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ORIGINAL ARTICLE

# Can Cholinesterase Inhibitors Provide Additional Effects to Cholinergic Neurotransmission Enhancement?

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## Introduction

The most frequent of the primary degenerative dementias is Alzheimer's disease (AD). The gradual loss of memory and attention in patients suffering from this illness are accompanied by aphasia, apraxia, agnosia, and alterations in visual-spatial perception. This group of symptoms is completed by emotional alterations, psychic instability, and changes in personality that appear in advanced phases of the illness. Different histopathological alterations have been described, like marked atrophy of the cerebral cortex with loss of cortical and subcortical neurons. Other histopathological hallmarks are the formation of senile plaques composed of β-amyloid (Aβ) and neuro fibrillary tangles composed of hyperphosphorylation of tau protein.

The role of cholinergic neurotransmission in memory processing and storage is the basis of the widely accepted cholinergic hypothesis. In AD there is a loss of cholinergic neurons in the basal forebrain and of cholinergic innervation of the cerebral cortex (Perry et al., 1994). In addition, there is a severe loss of nicotinic acetylcholine receptors (nAChRs), which correlates with the severity of the disease at the time of death (Perry et al., 2000). During the past two decades, cholinesterase (ChE) inhibition has become the most widely studied and effective clinical approach to treat the symptoms of AD (Lahiri and Farlow, 1996; Soreq and Seidman, 2001). Four acetylcholinesterase inhibitors (AChEIs), tacrine, donepezil, rivastigmine, and galantamine, have been approved by the FDA and the EMEA (EU) for treating the symptoms of AD. It is postulated that the most important therapeutic effect of ChE inhibitors for AD patients is to stabilize cognitive function, at least during 6 mo of the clinical trial (Giacobini, 2003).

On the other hand, signaling through neuronal nAChRs is being increasingly recognized as playing an important role in different processes such as neurite outgrowth, synaptic transmission, control and synthesis of neurotrophic factors, and neuroprotection (Donnelly-Roberts and Brioni, 1998; Belluardo et al., 2000). Therefore, indirectly, AChEIs, by increasing cholinergic neurotransmission, could provide a neuroprotective signal. Several preclinical studies from our group and others have shown that some of the AChEI drugs used in clinic are neuroprotective (Francis et al., 2005).

If AChEIs could provide neuroprotection besides enhancement of cholinergic neurotransmission, they could not only offer symptomatic improvement but they could also modify the course of the disease in these patients. In this study we present the neuroprotective properties of different AChEIs used in clinic to treat AD patients (donepezil, rivastigmine, and galantamine) in models that can be relevant to the pathogenesis of this disease, such as hyperphosphorylation of tau by okadaic acid, Aβ peptide, overexpression of amyloid precursor protein (APP), or cerebral ischemia.

## Materials and Methods

We have used different models to evaluate the neuroprotective properties of different AChEIs.

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### Human Neuroblastoma Cell Line SH-SY5Y

Cell death was induced by treating SH-SY5Y cells with okadaic acid (30 n*M* for 24 h) or $A\beta_{25-35}$ (10 μ*M* for 24 h). The AChEIs were incubated 24 h before and during the 24-h exposure to toxic stimuli; then cell death was evaluated by measuring lactose dehydrogenase (LDH) release or apoptosis, quantified by analyzing the cell cycle by flow cytometry in propidium iodide–stained cells (Arias et al., 2004).

### Human Neuroblastoma Cell Line SKNMC Overexpressing APP

Because of the overexpression of APP, SKNMC cells show higher spontaneous apoptosis in comparison to control cells (Recuero et al., 2003). Cells were plated, left for 48 h and treated for 48 h with the AChEI; then apoptosis was quantified by analyzing the cell cycle by flow cytometry in propidium iodide–stained cells.

### Rat Hippocampal Slices Subjected to Oxygen and Glucose Deprivation

Slices were prepared as described by Sobrado and coworkers (2004). Briefly, hippocampal slices were subjected to oxygen and glucose deprivation (OGD) for 1 h, and oxygen and glucose were reintroduced for 3 h. Viability was assessed by measuring the amount of LDH released to the exrtracellular media.

### Transient Global Cerebral Ischemia in Gerbils

Adult male Mongolian gerbils weighing 60–80 g were used. Experimental procedures were performed following the rules of the ethical committee for the care and use of animals in research of our medical school. Animals were anesthetized with 1.5% halothane under spontaneous respiration. A midline ventral incision was made in the neck, and the common carotid arteries (CCAs) were carefully isolated and occluded for 5 min using silk sutures. Blood flow during the occlusion and reperfusion was confirmed visually, and the incision was closed. The sham-operation group was treated in the same way without occlusion of the CCAs. The gerbils were sacrificed 3 d after ischemia. Galantamine was administered 24 h prior to occlusion of both carotids and for 3 consecutive days thereafter. Then, they were anesthetized with 4% halothane, transcardially perfused with 0.9% saline solution, and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4). The brains were then removed, postfixed in the same fixative at 4°C, and embedded in paraffin to be sectioned with a rotary microtome at 5 μm representative coronal sections, which included the dorsal hippocampus (1.0–2.2 mm posterior to bregma), and were then stained with hematoxylin and eosin. The number of viable pyramidal neurons in CA1 was counted, as this number was normalized per millimeter. In parallel, in slices stained with TUNEL and active caspase-3, positive neurons were also quantified.

## Results and Discussion

We have found some analogies but also some differences in the extent of neuroprotection, as well as in the mechanism involved, among galantamine, donepezil, rivastigmine, and tacrine. Maximum protection afforded by galantamine, donepezil, and rivastigmine was achieved at concentrations of 0.3, 1, and 3 μ*M*, respectively; these concentrations differ from their $IC_{50}$ to block AChE or butyrylcholinesterase (Greig et al., 2003). It therefore seems that the neuroprotective effects are not directly related to their capacity to block these enzymes. For example, tacrine, a potent blocker of AChE, did not afford any protection in our model. A new hypothesis postulates that inhibition of a peripheral site of AChE might be related to neuroprotection (Dorronsoro et al., 2005); this peripheral site seems to be related to the formation and deposit of Aβ in the brain. Considering this hypothesis, perhaps the interaction with the peripheral site correlates better with the neuroprotective effects of these drugs than with its interaction with the active site of the enzyme; however, this still remains to be proved.

To determine if the neuroprotective effects of these drugs were mediated through nAChRs, we used different nAChR antagonists. Methyllicaconitine, an α7 nicotinic antagonist, but not dihydro-β-erithroidine, an α4β2 nicotinic antagonist, reverted the protective effects of donepezil, galantamine, and nicotine. In the case of rivastigmine, neither nAChR antagonist used significantly reverted its neuroprotective effect. Therefore, these results indicate that the mechanism of donepezil and galantamine, as for nicotine, seems to be mediated by the α7 nAChR subtype. As to the signaling pathway, the PI3K/AKt blocker LY294002 reversed the protective effects of galantamine, donepezil, and nicotine but not that of rivastigmine. However, the Bcl-2 antagonist HA 14-1 reversed the protective effects of the three AChEIs and that of nicotine. Therefore, it seems that the three AChEIs

used have an antiapoptotic mechanism related to the overexpression of Bcl-2, although the signaling pathway differs.

The other model that has been used to induce neuronal cell death is ischemia. Cerebrovascular disease (CVD) and ischemic brain injury secondary to cardiovascular disease are common causes of dementia and cognitive decline in the elderly. CVD also contributes to cognitive loss in AD. For example, in a 4-yr follow-up study, the incidence of dementia increased gradually, shifting from an AD-type picture in the first years to a vascular dementia type later, in years 2–4, in patients that had suffered stroke (Altieri et al., 2004). Cholinergic deficits in vascular dementia due to ischemia of basal forebrain nuclei and cholinergic pathways can be treated with ChE inhibitors used in AD. Against ischemia we have used galantamine (an AChEI drug with allosteric-modulating properties of the nAChR used to treat mild to moderate AD patients) and memantine (a noncompetitive blocker of NMDA receptors used to treat moderate to advanced AD patients). In rat hippocampal slices subjected to OGD, we found that galantamine was more potent that memantine in preventing cell death measured as LDH release (Sobrado et al., 2004). These results were rather surprising; however, they correlated with the finding that galantamine significantly reduced glutamate release after the OGD period in comparison to memantine.

In a transient global cerebral ischemia model in gerbils, we have studied the neuroprotective effects of galantamine at 1 and 10 mg/kg. Viability of pyramidal neurons in CA1 was counted in hematoxylin-eosin- or TUNEL-stained slices. Galantamine (10 mg/kg) significantly increased the number of viable pyramidal neurons in CA1. However, both 1 and 10 mg/kg showed dose-dependent and significant reduction of TUNEL-positive neurons in CA1. A significant reduction in the number of pyramidal neurons positive for active caspase-3 showed an antiapoptotic effect. Galantamine's neuroprotective effect was prevented by treating the animals with mecamylamine (10 mg/kg). Therefore, galantamine preserved its neuroprotective action in vivo, and part of its mechanism was related to nicotinic receptors.

## Conclusions

The results of this study show that all of the AChEIs currently used in the clinic for AD can provide different degrees of neuroprotection in cytotoxic models that are relevant to AD pathology. In the case of galantamine, its neuroprotective action was also maintained in vivo. Whether these neuroprotective effects are clinically relevant or not and whether they can modify the course of the disease is still to be demonstrated with functional long-term clinical trials, together with imaging and biochemical probes in AD patients.

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