Galantamine post-ischemia provides neuroprotection and memory recovery against transient global cerebral ischemia in gerbils

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ABSTRACT

Galantamine, currently used in Alzheimer’s patients, has shown neuroprotection in hippocampal slices subjected to oxygen-glucose deprivation. Here we present an in vivo study to evaluate the potential neuroprotective effects of galantamine in a transient global cerebral ischemia model in gerbils. Three treatment protocols were used. In the pretreatment protocol gerbils were treated before ischemia and for three consecutive days thereafter. Eight groups of animals were included: sham operation plus placebo, mecamylamine 10 mg/kg and galantamine 10 mg/kg, respectively, and ischemia plus placebo, mecamylamine 10 mg/kg, galantamine 1 mg/kg, galantamine 10 mg/kg and mecamylamine plus galantamine 10 mg/kg, respectively. Post-ischemia protocols included three groups of animals: sham operation, ischemia plus placebo, and ischemia plus galantamine 10 mg/kg; substances were administered 3 or 6 h after ischemia and for two consecutive days thereafter. Pyramidal neurons surviving in the CA1 region of the hippocampus were evaluated 72 h after reperfusion, TUNEL histochemistry, caspase-3 and SOD-2 immunohistochemistries and western blottings were performed, and object-placement tests were carried out. Galantamine significantly increased the number of living pyramidal neurons after ischemia-reperfusion injury. Galantamine significantly reduced TUNEL, active caspase-3 and SOD-2 immunoreactivity. The nicotinic antagonist mecamylamine blocked the protective effects of galantamine. The neuroprotective effects of galantamine were preserved even when first administered at 3 h post-ischemia. These results correlated with the performance in the object-placement test. This study shows that galantamine provides in vivo neuroprotection and memory recovery against global cerebral ischemia, even when administration begins 3 h post-ischemia.
INTRODUCTION

Ischemic brain injury secondary to cardiovascular disease is a common cause of dementia and cognitive decline in the elderly. Cerebrovascular disease also contributes to cognitive loss in Alzheimer’s disease. Cholinergic deficits in vascular dementia produced by ischemia of basal forebrain nuclei and cholinergic pathways are currently treated with the same cholinesterase inhibitors used to treat Alzheimer’s disease. Controlled clinical trials with galantamine in patients with vascular disease, as well as in patients with Alzheimer’s plus cerebrovascular disease, have demonstrated improvement in cognition, behavior and activities of daily living (Erkinjuntti et al., 2003).

Galantamine is an acetylcholinesterase inhibitor that also shows allosteric potentiation of the nicotinic receptor (Schrattenholz et al., 1996), of the N-methyl-D-aspartate (NMDA) receptor (Moriguchi et al., 2004), and facilitation of synaptic transmission (Santos et al., 2002; Schilström et al., 2007). Our laboratory demonstrated the antiapoptotic and neuroprotective effects of galantamine, which acts on alpha-7 neuronal nicotinic receptors and also induces antiapoptotic protein Bcl-2 expression in cultured chromaffin cells and human neuroblastoma cells (Arias et al., 2004; 2005). This was corroborated by Kihara and colleagues in cultured cortical neurons (Kihara et al., 2004). Neuroprotection afforded by galantamine in vivo has been reported in anti-nerve growth factor mice (Capsoni et al., 2002) and after global ischemia (Ji et al., 2007). Galantamine has shown to improve cognition in rabbits (Woodruff-Pak et al., 2001; Weible et al., 2004), rats (Briley and Chopin, 1992; Fishkin et al., 1993; Bores et al., 1996; Barnes et al., 2000; Iliev et al., 2000) and mice (Sweeney et al., 1988; Gould and Feiro, 2005; Van Dam and De Deyn, 2006; de Bruin and Pouzet, 2006) with cognitive deficits.
We had previously observed the neuroprotective actions of galantamine in a model of hippocampal slices subjected to oxygen and glucose deprivation (Sobrado et al., 2004). Therefore, our next step has been to test whether galantamine protected hippocampal neurons from ischemia *in vivo* in a transient global cerebral ischemia model. Experiments to test neuroprotective drugs in animals become even more relevant when treatments are administered after the injury and behavioral assessments are performed and correlated. Therefore, we carried out post-ischemic treatment protocols, as well as spatial memory tests, using the neuroprotective dose resulting from previous pre-treatment experiments.

The results of the present study show that galantamine notably protected pyramidal neurons in the hippocampal CA1 region of gerbils subjected to transient cerebral ischemia, and that it led to the recovery of spatial memory. These protective effects were mediated by nicotinic receptors and were present even when galantamine was administered after the ischemic insult.

**MATERIALS AND METHODS**

**Induction of global ischemia**

180 adult male Mongolian gerbils (*Meriones unguiculatus*; weight, 60 to 80 g) were used. The experimental procedures were performed following the rules of our medical school’s ethical committee for the care and use of animals in research, in accordance with the Guide for the Care and Use of Laboratory Animals. Gerbils were housed individually under controlled temperature and lighting conditions with food and water provided *ad libitum*.

To induce ischemia, animals were anesthetized with 1.5% halothane in oxygen under spontaneous respiration. Brain and body temperature were maintained at 37±0.5°C using a temporalis muscle probe and a servo-controlled rectal probe-heating pad (Cibertec RTC-1). A
catheter was inserted into the right femoral artery to continuously monitor arterial blood pressure and glucose concentration. Measurements (Schiller CM-8) were performed before, during and after occlusion. A midline neck incision was made and the common carotid arteries (CCAs) were carefully isolated and transiently occluded for 5 min using silk sutures. Blood flow during the occlusion and reperfusion was confirmed visually under a surgical microscope (Leica Wild-M650) and the incision was closed. The sham operation group was treated in the same way, but without CCAs occlusion. When the brains of the animals subjected to the CCAs occlusion are going through ischemia, a brief period of panting breathing and body movements followed by quiescence is observed (Martínez et al., 2001). Only animals showing these behaviors were considered in the study. It is not until 3 days after reperfusion that a stabilized and reproducible lesion is obtained (Kirino, 1982), and neuroprotection as well as some involved mechanisms can be studied. Therefore, gerbils were sacrificed three days after ischemia by perfusion with saline solution and then fixed with freshly prepared 4% paraformaldehyde in 0.1 mol/L phosphate buffer pH 7.4. The brains were embedded in paraffin prior to sectioning on a rotary microtome (Shandon AS-325-Retraction) in 5 µm coronal sections that included the dorsal hippocampus. Sections were stained with hematoxylin-eosin or used for immunohistochemistry and the pyramidal neurons in CA1 were examined, as they are the most vulnerable to ischemia-reperfusion injury (Kirino, 1982).

**Drug administration protocol**

Gerbils were randomly divided into eight groups (n=8): the sham operation group (sham), the sham 10 mg/kg mecamylamine group (sham mecamylamine), the sham 10 mg/kg galantamine group (sham galantamine), the ischemia placebo treated group (ischemia), the
ischemia 10 mg/kg mecamylamine group (mecamylamine), the ischemia 1 mg/kg galantamine group (galantamine 1), the ischemia 10 mg/kg galantamine group (galantamine 10), and the ischemia 10 mg/kg mecamylamine plus 10mg/kg galantamine group (mecamylamine plus galantamine). Galantamine (Tocris 0686 or kindly provided by Johnson and Johnson) was given subcutaneously twice a day, starting the day before ischemia and ending the day before sacrifice. Mecamylamine (Sigma M9020) was administered by intraperitoneal injection 30 min before the 10 mg/kg galantamine dose. A vehicle (0.9% NaCl saline solution) was substituted for the drugs when necessary. From the results obtained with the described pre-treatment protocol, post-ischemia treatment protocols were designed with the administration of the neuroprotective dose. Therefore, the post-ischemia treatment groups (n=8) were: the sham operation group (sham), the ischemia placebo treated group (ischemia) and the ischemia 10 mg/kg galantamine group (galantamine). Galantamine was given subcutaneously 3 h (one set) or 6 h (the other set) after ischemia and at subsequent 12 h intervals until sacrifice (figure 4A).

Evaluation of hippocampal damage

Hippocampal damage was determined by counting the number of viable neurons in the *stratum pyramidale* within the dorsal CA1 subfield at a magnification of 1000X (Zeiss Axioplan) in the hematoxylin-eosin stained sections. Only neurons with visibly normal nuclei were counted. The number of viable CA1 pyramidal neurons per 1-millimeter length (Image ProPlus® analyzer) was calculated for both hemispheres, and the mean of two sections was calculated for each animal. In some cases asymmetrical injury between the left and right hippocampi was found; these animals were excluded from the study. A researcher who was blinded as to the treatments received by the animals assessed histological sections.
TUNEL staining and analysis

The detection of DNA fragmentation was assessed with a nick end-labeling assay kit (TdT-FragEL DNA fragmentation detection kit [TUNEL], Oncogene Research Products), following the manufacturer’s indications. Coronal sections stained with the TUNEL method were also counterstained with methyl green. TUNEL-positive neurons per 1-mm length at dorsal CA1 stratum pyramidale of both hemispheres were counted at 1000X.

Immunohistochemistry and analysis of active caspase-3 and SOD-2

After deparaffination and rehydration, sections were immersed in 0.01 mol/L citrate pH 6.0 buffer and boiled for 20 min. Sections were then blocked with 1.5% bovine serum albumin and incubated overnight with the primary antibodies (anti-active caspase-3, Alexis Biochemicals, 1:10; anti-Mn superoxide dismutase, Stressgen, 1:100). Sections were immersed in 3% H₂O₂ in methanol and incubated with a secondary-biotinylated universal antibody (1:200) followed by streptavidine-peroxidase complex (Vectastatin® Universal Quick Kit, Vector Laboratories) and 0.06% diaminobenzidine, 0.2% H₂O₂. Negative control sections were incubated without the primary antibodies. Sections were counterstained with hematoxylin. Positive neurons per 1-mm length at dorsal CA1 stratum pyramidale in both hemispheres were counted at 1000X.

Western blot analysis

5 animals were decapitated, and the hippocampi quickly extracted and frozen in liquid nitrogen. Samples were resuspended in Tris/HCl (50 mmol/L, pH 7.8), EDTA (1 mmol/L), DTT (1 mmol/L), PMSF (100 µg/ml), pepstatin A (2 µg/ml), leupeptin (2 µg/ml), trypsin inhibitor (10
µg/ml), benzamidine (0.2 mmol/L) and mechanically dissociated. Total protein concentration was measured by the BCA protein assay and equal quantities of protein were run on a 10% or 15% SDS-polyacrilamide gel electrophoresis and electrotransfered to nitrocellulose membranes (Pall; Life Sciences) that were blocked with 5% nonfat dry milk in TBST for 1 h and incubated with the primary antibodies (rabbit anti-caspase-3, 1:500, Cell Signaling; rabbit anti-Mn superoxide dismutase, Stressgen, 1:50000) overnight at 4°C. Protein loading was monitored by comparison with a mouse anti-beta-actin (1:10000; Sigma). Membranes were incubated with the corresponding anti-rabbit or anti-mouse peroxidase conjugate (1:5000; Santa Cruz, CA). Chemiluminescence was detected with an ECL western blot detection kit (Amersham Pharmacia) according to the manufacturer’s recommendation.

Object placement test

Object placement is a hippocampal-dependent spatial memory task. The current method of object placement was adapted for gerbils from methods used in rats (Ennaceur et al., 1997). Animals were placed for 5 min on a field (40 cm x 40 cm x 40 cm made up of gray PVC) for five days previous to the day of ischemia, and for the two following days, in order to reduce neophobic responses (see protocol on figure 6). On the third day of reperfusion, subjects were placed on the field with two identical objects (cylindrical glass bottles, heavy enough to prevent gerbils from moving; height: 22 cm; diameter: 9 cm) and allowed to explore them for 30 s (T1, sample trial). Exploration of the objects was timed with stopwatches when subjects sniffed at, whisked at, or looked at the objects from no more than 2 cm away. On the fourth day of reperfusion, one object was moved to a new location. The time spent exploring the objects in new (novel) and old (familiar) locations (T2, recognition trial) was observed visually and timed.
with stopwatches for 3 min by an observer who was unaware of the treatments. All locations for the objects were counterbalanced among groups and objects and field were washed with 0.1% acetic acid between trials to equate olfactory cues. The time measured as an exploration behavior was used to calculate a memory discrimination index (DI) as reported by Blalock and colleagues (2003): \[ DI = \frac{(N - F)}{(N + F)} \], where N is the time spent exploring the new located object and F is the time spent exploring the familiar located object. Higher DI is considered to reflect greater memory ability. For the object placement test a treatment protocol from the previously obtained data on neuroprotection was designed (figure 6A). Therefore animals were randomly divided into six groups (n=10): the sham operation group (sham), the sham 10 mg/kg mecamylamine treated group (sham mecamylamine), the sham 10 mg/kg galantamine treated group (sham galantamine), the ischemia placebo treated group (ischemia), the ischemia 10 mg/kg 3 h post-ischemia galantamine group (galantamine 10) and the ischemia 10 mg/kg mecamylamine plus 10 mg/kg 3 h post-ischemia galantamine group (mecamylamine plus galantamine). The last administration of the drugs is performed at least 12 h before sample trials, so differences in the discrimination index are not due to acute drug effects.

**Statistical analysis**

Results were expressed as mean±standard error of the mean. Statistical differences were determined with Kruskal-Wallis and ANOVA followed by Bonferroni post hoc. Statistical significance was set at p<0.05.

**RESULTS**
Physiological parameters (weight, brain and body temperature, arterial blood pressure and glucose concentration) remained within normal levels and basal values did not show statistical differences between groups. Morbidity was evaluated at 30, 60 and 120 min after the recovery of the animals, using the method of McGraw (1977); no statistical differences were found among groups.

**Assessment of CA1 pyramidal viable neurons**

Ischemia provoked a pronounced neuronal loss (239±51.8 neurons/mm in sham to 43±15.1 in ischemia) (figures 1A, B, and F) that was much less in animals treated with galantamine in a dose-dependent manner (61±18.8 neurons/mm at 1 mg/kg and 171±30.2 neurons/mm at 10 mg/kg) (C, D and F). Animals treated with mecamylamine plus galantamine did not show the neuroprotective effect (34±8.5 neurons/mm) (E and F). Several control groups were performed to exclude the possibility of drug effect on the viability of CA1 pyramidal neurons. These groups included sham animals treated with mecamylamine (230±33.8 neurons/mm), sham animals treated with galantamine (252±9.4 neurons/mm) and ischemic animals treated with mecamylamine (30±20.0 neurons/mm).

From the results described above, the following assays were performed to investigate the mechanisms through which galantamine exerted the neuroprotective effect.

**TUNEL-stained pyramidal neurons in CA1**

After ischemia-reperfusion injury numerous TUNEL-positive neurons appeared (252±42.4 neurons/mm) (figure 2A-E). Galantamine decreased DNA fragmentation in a dose-dependent manner (60±22.9 neurons/mm at the dose of 1 mg/kg and to 7±4.9 neurons/mm at the
dose of 10 mg/kg). Animals treated with both mecamylamine and galantamine again showed numerous TUNEL-positive neurons (174±19.6 neurons/mm).

**Caspase-3 expression in the hippocampus**

We analyzed active caspase-3 in CA1 pyramidal neurons and by western blot in the whole hippocampus (figure 2F-K). Sham animals showed low active caspase-3 staining (6±2.2 neurons/mm). Transient global ischemia induced a marked increase (67±23.9 neurons/mm), detectable by western blotting. Galantamine treatment reduced active caspase-3 levels in a dose-dependent manner (16±4.8 neurons/mm at 1 mg/kg and 14±3.7 neurons/mm at 10 mg/kg). In the latter case mecamylamine did not block the galantamine effect (19±5.3 neurons/mm).

**SOD-2 expression in the hippocampus**

The levels of SOD-2 expression in the hippocampus were measured by western blot and immunohistochemistry (figure 3). After ischemia, SOD-2 markedly increased over sham animals (95±28.6 neurons/mm) (panels A, B, E and F) and was reduced by galantamine (5±2.5 neurons/mm at 1 mg/kg and 1±0.6 neurons/mm at 10 mg/kg) (panels C, E and F). Once more, mecamylamine markedly blocked these galantamine effects (56±15.7 neurons/mm) (panels D, E and F).

**Neuroprotective effects of galantamine when given after the ischemic insult**

The protocol used to test the neuroprotective effects of galantamine when given post-ischemia is shown in figure 4A. Ischemia reduced the number of viable pyramidal neurons/mm...
from 202±26.4 to 46±13 (figures 4B and 5A); when administered 3 h post-ischemia at the dose of 10 mg/kg, galantamine significantly increased this number up to 148±23 neurons/mm.

These results were corroborated by the TUNEL assay (figures 4B and 5B) and active caspase-3 staining (figures 4B and 5C). No TUNEL-positive neurons were observed in sham animals whereas 230±53.5 neurons/mm appeared in the ischemia group. Galantamine reduced this number to 25±9.4 neurons/mm. No caspase-3-positive neurons were observed in sham animals whereas 98±31.1 appeared in the ischemia group. Galantamine reduced this number to 6±3.3 neurons/mm.

In the case of SOD-2, the number of positive pyramidal neurons rose from 2±0.8 in sham animals to 80±27.3 neurons/mm after ischemia; once more galantamine significantly reduced this number to 5±1.5 neurons/mm (figures 4B and 5D).

When galantamine was administered 6 h after the ischemia-reperfusion injury the neuroprotective effect was not statistically significant, although a positive trend was observed. Sham animals had 182±4.5 neurons/mm, ischemic animals had 12±6.4 neurons/mm and galantamine treated animals had 63±23.1 neurons/mm.

**Spatial memory: object placement test**

We thought of interest to correlate the histological findings of neuroprotection with a functional test, in this case we used a spatial memory test that depends upon hippocampal functionality i.e. the object placement test (Ennaceur et al., 1997). We performed these experiments following the protocol shown in figure 6A.

No differences among groups and within each of the separate groups in time exploring each object (T1) were found, thus excluding the possibility of different exploratory activity of
the animals. No differences among groups and within each of the separate groups in time exploring the objects (T2) were found, thus excluding the possibility of having a preference for a specific object or place that is independent of the familiarity/novelty of the location of the items.

A clear treatment difference in the performance of animals was observed; sham animals discriminated between the old and new location, whereas animals subjected to ischemia did not (figure 6B). However, animals treated with galantamine recovered their ability to discriminate old and new location, but this ability was impaired in animals treated with mecamylamine plus galantamine. No statistical differences were found among sham animals treated with placebo and sham animals treated either with galantamine or with mecamylamine, so neither of these drugs alters discrimination index by itself.

**DISCUSSION**

The results of the present study show that galantamine has a neuroprotective effect in a model of transient global cerebral ischemia in the gerbil. This effect was related to nicotinic receptors because of the blocking effect of mecamylamine, a nicotinic antagonist.

The pharmacological profile of galantamine is actually more complex than previously thought. Its classic acetylcholinesterase inhibitory effect is well-documented, as well as its allosteric potentiating actions on neuronal nicotinic receptors (Arroyo et al., 2002). In the present study, we found that the nicotinic receptor blocker mecamylamine suppressed the neuroprotective effects of galantamine. Thus, nicotinic receptors are necessary for galantamine to exert neuroprotection. These results are in line with our previous observations in human neuroblastoma cells and bovine chromaffin cells, where galantamine protected from both beta-
amyloid- and reticular stress-induced toxicity; this protection was linked to nicotinic receptors (Arias et al., 2004; 2005).

It is well known that transient global cerebral ischemia causes delayed cell death in hippocampal CA1 pyramidal neurons (Kirino, 1982) although the mechanisms involved are not fully understood. Apoptotic cell death has been confirmed by DNA laddering, nick-end labeling and light microscopy. However, the morphological changes of typical apoptosis differ from those of delayed neuronal death, in which necrotic features are revealed by electron microscopy. Results described in the present paper show caspase-3 activation after ischemia as described previously (Niwa et al., 2001), so apoptosis is contributing to programmed cell death in this model. Galantamine significantly reduced active caspase-3 in CA1 pyramidal neurons and TUNEL-positive pyramidal neurons after transient ischemia, which is consistent with the previously described in vitro antiapoptotic effect (Arias et al., 2004). Galantamine reduced caspase-3 activation, independently of nicotinic receptors. However, reduction of DNA fragmentation did depend, at least in part, on nicotinic receptors (see figure 2). Therefore caspase-3 independent mechanisms also seem to be involved in galantamine’s neuroprotective action. Caspase-3 independent mechanisms have also been described in the same experimental model by other authors (Nitatori et al., 1995; Colbourne et al., 1999).

Although Ji and colleagues (2007) and Iliev and colleagues (2000) found that galantamine was protective given 30 and 25 min, respectively, after ischemia, an interesting clinical and practical finding of this study is that the neuroprotective actions of galantamine were effective even when treatment was initiated 3 h after the ischemic episode; this is the therapeutic window recommended for thrombolytic treatment of acute ischemic stroke patients (NINDS Trial, 1995). Considering that the first administration of the drug in this protocol was not up to 3
h after ischemia, galantamine could be protecting against the cell death caused by oxygen radicals generated during the reperfusion period. In fact, galantamine is a molecule with antioxidant properties (Traykova et al., 2003). It is well documented that reperfusion after brain ischemia triggers the overproduction of reactive oxygen species that can exacerbate cell death (McCord, 1985). Thus, the antioxidant enzyme activity in the tissue affected by ischemia-reperfusion is particularly important, as this activity is the primary endogenous defense against free radical-induced injury. The enzymatic defense against activated oxygen species involves the cooperative action of different antioxidant enzymes and this study has focused on the inducible mitochondrial Mn SOD or SOD-2.

We have confirmed that SOD-2 immunoreactivity was significantly increased in animals subjected to ischemia-reperfusion injury, as previously reported in this animal model (Garnier et al., 2001). In our experiments, animals treated with galantamine prior to or post ischemia-reperfusion injury showed restored SOD-2 immunoreactivity to sham levels and this effect was blocked by mecamylamine; these results suggest that the nicotinic receptor mediated effects of galantamine could play an important role in preventing the detrimental effects of superoxide radicals. In fact, Li and colleagues (2000) reported that alpha-7 receptor activation by GTS-21 ([2,4]-dimethoxybenzyldene anabaseine dihydrochloride) attenuated the intracellular oxidative stress produced by ethanol; similarly Ravikumar and colleagues (2004) reported in rats with spinal cord injury that nicotine can protect against activation of the redox-responsive transcription factors NF-κB, AP-1 and CREB thus reducing mRNA levels of TNFα and MCP-1, which was reversed by mecamylamine.

The fact that the neuroprotection afforded by galantamine translated into spatial memory recovery and therefore hippocampal function recovery strengthens the significance of the
histopathological findings observed in galantamine treated animals, and is in accordance with the previous literature in rodents (Iliev et al., 2000; Van Dam and De Deyn, 2006; de Bruin and Pouzet, 2006) and humans (Wilcock et al., 2000; Erkinjuntti et al., 2003). The elimination half-life of galantamine is 40-50 min both in rats (Mihailova and Yamboliev, 1986) and mice (Bickel et al, 1991) after i.v. injection, so we administered galantamine at least 12 h before sample and recognition trial, in order to exclude acute drug effects on behavioural data.

In conclusion, this study shows that galantamine treatment prevented neuronal death and improved spatial memory that was impaired after transient global cerebral ischemia, and could therefore benefit patients suffering vascular dementia or stroke. This supports the view that clinical trials could be conducted to proof the therapeutic potential of post-ischemic administration of galantamine to patients suffering stroke.

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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

**Figure 1:** Galantamine, when given 24 h before ischemia, increased the number of viable pyramidal neurons in CA1 after transient global ischemia. Photomicrographs of CA1 pyramidal layer stained with hematoxylin-eosin of the most relevant treatment groups: sham (A), ischemia (B), galantamine 1 mg/kg (C), galantamine 10 mg/kg (D), mecamylamine 10 mg/kg plus galantamine 10 mg/kg (E). Scale bar, 40 µm. Treatment was initiated before the onset of ischemia. (F) Histogram representing the data for each treatment group. Statistical differences were determined with Kruskal-Wallis and ANOVA followed by Bonferroni post hoc. ***p<0.001 compared to sham; ##p<0.01 compared to ischemia; $$ p<0.01 compared to galantamine 10 mg/kg.

**Figure 2:** Galantamine 1 and 10 mg/kg, given 24 h before ischemia, significantly reduced the number of TUNEL- and active caspase-3-positive pyramidal neurons in CA1. Top: photomicrographs of CA1 pyramidal layer show TUNEL-stained sections corresponding to sham (A), ischemia (B), galantamine 10 mg/kg (C) and mecamylamine 10 mg/kg plus galantamine 10 mg/kg (D) treated animals. E: histogram representing the TUNEL data for each treatment group. Bottom: photomicrographs of CA1 pyramidal layer show active caspase-3-stained sections corresponding to sham (F), ischemia (G), galantamine 10 mg/kg (H) and mecamylamine 10 mg/kg plus galantamine 10 mg/kg (I) treated animals. J: histogram representing the active caspase-3 data for each treatment group. K: caspase-3 western blot. Statistical differences were determined with Kruskal-Wallis and ANOVA followed by Bonferroni post hoc. ***p<0.001 compared to sham; ###p<0.001 compared to ischemia; $$$p<0.001 compared to galantamine 10 mg/kg. Scale bar, 40 µm.
**Figure 3:** Effects of galantamine, given 24 h before ischemia, on SOD-2 immunoreactivity after transient global ischemia. Photomicrographs of CA1 pyramidal layer show SOD-2-stained sections corresponding to sham (A), ischemia (B), galantamine 10 mg/kg (C) and mecamylamine 10 mg/kg plus galantamine 10 mg/kg (D) treated animals. E: histogram representing the SOD-2 positive neurons for each treatment group. F: SOD-2 western blot. Statistical differences were determined with Kruskal-Wallis and ANOVA followed by Bonferroni post hoc. ***p<0.001 compared to sham; ###p<0.001, compared to ischemia; $$$p<0.001, compared to galantamine 10 mg/kg.

**Figure 4:** Galantamine (10 mg/kg) administered 3 h post-ischemia afforded neuroprotection and reduced DNA fragmentation, active caspase-3 and SOD-2. A: schematic representation showing the protocol used in the 3 h post-ischemia experiments. B: photomicrographs of CA1 pyramidal neurons stained with hematoxylin-eosin, TUNEL, caspase-3 and SOD-2 of sham, ischemia and galantamine treated animals. Scale bars, 40 µm.

**Figure 5:** Galantamine (10 mg/kg) administered 3 h post-ischemia afforded neuroprotection and reduced DNA fragmentation, active caspase-3 and SOD-2. A: histogram showing the number of viable pyramidal neurons in CA1. B: histogram representing the TUNEL data for each treatment group. C: histogram representing the active caspase-3 data for each treatment group. D: histogram representing the number of SOD-2 data for each treatment group. Statistical differences were determined with Kruskal-Wallis and ANOVA.
followed by Bonferroni post hoc. *p<0.05, **p<0.01, ***p<0.001 compared to sham; ##p<0.01, compared to ischemia.

**Figure 6: Spatial memory recovery after galantamine 10 mg/kg administered 3 hours post-ischemia.** A: schematic representation showing the protocol used for the object placement test. B: histogram representing the memory discrimination index. Statistical differences were determined with Kruskal-Wallis and ANOVA followed by Bonferroni post hoc. **p<0.01, compared to sham; #p<0.05, compared to ischemia; $$p<0.01, compared to galantamine.
Figure 3

E

Number of SOD-2-positive neurons/mm

Sham  Ischemia  Gal 1  Gal 10  Meca+Gal

Global ischemia

F

SOD-2
18 kD

beta-Actin

Global ischemia

Sham  Isch  Gal 1  Gal 10  Meca + Gal
3 HOURS POST ISCHEMIA TREATMENT

Figure 5

A
Number of CA1 viable pyramidal neurons (neurons/mm)

Sham | Ischemia | Gal 10
Global ischemia

B
Number of TUNEL-positive (neurons/mm)

Sham | Ischemia | Gal 10
Global ischemia

C
Number of active caspase-3-positive (neurons/mm)

Sham | Ischemia | Gal 10
Global ischemia

D
Number of SOD-2-positive (neurons/mm)

Sham | Ischemia | Gal 10
Global ischemia