Galantamine Postischemia 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 3 consecutive days thereafter. Eight groups of animals were included: sham operation plus placebo, 10 mg/kg mecamylamine and 10 mg/kg galantamine, respectively; and ischemia plus placebo, 10 mg/kg mecamylamine, 1 mg/kg galantamine, and 10 mg/kg galantamine and 10 mg/kg mecamylamine plus galantamine, respectively. Postischemia protocols included three groups of animals: sham operation, ischemia plus placebo, and ischemia plus 10 mg/kg galantamine; substances were administered 3 or 6 h after ischemia and for 2 consecutive days thereafter. Pyramidal neurons surviving in the cornus ammonis 1 region of the hippocampus were evaluated 72 h after reperfusion, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) histochemistry, caspase-3 and superoxide dismutase (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 postischemia. 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 postischemia.

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) and N-methyl-D-aspartate recep-

ABBREVIATIONS: CA1, cornus ammonis 1; CCA, common carotid artery; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; SOD, superoxide dismutase; DI, discrimination index; ANOVA, analysis of variance; GTS-21, (2.4)-dimethoxybenzylidene anabaseine dihydrochloride.

tor (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 α-7 neuronal nicotinic receptors and also induces antiapoptotic protein B-cell leukemia/lymphoma 2 expression in cultured chromaffin cells and human neuroblastoma cells (Arias et al., 2004, 2005). This was corroborated by Kihara et al. (2004) in cultured cortical neurons. Neuroprotection afforded by galantamine in vivo has been reported in antinerve 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; de Bruin and Pouzet, 2006; Van Dam and De Deyn, 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 postischemic treatment protocols, as well as spatial memory tests, using the neuroprotective dose resulting from previous pretreatment 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. One hundred eighty adult male Mongolian gerbils (*Meriones unguiculatus*; weight, 60–80 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 $37 \pm 0.5°C$ using a temporalis muscle probe and a servo-controlled rectal probe heating pad (RTC-1; Cibertec, Madrid, Spain). A catheter was inserted into the right femoral artery to continuously monitor arterial blood pressure and glucose concentration. Measurements (Schiller CM-8, Baar, Switzerland) 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; Leica, Wetzlar, Germany), and the incision was closed. The sham operation group was treated in the same way, but without CCA 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 3 days after ischemia by perfusion with saline solution and then fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were embedded in paraffin before sectioning on a rotary microtome (Shandon AS-325-Retraction; Thermo Electron Corporation, Waltham, MA) 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 because 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 10 mg/kg galantamine group (mecamylamine plus galantamine). Galantamine (Tocris 0686; Tocris Cookson Inc., Bristol, UK; or kindly provided by Johnson & Johnson, New Brunswick, NJ) was given s.c. twice a day, starting the day before ischemia and ending the day before sacrifice. Mecamylamine (Sigma M9020; Sigma-Aldrich, St. Louis, MO) was administered by i.p. 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 pretreatment protocol, postischemia treatment protocols were designed with the administration of the neuroprotective dose. Therefore, the postischemia 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 s.c. 3 (one set) or 6 (the other set) h after ischemia and at subsequent 12-h intervals until sacrifice (see Fig. 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 1000× (Zeiss Axioplan; Carl Zeiss GmbH, Jena, Germany) in the hematoxylin-eosin-stained sections. Only neurons with visibly normal nuclei were counted. The number of viable CA1 pyramidal neurons per 1-mm length (Image-Pro Plus analyzer; Media Cybernetics, Inc., Silver Spring, MD) 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); Calbiochem, San Diego, CA], 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 1000×.

Immunohistochemistry and Analysis of Active Caspase-3 and SOD-2. After deparaffination and rehydration, sections were immersed in 0.01 M citrate buffer, pH 6.0, and boiled for 20 min. Sections were then blocked with 1.5% bovine serum albumin and incubated overnight with the primary antibodies [antiactive caspase-3 (1:10; Alexis Corporation, Läufelfingen, Switzerland) and anti-Mn superoxide dismutase (1:100; StressGen Biotechnologies,

San Diego, CA)]. Sections were immersed in 3% H_2O_2 in methanol and incubated with a secondary-biotinylated universal antibody (1: 200) followed by streptavidin-peroxidase complex (Vectastain Universal Quick Kit; Vector Laboratories, Burlingame, CA) and 0.06% diaminobenzidine and 0.2% H_2O_2. 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 1000×.

Western Blot Analysis. Five animals were decapitated, and the hippocampi were quickly extracted and frozen in liquid nitrogen. Samples were resuspended in 50 mM Tris/HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 0.2 mM benzamidine and mechanically dissociated. Total protein concentration was measured by the bicinchoninic acid protein assay, and equal quantities of protein were run on a 10% or 15% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Pall; Thermo Electron Corporation) that were blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 for 1 h and incubated with the primary antibodies [rabbit anticaspase-3 (1:500; Cell Signaling Technology Inc., Beverly, MA); rabbit anti-Mn superoxide dismutase (1:50,000; StressGen] overnight at 4°C. Protein loading was monitored by comparison with a mouse anti-β-actin (1:10,000; Sigma-Aldrich). Membranes were incubated with the corresponding anti-rabbit or antimouse peroxidase conjugate (1:5000;

Santa Cruz Biochemicals, Santa Cruz, CA). Chemiluminescence was detected with an enhanced chemiluminescence Western blot detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) 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 × 40 × 40 cm made up of gray polyvinyl chloride) for 5 days previous to the day of ischemia and for the 2 following days to reduce neophobic responses (see protocol in Fig. 6). On the 3rd 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 4th 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 et al. (2003): DI = $(N - F)/(N + F)$, where N is the time spent exploring the new located
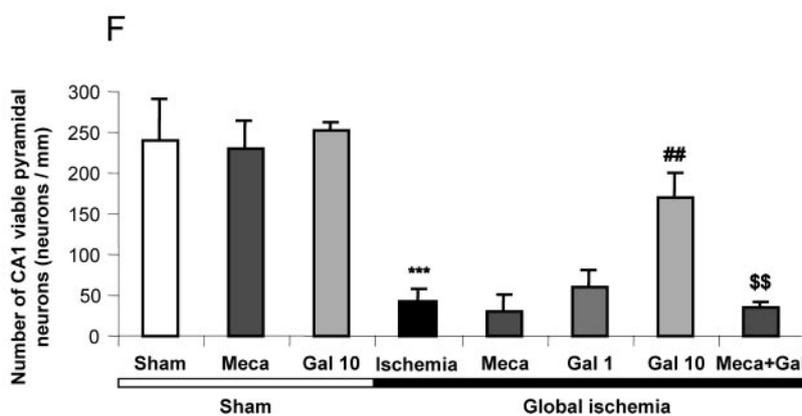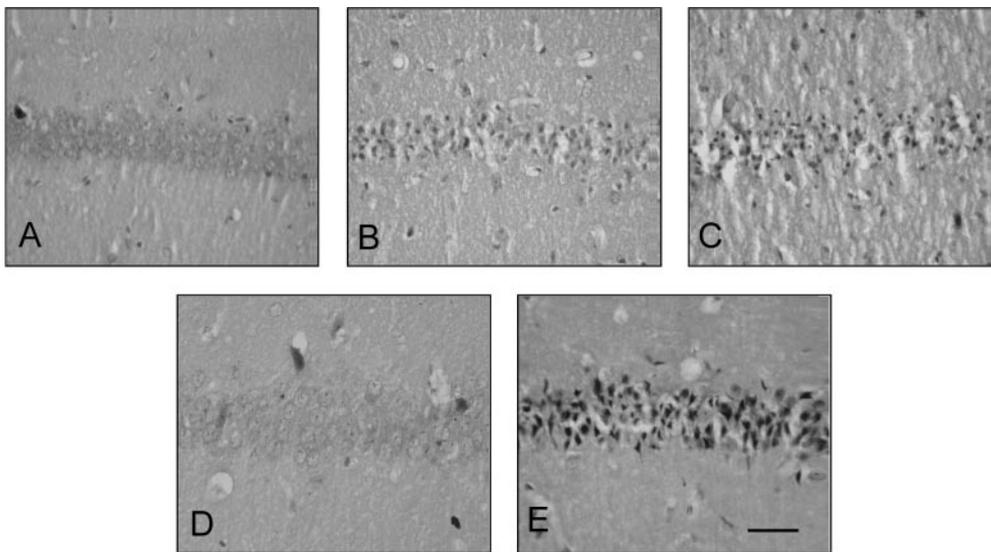

Fig. 1. Galantamine, when given 24 h before ischemia, increased the number of viable pyramidal neurons in CA1 after transient global ischemia. Photomicrographs of CA1 pyramidal layer stained with hematoxylin-eosin of the most relevant treatment groups: sham (A), ischemia (B), 1 mg/kg galantamine (C), 10 mg/kg galantamine (D), and 10 mg/kg mecamylamine plus 10 mg/kg galantamine (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 with sham; ##, $p < 0.01$ compared with ischemia; $$, $p < 0.01$ compared with 10 mg/kg galantamine.

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 (see Fig. 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 postischemia galantamine group (galantamine 10), and the ischemia 10 mg/kg mecamylamine plus 10 mg/kg 3-h postischemia galantamine group (mecamylamine plus galantamine). The last administration of the drugs was performed at least 12 h before sample trials, so differences in the discrimination index were not due to acute drug effects.

Statistical Analysis. Results were expressed as mean ± S.E.M. 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) (Fig. 1, A, 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) (Fig. 1, C, D, and F). Animals treated with mecamylamine plus galantamine did not show the neuroprotective effect (34 ± 8.5

Fig. 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 the CA1 pyramidal layer show TUNEL-stained sections corresponding to sham (A), ischemia (B), 10 mg/kg galantamine (C), and 10 mg/kg mecamylamine plus 10 mg/kg galantamine (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), 10 mg/kg galantamine (H), and 10 mg/kg mecamylamine plus 10 mg/kg galantamine (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 with sham; ###, $p < 0.001$ compared with ischemia; $$$, $p < 0.001$ compared with 10 mg/kg galantamine. Scale bar, 40 μm.
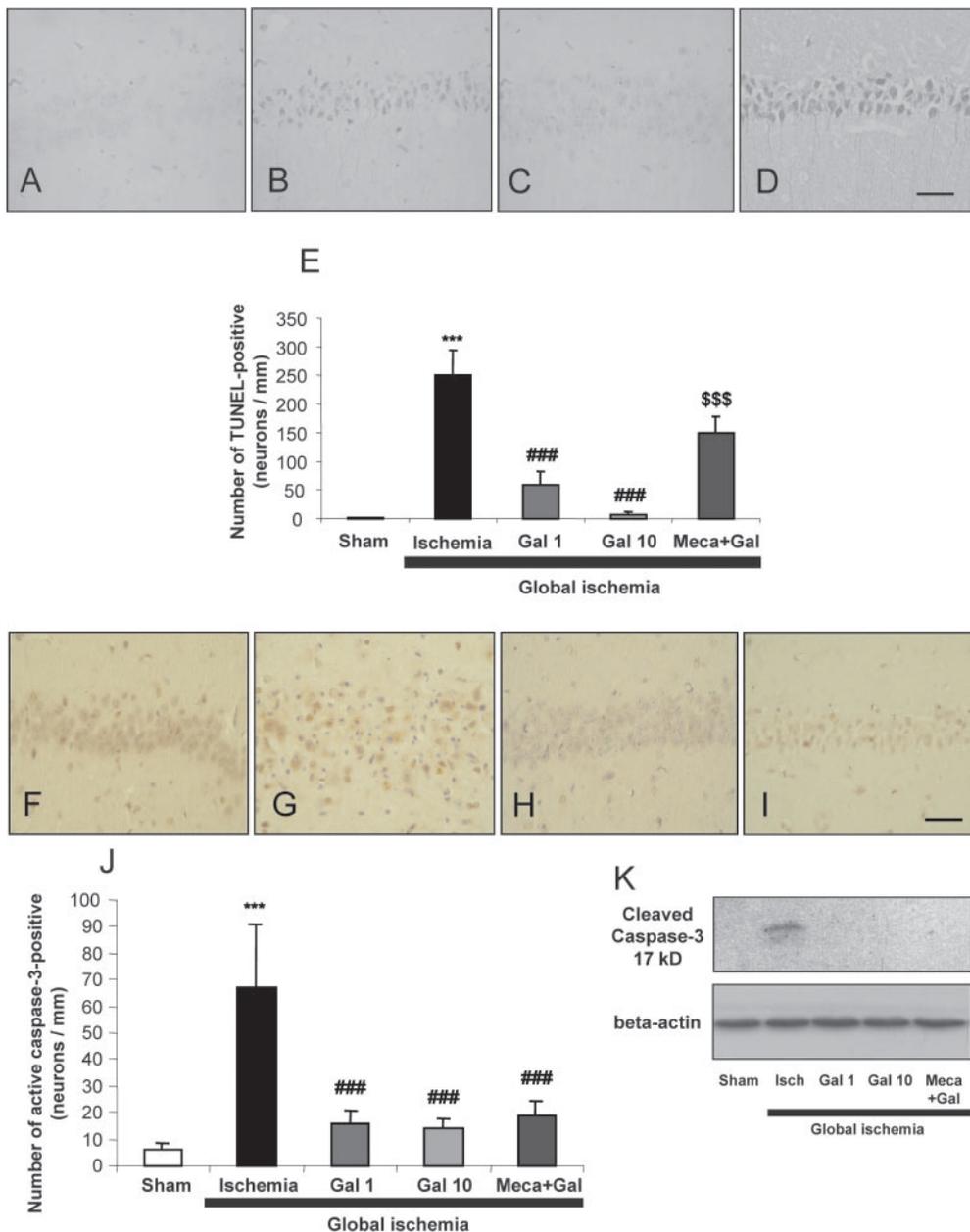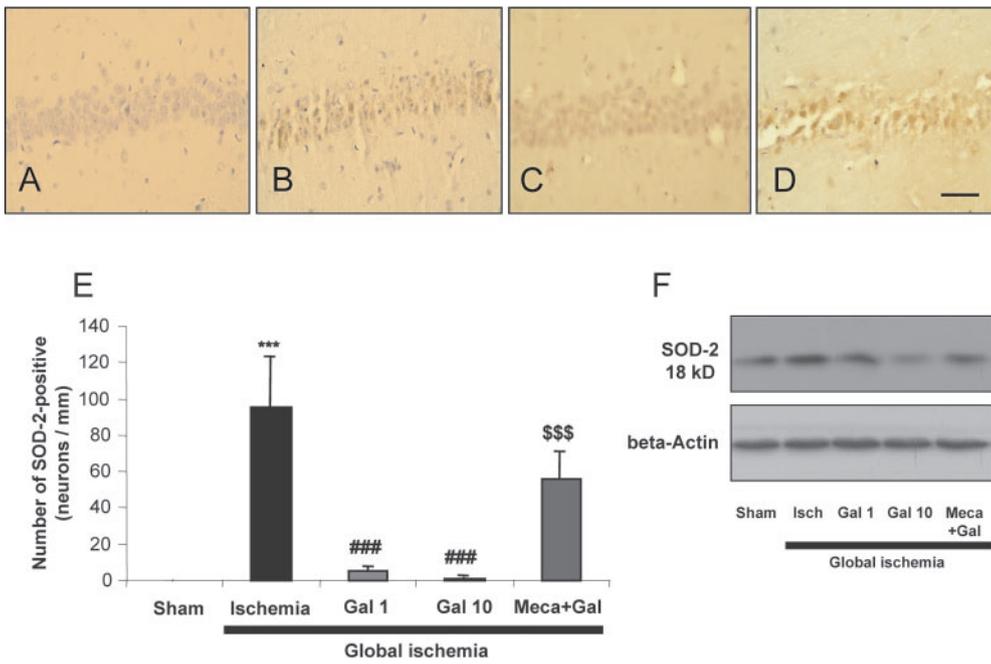
Fig. 3. Effects of galantamine, given 24 h before ischemia, on SOD-2 immunoreactivity after transient global ischemia. Photomicrographs of CA1 pyramidal layer show SOD-2-stained sections corresponding to sham (A), ischemia (B), 10 mg/kg galantamine (C), and 10 mg/kg mecamylamine plus 10 mg/kg galantamine (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 with sham; ###, $p < 0.001$ compared with ischemia; \$\$\$, $p < 0.001$ compared with 10 mg/kg galantamine.

Fig. 4. Galantamine (10 mg/kg) administered 3 h postischemia afforded neuroprotection and reduced DNA fragmentation, active caspase-3, and SOD-2. A, schematic representation showing the protocol used in the 3-h postischemia 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.

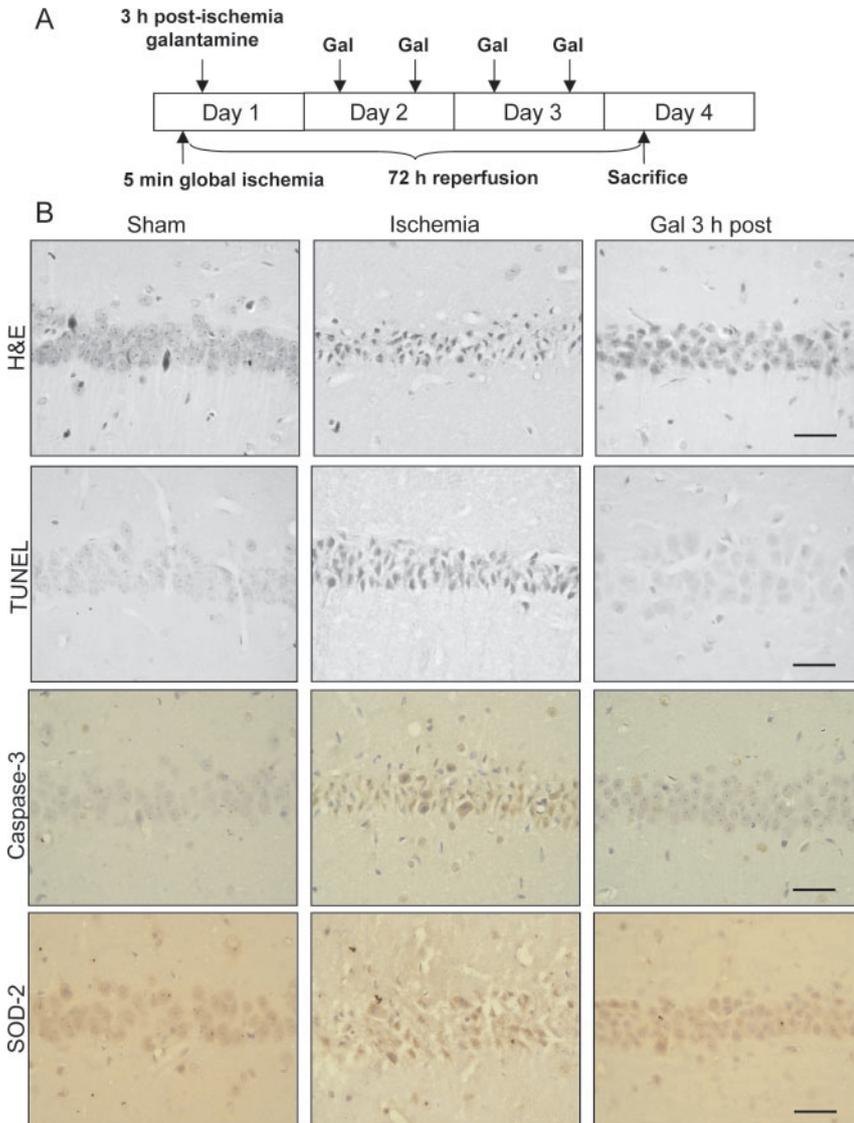neurons/mm) (Fig. 1, E and F). Several control groups were performed to exclude the possibility of drug effect on the viability of CA1 pyramidal neurons. These groups included sham animals treated with mecamylamine (230 ± 33.8 neurons/mm), sham animals treated with galantamine (252 ± 9.4 neurons/mm), 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) (Fig. 2, A–E). Galantamine decreased DNA fragmentation in a dose-dependent manner (60 ± 22.9 neurons/mm at the dose of 1 mg/kg 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 (Fig. 2, F–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 (Fig. 3). After ischemia, SOD-2 markedly increased over sham animals (95 ± 28.6 neurons/mm) (Fig. 3, 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) (Fig. 3, C, E, and F). Once more, mecamylamine markedly blocked these galantamine effects (56 ± 15.7 neurons/mm) (Fig. 3, D–F).

Neuroprotective Effects of Galantamine When Given after the Ischemic Insult. The protocol used to test the neuroprotective effects of galantamine when given postischemia is shown in Fig. 4A. Ischemia reduced the number of viable pyramidal neurons/mm from 202 ± 26.4 to 46 ± 13 (Figs. 4B and 5A); when administered 3 h postischemia 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 (Figs. 4B and 5B) and active caspase-3 staining (Figs. 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 neurons/mm 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 (Figs. 4B and 5D).

When galantamine was administered 6 h after the ischemia-reperfusion injury, the neuroprotective effect was not
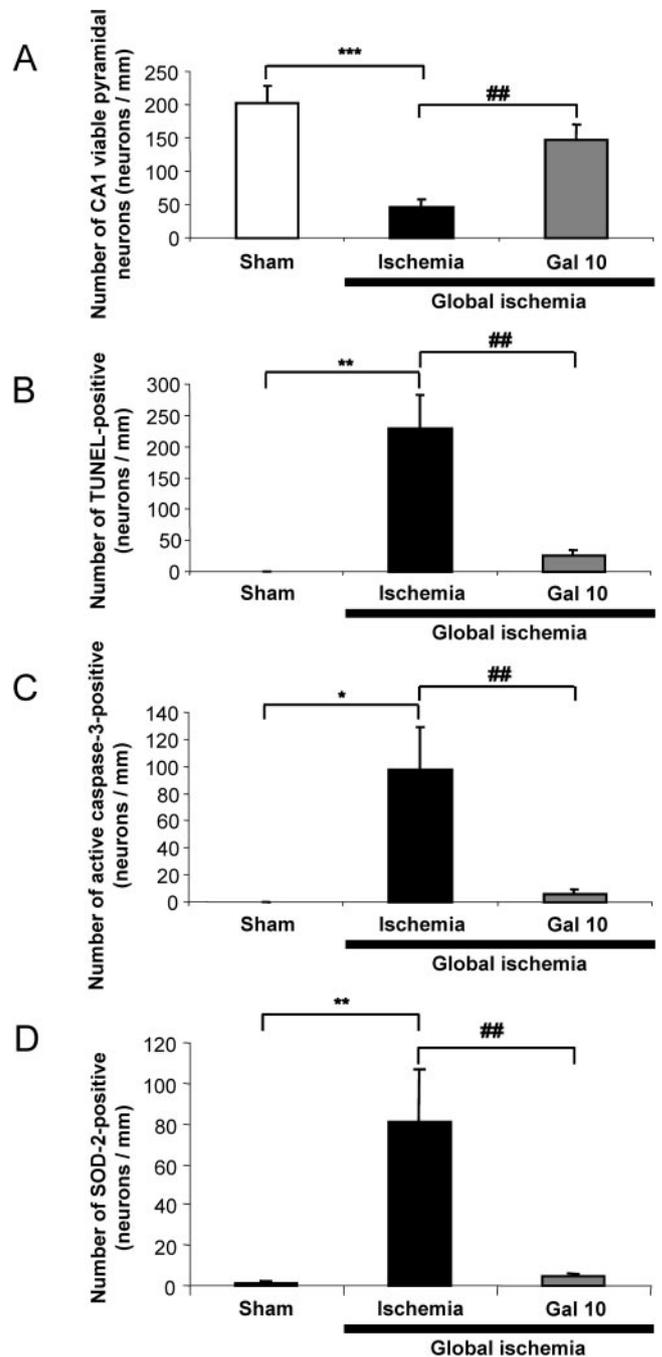
Fig. 5. Galantamine (10 mg/kg) administered 3 h postischemia afforded neuroprotection and reduced DNA fragmentation, active caspase-3, and SOD-2. A, histogram showing the number of viable pyramidal neurons in CA1. B, histogram representing the TUNEL data for each treatment group. C, histogram representing 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 with sham; ##, $p < 0.01$, compared with ischemia.

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 it was 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 Fig. 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 (Fig. 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.
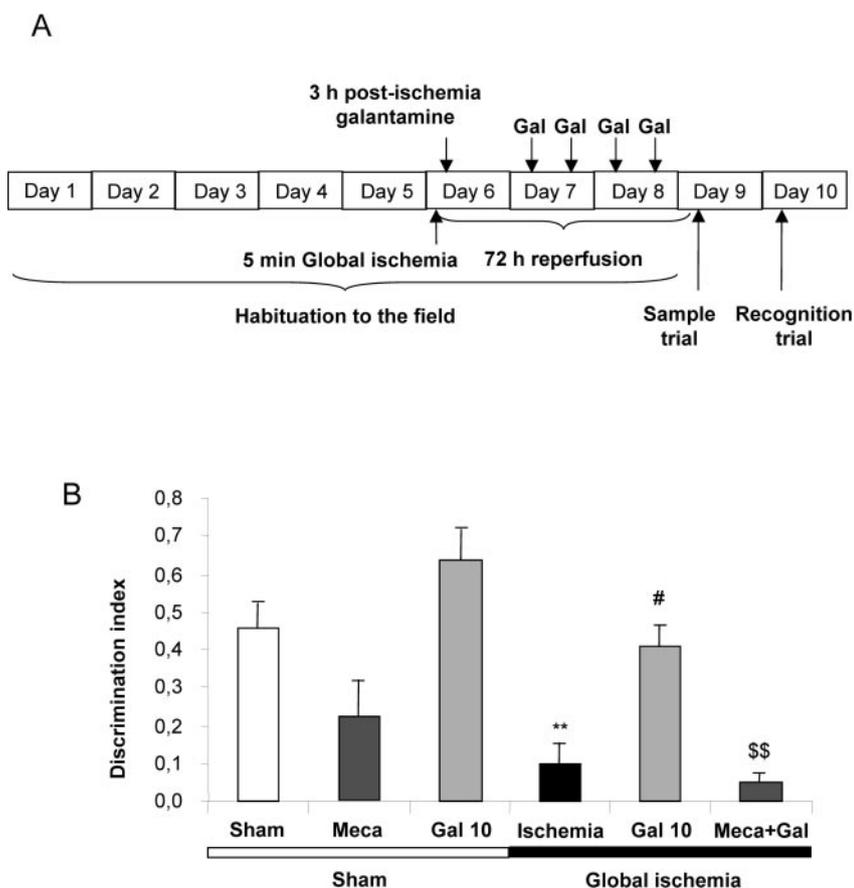
The pharmacological profile of galantamine is actually more complex than previously thought. Its classic acetylcholinesterase inhibitory effect is well documented, as well as its allosteric potentiating actions on neuronal nicotinic receptors (Arroyo et al., 2002). In the present study, we found that 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 β-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,

Fig. 6. Spatial memory recovery after 10 mg/kg galantamine administered 3 h postischemia. 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 with sham; #, $p < 0.05$ compared with ischemia; $$, $p < 0.01$ compared with galantamine.

reduction of DNA fragmentation did depend, at least in part, on nicotinic receptors (see Fig. 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 et al. (2007) and Iliev et al. (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 (National Institute of Neurological Diseases and Stroke rt-PA Stroke Study Group, 1995). Considering that the first administration of the drug in this protocol was not until 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 because 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 MnSOD 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 postischemia-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 et al. (2000) reported that α-7 receptor activation by GTS-21 attenuated the intracellular oxidative stress produced by ethanol; similarly, Ravikumar et al. (2004) reported in rats with spinal cord injury that nicotine can protect against activation of the redox-responsive transcription factors nuclear factor-κB, activating protein-1, and cAMP response element-binding protein, thus reducing mRNA levels of tumor necrosis factor α and monocyte chemotactic protein-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; de Bruin and Pouzet, 2006; Van Dam and De Deyn, 2006) and humans (Wilcock et al., 2000; Erkinjuntti et al., 2003). The elimination half-life of galantamine is 40 to 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 to exclude acute drug effects on behavioral 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 prove the therapeutic potential of postischemic administration of galantamine to patients suffering stroke.

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