine is increased by donepezil even when the oral administration route is used.

Donepezil at a dose of 2.5 mg/kg was somewhat more potent than tacrine at a dose of 5 mg/kg. Kawashima et al. (1994) also examined the effect of tacrine (1.65 and 5

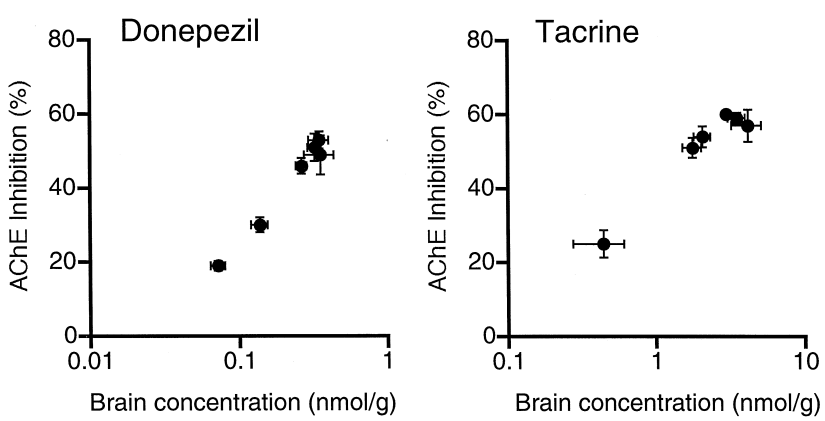

Fig. 6. Relation between AChE inhibition and the brain concentration of donepezil or tacrine after oral administration. AChE inhibition is expressed as percent inhibition, calculated based on the AChE activity of the intact group shown at 0 h as 100%. Values are means ± S.E.M. $n = 5$.

mg/kg, i.p.) under the same experimental conditions, and reported that donepezil increased the extracellular acetylcholine concentration more potently than did tacrine. The result of the present study was in agreement with theirs, although the routes of drug administration were different in the two experiments. On the other hand, the effect of ENA-713 on the extracellular acetylcholine concentration was shorter than that of donepezil or tacrine, but was the most potent. Ohara et al. (1997) reported that oral administration of ENA-713, at a dose of 1 mg/kg, increased the extracellular acetylcholine concentration by 250% at 20–30 min after administration, and the effect continued for only about 60 min. However, in their experiment, the effect of ENA-713 was not compared with that of other cholinesterase inhibitors and the extracellular concentration of acetylcholine measured by them was not a physiological level because of the addition of 10^{-7} M physostigmine in the perfusion solution. Our results showed that ENA-713 is a shorter-acting cholinesterase inhibitor than is donepezil or tacrine.

The acetylcholine-increasing action in the rat hippocampus produced by donepezil, tacrine and ENA-713 was well-correlated to the ex vivo AChE inhibition in the rat brain at the same dose of each drug. Both the acetylcholine-increasing action and the AChE inhibition of donepezil at 2.5 mg/kg were somewhat less potent than those of tacrine at 10 mg/kg. Moreover, donepezil and tacrine showed long-lasting effects on both the extracellular acetylcholine concentration and the brain AChE activity. On the other hand, the effects of ENA-713 on the extracellular acetylcholine concentration and brain AChE were of short duration. These results are consistent with the idea that the effect of these compounds on the extracellular acetylcholine concentration is based on the inhibition of AChE in the brain.

The findings on AChE inhibition by donepezil and tacrine are consistent with the observed brain concentrations of the two drugs. There was a linear relation between AChE inhibition and the drug concentration in the brain after oral administration of these compounds. The maximal concentration of tacrine (4.19 nmol/g) was about 12-fold higher than that of donepezil (0.355 nmol/g). Considering that the IC_{50} value of donepezil (5.7 nM) for in vitro AChE inhibition is about 14-fold lower than that of tacrine (80.6 nM) (Yamanishi et al., 1988; Rogers et al., 1991), these concentrations of donepezil and tacrine are expected to show roughly equipotent in vivo effects. Moreover, 1/5 and 1/9 of the maximal concentrations of donepezil and tacrine remained even at 12 h after administration, respectively. These results suggest that orally administered donepezil and tacrine penetrate into the brain and inhibit AChE activity there, resulting in an increase of extracellular acetylcholine.

In spite of the clear pharmacological effects, the brain concentration of ENA-713 was below the quantification limit at all time points measured. ENA-713 is a carbamate-based cholinesterase inhibitor like physostigmine or neostigmine (Anand and Gharabawi, 1996). This type of cholinesterase inhibitor associates with and carbamylates the active site of AChE, thereby deactivating the enzyme, and is itself hydrolyzed. In contrast to the acetylated AChE, which is formed during hydrolysis of acetylcholine, carbamylated AChE is far more stable (Taylor, 1996). This may be one of the reasons why ENA-713 shows a potent extracellular acetylcholine-increasing action and brain AChE inhibition even though the brain concentration of the drug is very low.

The maximal changes in extracellular acetylcholine concentration and AChE activity produced by ENA-713 were not well-correlated as compared to those with donepezil and tacrine. The maximal AChE inhibition produced by ENA-713 at 0.5 h after administration was more potent than that by donepezil or tacrine, although the maximal increase in extracellular acetylcholine concentration after administration of ENA-713 was the smallest of the three. This may be explained by the rapid change of AChE inhibition by ENA-713 at the early phase after administration. In the microdialysis technique, especially when a very low level of basal acetylcholine is measured, several minutes are required to collect each sample. Thus, it is conceivable that the rapid changes of extracellular acetylcholine at the early phase after administration are leveled in each sample over the 20-min collection period. This is a technical limitation of the microdialysis method.

We noted slight discrepancies between the inhibition of brain AChE activity and the extracellular acetylcholine-increasing action of the cholinesterase inhibitors used. The duration of AChE inhibition may be slightly longer than that of the acetylcholine-increasing action. Similar inconsistencies of time course between extracellular acetylcholine concentration and AChE inhibition have also been reported by Messamore et al. (1993) and Ishii et al. (1994). Other regulatory mechanisms, in addition to AChE, appear to contribute to the control of acetylcholine concentration in the synaptic cleft, and may cause the change of acetylcholine concentration in the synaptic cleft to recover faster than that of AChE activity. Extracellular acetylcholine concentration measured by microdialysis is a more reliable index of the functional activity of the cholinergic system in the brain than is AChE activity for studying the effects of drugs on central cholinergic transmission.

5. Conclusion

The findings of this study demonstrated that centrally acting cholinesterase inhibitors, donepezil, tacrine and ENA-713, potently increase the extracellular acetylcholine concentration in the synaptic cleft of the hippocampus of rats mostly through AChE inhibition, and that donepezil has a more potent activity than tacrine and a longer-lasting

effect than ENA-713 on the central cholinergic system. Donepezil may be one of the more useful cholinesterase inhibitors for the treatment of Alzheimer's disease.

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