DONEPEZIL DOSE-DEPENDENTLY INHIBITS ACETYLCHOLINESTERASE ACTIVITY IN VARIOUS AREAS AND IN THE PRESYNAPTIC CHOLINERGIC AND THE POSTSYNAPTIC CHOLINOCEPTIVE ENZYME-POSITIVE STRUCTURES IN THE HUMAN AND RAT BRAIN

P. KASA,*† H. PAPP,* P. KASA JR‡ and I. TOROK§

*Alzheimer’s Disease Research Centre and †Department of Pharmaceutical Technology, University of Szeged, H-6720 Szeged, Hungary
‡Erzsebet Hospital, H-6800 Hódmezovásárhely, Hungary

Abstract—In the symptomatic treatment of mild to moderately severe dementia associated with Alzheimer’s disease, donepezil (E2020) has been introduced for the inhibition of acetylcholinesterase activity in the human brain. However, there is no morphological evidence as to how this chemical agent affects the acetylcholinesterase-positive structures in the various areas of the human and the rat CNS. This study demonstrates by histochemical means that donepezil exerts a dose-dependent inhibitory effect in vitro on acetylcholinesterase activity. The most sensitive areas were the cortex and the hippocampal formation. Within the different layers of the cortex, the cholinoceptive acetylcholinesterase-positive postsynaptic pyramidal cell bodies were more sensitive than the presynaptic cholinergic axonal processes. In the cortex, the cell body staining was already abolished by even 2 × 10⁻⁸ M donepezil, whereas the axonal staining could be eliminated only by at least 5 × 10⁻⁸ M donepezil. In the hippocampus, the axonal acetylcholinesterase reaction end-product was eliminated by 5 × 10⁻⁷ M donepezil. The most resistant region was the putamen, where the staining intensity was moderately reduced by 1 × 10⁻⁸ M donepezil. In the rat brain, the postsynaptic cholinoceptive and presynaptic cholinergic structures were inhibited by nearly the same dose of donepezil as in the human brain. These histochemical results provide the first morphological evidence that, under in vitro circumstances, donepezil is not a general acetylcholinesterase inhibitor in the CNS, but rather selectively affects the different brain areas and, within these, the cholinoceptive and cholinergic structures. The acetylcholinesterase staining in the nerve fibers (innervating the intracerebral blood vessels of the human brain and the extracerebral blood vessels of the rat brain) and at the neuromuscular junction in the diaphragm and gastrocnemius muscle of rat, was also inhibited dose-dependently by donepezil.

It is concluded that donepezil may be a valuable tool with which to influence both the pre- and the postsynaptic acetylcholinesterase-positive structures in the human and rat central and peripheral nervous systems. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: acetylcholinesterase, Alzheimer’s disease, CNS, donepezil, histochemistry, peripheral nervous system.

Donepezil (R,S-1-benzyl-4-[(5,6-dimethoxy-1-indan)-2-yl]methylpiperidine hydrochloride) (E2020) is a centrally acting second-generation cholinesterase inhibitor that is used in the treatment of Alzheimer’s disease (AD). Neuropathologically, the affected brain is characterized by the presence of senile plaques and neurofibrillary tangles, and the loss of different transmitter-containing axons. Brain regions that are associated with cognitive, learning and memory functions, and particularly the neocortex and hippocampus, are most affected by the characteristic neuropathology of AD. It has been proposed that the degeneration of cholinergic neurons that is associated with the loss of cholinergic neurotransmission in the neocortex and other brain areas may contribute significantly to the cognitive function deterioration observed in subjects with AD. It has therefore been suggested that pharmacological enhancement of cholinergic neurotransmission may alleviate the symptoms in AD. According to the cholinergic hypothesis of the disease, the inhibition of acetylcholinesterase (AChE) activity may be one of the most realistic approaches to the symptomatic treatment of AD.

Many neuropharmacological, pharmacokinetic and clinical studies have been performed on the effects of donepezil on the cognitive functions. Its mechanism of action is to increase the central cholinergic activity by inhibiting AChE activity in the brain, thereby increasing the level of acetylcholine (ACh) at the synaptic sites. Donepezil has been shown to be clinically effective and well tolerated in the treatment of the symptoms of mild to moderately severe AD. Although the use of AChE inhibitors is the most highly developed approach for the treatment of AD, there has as yet been no demonstration of the precise morphological site(s) of its action. Nor is it known how AChE inhibitors with known central effects influence the enzyme activities in the peripheral nervous system (PNS). Biochemically, donepezil has demonstrated to inhibit AChE activity in the cortex, the hippocampus, the striatum and the hypothalamus of the rat brain. Histochemical procedures, however, display advantages over biochemical methods because they allow detection of the effects of donepezil at a single cell level and at the single synaptic site, such as the peripherally located neuromuscular junction. In the present histochemical study, therefore, our aims were: (i) to investigate the regional selectivity of donepezil on AChE activity in the non-demented human brain; (ii) to compare the AChE inhibitory effects of donepezil in the non-demented human brain with those observed in the rat brain; and (iii) to investigate the AChE inhibitory effect of donepezil at the myoneuronal
juncture. It was also necessary to establish the effects of this agent on the intensity of AChE staining, in the cholinergic axons innervating the extra- and intracerebral blood vessels.

**EXPERIMENTAL PROCEDURES**

**Materials**

Donepezil (R,S-1-benzyl-4-[5,6-dimethoxy-1-indanon]-2-yl)methyl-piperidine hydrochloride) was generously donated by Y. Kay (Eisai Co., Tsukuba Research Laboratory, Ibaraki, Japan). Acetylthiocholine iodide, copper sulfate, potassium ferricyanide, sodium maleate, 3,3' -diaminobenzidine-4HCl, nickel chloride, hydrogen peroxide (H$_2$O$_2$), sodium citrate and Tris–HCl buffers were from Sigma Chemical Co. (St Louis, MO, USA). Parafomaldehyde was purchased from Merck KGaA (Darmstadt, Germany). The DPX histological mountant was from Fluka Chemie Ag (Buchs, Germany).

**Tissue preparation from human brains**

Five brains with no history of previous mental disturbance were obtained at autopsy (mean age 64.8 ± 3.6 years). The interval between death and autopsy was less than 4 h. The left half of the human brain was sliced coronally into approximately 1 cm slabs, which were fixed by immersion for 24–36 h in an ice-cold 0.1 M phosphate-buffered (pH 7.4) 4% paraformaldehyde solution. After fixation, the samples were cryoprotected in 30% sucrose solution until they sank to the bottom of the container. The samples contained cortical areas, the hippocampal complex and the basal part of the brain. Forty-micrometer-thick sections were cut on a freezing microtome and incubated for AChE activity. 29,67

**Tissue preparation from rat brains**

These investigations were performed in accordance with the ethical guidelines for animal investigations of the Hungarian Ministry of Welfare. Experiments were carried out in accordance with the European Communities Council Directive (24 November 1986; 86/609/EEC) and the Albert Szent-Györgyi Medical University Guidelines for Ethics in Animal Experiments. All efforts were made to minimize the number of animals used (a total of 30 animals was used). After ether anesthesia, adult Sprague–Dawley rats (weighing 200–250 g) were perfused transcardially for 30 min with cold 200–250 ml, freshly prepared from Merck, Darmstadt, Germany. The DPX histological mountant was from Fluka Chemie AG (Buchs, Germany).

**Acetylcholinesterase histochemistry**

To determine the possible inhibitory effects of donepezil on AChE histochemical staining, sections from human brain, rat cerebra and different muscles (diaphragm and gastrocnemius muscle) were pre-incubated for 20 min in a 0.1 M phosphate buffer solution (pH 7.4) which contained different doses of donepezil (5 × 10$^{-3}$, 2 × 10$^{-4}$, 5 × 10$^{-5}$, 5 × 10$^{-6}$ and 1 × 10$^{-7}$ M). Thereafter, without washing, the control and treated brain and muscle samples were placed in an incubation medium that contained the same dose of donepezil as was present in the preincubation solution. Control samples were incubated in the absence of donepezil, since it has been demonstrated that donepezil interacts with Trp279 and Phe330, which are present in AChE but not in the butyrylcholinesterase. 24 The muscles were incubated for 7 min, the rat brain tissues for 60 min and the human brain samples for 2 h at 25°C with constant agitation in a dark box. The incubation solution was prepared as described previously. 29,67 The medium consisted of acetylthiocholine iodide (1.8 mM), sodium citrate (0.1 M), copper sulfate (0.03 M), potassium ferricyanide (50 μM) and sodium maleate (0.1 M; pH 6.0). After incubation the sections were placed for 30 s at room temperature in a solution containing 0.05% 3,3’-diaminobenzidine-4HCl, 0.3% nickel chloride and 0.03% hydrogen peroxide in Tris–HCl buffer. All mounted sections were dehydrated in graded alcohols, cleared in xylene and mounted in DPX.

**Image analysis**

The intensity of AChE histochemical staining was evaluated by measuring the optical density (OD). The OD in an area comprising the stained neuronal perikarya, dendrites and axons was determined with a Quantimet 500HC Image Analysis System (Leica Cambridge) linked to a JVC-Color camera mounted on a Leica Laborlux “S” Leitz microscope.

The histochemical reactions were visualized and the digitized image (256 gray levels) was displayed on a color monitor with 1024×768 pixel resolution. The inhibitory effect of donepezil on AChE staining was evaluated. Each site was sampled within a rectangular box created on the computer monitor. In the case of the cortical area, the long axis of the box extended from the cortical surface to the white matter. Five fields of each site were chosen at random. The fields of interest were measured and the OD measurements were averaged. The OD of the white matter in the same section served as the background value. The absolute OD of the area of interest was obtained by subtracting the OD of the background from the OD of the area of interest. All measurements were made under the same optical and light conditions. The OD values obtained are expressed as percentages of the AChE-stained untreated control section (taken as 100%).

**RESULTS**

**Inhibition of acetylcholinesterase activity in the human brain**

The histochemical localization of AChE activity in the different areas of the human CNS has been well described in several reports. 15,17,30,35,40–43,50,54,59 We confirmed the findings of these previous studies, demonstrating that regional and laminar variations of the AChE-positive structures can be observed in non-demented control brain samples. The areas studied contained different amounts of AChE-positive fibers and various numbers of stained neuronal perikarya in different cortical layers (Fig. 1A). In the hippocampal complex very dense AChE staining was present in sectors CA3 and CA2. Moderate AChE activity was observed in areas CA4 and CA1 (Fig. 2A). Intensive axonal and perikaryonal staining was exhibited in the putamen (Fig. 2E) and in the nucleus basalis of Meynert (NBM) (Fig. 3A). There was strong cell body AChE positivity in the globus pallidus externus, the diagonal band of Broca (vertical and horizontal parts) and the supraoptic nucleus (SO) (Fig. 3B).

The AChE-positive structures in the different areas of the human brain were sensitive to donepezil in a dose-dependent manner. Donepezil (2 × 10$^{-6}$ M) inhibited most of the AChE-positive neurons and reduced the axonal staining in the temporal cortex (Fig. 1B) and slightly decreased the staining intensity in hippocampal areas CA1 and CA4 (Fig. 2B). The application of 2 × 10$^{-5}$ M donepezil, however, did not affect the AChE activity in the basal part of the forebrain. At 5 × 10$^{-5}$ M, donepezil severely reduced the axonal staining in sector CA1 and inhibited the enzyme activity in the area CA4 (Fig. 2C). At 5 × 10$^{-4}$ M, donepezil abolished the perikaryonal and axonal staining in the hippocampal formation (Fig. 2D). The AChE activity in the cholinergic neuronal
perikarya and axons in the putamen was resistant to $5 \times 10^{-8}$ M donepezil (Fig. 2F) and slightly decreased by $5 \times 10^{-7}$ M donepezil (Fig. 2G). At $1 \times 10^{-6}$ M, however, the inhibitor moderately reduced the AChE positivity in the axons in the putamen, but intense staining persisted in the cholinergic cell body (Fig. 2H).

The AChE-positive neuronal perikarya present in the diagonal band of Broca (vertical and horizontal parts), the NBM, the globus pallidus (external and internal parts) and the SO nucleus were more resistant than the cholinergic axons in the same area to different concentrations of donepezil. At $5 \times 10^{-9}$ M, donepezil had no effect, $5 \times 10^{-8}$ M slightly inhibited the AChE staining, while $5 \times 10^{-7}$ M donepezil had a very pronounced reducing effect on the axonal AChE staining. Despite the reduction in the staining intensity in the cell processes, the reaction end-product was significantly reduced in the cortical cholinergic axons and abolished from the neuronal perikarya with $2 \times 10^{-8}$ M donepezil (Fig. 4B). All the axonal AChE staining disappeared from the cortex and the cell bodies in hippocampal areas CA1 and CA4 on inhibition with $5 \times 10^{-7}$ M donepezil. However, in other areas of the hippocampus, and especially in CA3, CA2 and the supragranular zone of the dentate gyrus, many AChE-positive axons remained (Fig. 4D). On application of $5 \times 10^{-7}$ M donepezil, all of the AChE positivity disappeared from the axons in the hippocampus (Fig. 4E), but AChE staining surprisingly still remained in some cell bodies of the medial septum, the diagonal band nucleus, the NBM and the axons and cholinergic neurons in the caudate–putamen (Fig. 4G). AChE-positive structures could be detected in the caudate–putamen and in the basolateral nucleus of amygdala even after $1 \times 10^{-6}$ M donepezil treatment (Fig. 4H).

Details of the inhibitory effects of various doses of donepezil on AChE-positive structures in the different areas of the rat brain are provided in Table 3.

**Inhibition of acetylcholinesterase activity in the rat brain**

In general, our study accords well with the earlier histochemical results in revealing the AChE-positive structures in the different areas of the control rat brain.\(^{30}\) In the coronal section of the control, untreated brain at the interaural 6.2 mm level,\(^{28}\) AChE staining is present (among others) in the frontoparietal cortex, the hippocampus, and the caudate–putamen. The various concentrations of donepezil ($5 \times 10^{-9}$, $2 \times 10^{-8}$, $5 \times 10^{-8}$, $5 \times 10^{-7}$ and $1 \times 10^{-6}$ M) dose-dependently inhibited the AChE staining in the various areas of the treated brain. In detail, the histochemical staining revealed that $5 \times 10^{-9}$ M donepezil reduced the reaction end-product in the cell bodies more effectively than that in the axons in the frontoparietal cortex. The AChE reaction end-product was significantly reduced in the cortical cholinergic axons and abolished from the neuronal perikarya with $2 \times 10^{-8}$ M donepezil (Fig. 4B). All the axonal AChE staining disappeared from the cortex and the cell bodies in hippocampal areas CA1 and CA4 on inhibition with $5 \times 10^{-7}$ M donepezil. However, in other areas of the hippocampus, and especially in CA3, CA2 and the supragranular zone of the dentate gyrus, many AChE-positive axons remained (Fig. 4D). On application of $5 \times 10^{-7}$ M donepezil, all of the AChE positivity disappeared from the axons in the hippocampus (Fig. 4E), but AChE staining surprisingly still remained in some cell bodies of the medial septum, the diagonal band nucleus, the NBM and the axons and cholinergic neurons in the caudate–putamen (Fig. 4G). AChE-positive structures could be detected in the caudate–putamen and in the basolateral nucleus of amygdala even after $1 \times 10^{-6}$ M donepezil treatment (Fig. 4H).

Details of the inhibitory effects of various doses of donepezil on the AChE-positive cell bodies and axons in the different areas of the rat brain are provided in Table 3.

**Inhibition of acetylcholinesterase activity in nerve fibers innervating the cerebral blood vessels**

The histochemical demonstration of cholinergic innervation...
Fig. 2. Photographs showing the effects of different doses of donepezil on AChE staining in the hippocampus (A–D) and putamen (E–H) of the human brain. In the control hippocampus intense perikaryonal staining is present in area CA4 (asterisk). Moderate AChE positivity may be seen in sector CA1 (star) (A). The application of $2 \times 10^{-8}$ M donepezil slightly reduced the axonal staining in the sectors CA1 and CA4 (B), while $5 \times 10^{-8}$ M donepezil eliminated most of the axonal AChE reaction in sector CA1 and severely reduced it in area CA4 (C). No AChE positivity could be discerned in any part of the hippocampus after the application of $5 \times 10^{-7}$ M donepezil (D). In the control putamen (pu), intense enzyme staining is present in the cholinergic fibers and cell bodies (E). No or a slight reduction in AChE activity could be detected after the application of $5 \times 10^{-8}$ M (F) or $5 \times 10^{-7}$ M donepezil (G). At $1 \times 10^{-6}$ M, donepezil slightly inhibited the AChE staining in the cholinergic axons, whereas intense AChE positivity remained in the neuronal perikarya (arrowheads) (H). Note the intense AChE staining in claustrum (cl) (E) and the dose-dependent reduction of the AChE reaction end-product (F–H). The identical areas in the different sections are labeled (stars). Scale bar $= 500 \, \mu m$. 
of the cerebral blood vessels is an important issue in the normal human brain, in individuals with AD and in animal models. This study has demonstrated the cholinergic innervation of the different extracerebral (Fig. 5A) and intracerebral (Fig. 5B, D) blood vessels in the rat and human. AChE histochemical staining revealed that donepezil dose-dependently inhibited the AChE staining reaction in the nerve fibers innervating the blood vessels. In rat brain samples treated in vitro with $2 \times 10^{-8}$ M donepezil, the number and intensity of AChE-positive nerve fibers were reduced in the extracerebral blood vessels (Fig. 5C), while on treatment with $5 \times 10^{-7}$ M donepezil they were abolished from the nerve fibers in the extracerebral blood vessels in the rat (Fig. 5E) and in the intracerebral vessels in humans (Fig. 5F).

Inhibition of acetylcholinesterase activity at the neuromuscular junction

In accord with the earlier findings, AChE histochemical staining is present at the control neuromuscular junction of the rat (Fig. 5G). With the same doses as in the brain, it was demonstrated that $2 \times 10^{-8}$ M donepezil only slightly (Fig. 5H), while $5 \times 10^{-7}$ M donepezil effectively (Fig. 5I) reduced the AChE-positive staining at the neuromuscular junction in the diaphragm and gastrocnemius muscle of the rat.

Densitometric demonstration of the inhibitory effect of donepezil on acetylcholinesterase staining in different areas of the human and the rat brain

For a quantitative analysis of the inhibitory effect of donepezil we measured the changes in the OD of the AChE histochemical staining. The results revealed a dose-dependent inhibitory effect of donepezil in the various areas of the cortex, the hippocampus, the caudate–putamen and the NBM. The quantitative OD results, relating to the inhibitory effects of donepezil, on the AChE histochemical staining in the human and the rat brain are summarized in Fig. 6A and 6B, respectively.

DISCUSSION

We confirmed the results of previous biochemical and neuropharmacological studies that donepezil inhibits AChE activity in the rat brain. In addition, the present histochemical study reveals for the first time the AChE-inhibitory effect of donepezil in various human brain areas and its effects on different cholinergic and cholinceptive neuronal structures in the human and rat CNS.

Our AChE histochemical study resulted in four principal findings. First, an important feature of this study is that AChE-positive postsynaptic neuronal perikarya in the cortex and the hippocampus are structures that are more sensitive to
donepezil than the presynaptic cholinergic fibers in the cortex and in the hippocampus. Second, the most resistant cholinergic fibers are present in the putamen, the basolateral nucleus of the amygdala, the olfactory tubercle and the lateral habenular nucleus. Third, the most resistant cholinergic neuronal perikarya are present in the NBM, the medial septum, the diagonal band (vertical and horizontal parts) and the globus pallidus. Fourth, the staining intensity in the cholinergic cell bodies of the putamen, which are marginally involved in AD, was only slightly inhibited with donepezil.

Effects of donepezil on the human CNS

In biochemical experiments, donepezil has been shown to inhibit AChE activity in the human brain and in human erythrocytes. Neurochemical studies have demonstrated that presynaptic cholinergic markers, such as choline acetyltransferase activity, the ACh level and the high-affinity choline uptake are significantly reduced in the cortex, the hippocampus and the NBM in AD. Since the neocortex and the hippocampal formation are known to be major sites of the neuropathological changes in AD, inhibition of the residual AChE activity in these areas might well be beneficial in alleviating the cholinergic hypofunction in AD. Indeed, in clinical applications, donepezil is used for the treatment of memory loss and the behavioral deterioration associated with the ACh deficit in AD.

Biochemical experiments have demonstrated that AChE and its different molecular forms (soluble and membrane-bound) are reduced in the brain in AD and anomalous AChE activity appears in the senile plaques and cerebrospinal fluid. This histochemical study revealed the noteworthy finding that the AChE activity present in the postsynaptic cholinceptive cortical pyramidal cell body is more sensitive than the presynaptic cholinergic axons to donepezil. (It is interesting to note that the cholinergic neurons present in the NBM, which receives cholinergic innervations and may therefore be regarded as cholinceptive, are not as sensitive to donepezil as the purely cholinceptive pyramidal cells within the cortical layers.) In other brain areas, such as the putamen, which is not involved in the AD neuropathology, the cholinergic axons and the cell bodies are very resistant to donepezil treatment. Our results therefore provide the first morphological evidence of a difference in the effects of donepezil on the presynaptic cholinergic and postsynaptic cholinceptive sites in the human brain. This study has demonstrated that the selectivity of donepezil is more pronounced towards the soluble form of AChE (G1 form), which is known to be located mainly in the cell body, than towards the membrane-bound AChE (G4 form), which is to be found predominately on the outer surface of axonal membranes of the cholinergic cells. Similarly to our results, in biochemical experiments where donepezil was added to soluble and crude mitochondrial fractions obtained from non-demented or AD brain, the AChE activity was inhibited in the soluble and the particulate fractions at a low concentration of the inhibitor. Donepezil inhibits the
Effects of donepezil on AChE-positive structures

Table 2. Microscopic evaluation of the inhibitory effects of different doses of donepezil on the histochemical staining in acetylcholinesterase-positive axons and cell bodies in the various areas of the human brain

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Inhibitory effect on</th>
<th>Dose of donepezil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5x10(^{-9})</td>
</tr>
<tr>
<td>Supraoptic nucleus</td>
<td>Axon</td>
<td>+</td>
</tr>
<tr>
<td>Globus pallidus (external part)</td>
<td>Cell body</td>
<td>+</td>
</tr>
<tr>
<td>Globus pallidus (internal part)</td>
<td>Cell body</td>
<td>+</td>
</tr>
<tr>
<td>Diagonal band (vertical part)</td>
<td>Cell body</td>
<td>+</td>
</tr>
<tr>
<td>Diagonal band (horizontal part)</td>
<td>Cell body</td>
<td>+</td>
</tr>
<tr>
<td>Nucl. basalis (Meynert)</td>
<td>Axon</td>
<td>+</td>
</tr>
<tr>
<td>Putamen Cell body</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The effects of various concentrations of donepezil on the AChE histochemical staining in the various human brain areas, axons and neuronal perikarya are indicated: no effect: +; slightly reduced: +/−; moderately reduced: −/+; severely reduced or abolished: −. The shaded area emphasizes the inhibitory effects of the different doses of donepezil.

Table 3. Microscopic evaluation of the inhibitory effects of different doses of donepezil on the histochemical staining in acetylcholinesterase-positive cell bodies and axons in various areas of the rat brain

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Inhibitory effect on</th>
<th>Dose of donepezil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5x10(^{-9})</td>
</tr>
<tr>
<td>Frontoparietal cortex</td>
<td>Axon</td>
<td>+</td>
</tr>
<tr>
<td>Hippocampus Cell body</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CA1 Axon</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Hippocampus Cell body</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CA4 Axon</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Septum Axon</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Cell body</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nucl. basalis (Meynert)</td>
<td>Axon</td>
<td>+</td>
</tr>
<tr>
<td>Putamen Axon</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cell body</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The effects of various concentrations of donepezil on the AChE histochemical staining in the various rat brain areas, axons and neuronal perikarya are indicated: no effect: +; slightly reduced: +/−; moderately reduced: −/+; severely reduced or abolished: −. The shaded area emphasizes the inhibitory effects of the different doses of donepezil.
Fig. 4. Photographs showing the AChE positivity in the frontoparietal cortex (A), hippocampus (C) and putamen (pu) (F) of control rat brain. Note the presence of intensely stained pyramidal cells (arrowheads) and the moderately enzyme-positive nerve fibers (A). At $2 \times 10^{-8}$ M, donepezil abolished the AChE positivity in the cortical pyramidal cells (B). In the control hippocampus intense axonal staining is present in area CA2 and CA3 (arrowheads) and in some neuronal perikarya in sector CA4 (asterisk) (C). On application of $5 \times 10^{-7}$ M donepezil all of the AChE positivity disappeared from the cells in area CA1 and CA4, but many enzyme stained axons remained in sector CA2, CA3 and the supragranular zone of the dentate gyrus (D). At $5 \times 10^{-7}$ M, donepezil abolished the AChE staining from the hippocampus (asterisk) (E) and slightly reduced it in the putamen (G). Enzyme-positive axons and neuronal cell bodies could be detected in the caudate–putamen even after $1 \times 10^{-9}$ M donepezil treatment (H). Scale bar = 100 μm.
Fig. 5. Photographs showing the cholinergic innervations of the extracerebral innervation of blood vessels in a control rat (A), an intracerebral blood vessel in the control human brain (B, D), the neuromuscular junction in the diaphragm of a control rat (G) and the same structures after treatment with different doses of donepezil (C, E, F, H, I). In the control sample of the rat brain, an extracerebral blood vessel is surrounded by a number of AChE-positive nerve fibers (A). At $2 \times 10^{-8}$ M, donepezil reduced (C), while $5 \times 10^{-8}$ M abolished the nerve fiber staining surrounding the blood vessels in the rat (E) and in the human brain (F). In the neuromuscular junction, heavy AChE reaction end-product is present in the diaphragm of the control sample (G). On application of $2 \times 10^{-8}$ M donepezil slightly (H), but $5 \times 10^{-7}$ M donepezil strongly reduced the enzyme staining in the motor end-plate in the diaphragm of the rat. Scale bar = 50 μm.
postsynaptic AChE activity better than the presynaptic AChE activity, which is quite remarkable. By slowing down the hydrolysis of ACh at the synaptic sites, donepezil will enhance the effect of the residual cholinergic presynaptic elements in AD. The result of this is similar to that found for heptyl-physostigmine.33

Effect of donepezil on the rat CNS

In biochemical experiments, donepezil has been shown to inhibit the AChE activity in the brain,48 the cortex,5,51 and the hippocampus5 of the rat. It elevates the extracellular levels of catecholamines39 and ACh in the cerebral cortex19 and the hippocampus.2 It will affect the N-methyl-p-aspartate receptors71 in the rat brain and interact with nicotinic receptors expressed in fibroblast cells.66 In vitro experiments revealed that the IC50 of donepezil on AChE activity present in the homogenate of the rat brain cortex is around 10−8 M,4,71 which is somewhat less than suggested by our OD experiments. The differences can be explained, however, in terms of the methods used in the various studies. In in vivo animal studies, donepezil has been demonstrated to improve the working memory.4,5

The in vivo inhibitory effect of donepezil on AChE activity has also been investigated in the frontal cortex, the hippocampus, the striatum and the hypothalamus of the rat.5 Biochemical studies suggested that the different doses of donepezil inhibited the AChE activity in the frontal cortex more markedly than that can be found in the striatum. Our histochemical results clearly demonstrate that the different neuronal elements (cell bodies and axonal processes) in the various brain regions are differently affected, even within the same region, such as the cortex, the hippocampus, the NBM and the striatum. This therefore provides firm morphological evidence that the cholinceptive pyramidal neuronal perikarya are more sensitive than the cholinergic presynaptic axons in the cortex and the hippocampus of the rat to a given concentration of donepezil. In contrast, the neuronal cell bodies in the NBM, from where the cholinergic axons emanate to various areas of the cortex, are moderately resistant to donepezil inhibition. We found that the most resistant AChE-positive neuronal perikarya are located in the putamen, the NBM, the diagonal band of Broca and some cells in the hypothalamus. An alternative interpretation is that higher concentrations of donepezil are needed to inhibit AChE in basal forebrain neurons and the striatum because of their higher AChE activity. Via the AChE-inhibitory effect of donepezil in neuropharmacological experiments, a microdialysis technique has shown that oral administration of donepezil leads to an increase in the level of ACh in the cerebral cortex of rats.31

Inhibitory effect of donepezil on the cholinergic nerves innervating the blood vessels to the brain

It is well documented that the intra- and extracerebral blood vessels and capillaries are innervated not only with amnergic, but also with cholinergic nerve fibers, in both the human3,14,24,68,74 and the rat brain.21,36,58,60,70 The brain cortical blood flow is largely dependent on the normal function of cholinergic neurons located in the basal forebrain and projecting their axons to the cortex36 (see also Refs 60 and 70). A dysfunction of these cholinergic neurons or their axon terminals innervating the blood vessels may therefore lead to pathology of the cortical microcirculation. Indeed, a disturbed cerebral blood flow has been demonstrated.8,14,51,74 The abnormalities may be dependent on cerebral endothelial cells, profound irregularities in the course of the vessels,69 thickening of the vascular basement membrane23 and a reduction or loss of innervation in the cerebral microvasculature.25 These factors may play significant roles in the dysfunction of the blood–brain barrier, in the cerebral microcirculation and in the pathogenesis of AD.6,9,63

Factors believed to stabilize or improve the blood circulation may result in an amelioration of the symptoms in AD. Alternatively, the modulation of the cholinergic neurotransmission may result in changes in memory performance in AD75 patients. Indeed, if AChE inhibitors (e.g., donepezil) are used to reduce the AChE activity in the cholinergic nerves, it might be suspected that the ACh remaining in the
cholinergic nerves innervating the intra- and extracerebral blood vessels may regulate the blood circulation in AD more effectively than without treatment. In our histochemical experiments, the AChE activity was inhibited by $2 \times 10^{-8}$ M donepezil, and totally abolished by $5 \times 10^{-8}$ M donepezil. Application of this drug and/or other AChE inhibitors to ameliorate the blood circulation in the treatment of AD is therefore an important possibility.

**Effect of donepezil on the rat neuromuscular junction**

Both human and animal in vivo studies have demonstrated a peripheral effect of donepezil. The oral administration of donepezil inhibited the pectoral muscle cholinesterase activity in young rats in a dose-dependent manner. Donepezil at or above 2.5 mg/kg caused fasciculation, a peripheral cholinergic sign of the activation of neuromuscular transmission. In nerve–hemidiaphragm preparations, an intracellular record more effectively than without treatment. In our histochemical experiments, the AChE activity in the neuromuscular junction was reduced by $2 \times 10^{-8}$ M donepezil, suggesting a local effect of donepezil on the postsynaptic membrane of the neuromuscular junction. In human studies, muscle cramps were registered as a side-effect in patients receiving a high dose of donepezil (10 mg daily during a 24 week trial), this could be attributed to the inhibition of AChE activity in the postsynaptic membrane folding at the neuromuscular junction. When the effects of donepezil on the cholinergic structures in the CNS and PNS are compared, however, the drug is observed histochemically to be more selective for the brain cholinopetetic neuronal perikarya than for the neuromuscular junction. In the cortex, the AChE positivity in the pyramidal cells can be abolished by $2 \times 10^{-8}$ M donepezil, while at the neuromuscular junction a similar level of inhibition can be achieved only with $5 \times 10^{-7}$ M. The results of this study reveal the importance of the regional, cellular and tissue specificity of the effects of donepezil on AChE activity.

**Acknowledgements**—We are grateful to Dr Z. Rakonczay for his generous help relating to the experiments. This work was supported by OTKA (T022883 and T032458), ETT (584/1996) and a Széchenyi Professorship to P.K.

**REFERENCES**


