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Research Report

Effects of memantine and galantamine given separately or in association, on memory and hippocampal neuronal loss after transient global cerebral ischemia in gerbils

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ABSTRACT

Galantamine is an acetylcholinesterase inhibitor and memantine is a non competitive antagonist of NMDA receptors that are being used to treat Alzheimer's disease (AD) patients. The fact that drugs with different mechanisms of action are available to treat AD introduces the prospect of prescribing drug combinations to amplify drug efficacy. This study was planed to evaluate the potential neuroprotective effects of galantamine combined with memantine in a transient global cerebral ischemia model in gerbils. Animal groups included in the study were: sham, ischemia, and ischemia plus galantamine (1 mg/kg and 10 mg/kg), memantine (10 mg/kg and 20 mg/kg), 1 mg/kg galantamine plus 10 mg/kg memantine, and 10 mg/kg galantamine plus 10 mg/kg memantine, respectively. Surviving pyramidal neurons in the CA1 subfield of the hippocampus, TUNEL, caspase-3 and SOD-2 immunohistochemistries, and the object placement test were evaluated 72 h after reperfusion. Memantine did not exert a clear neuroprotective effect, nor did it prevent spatial memory loss. In a previous study using the same experimental model, galantamine was neuroprotective and improved spatial memory. In this study, the association of 10 mg/kg memantine with 10 mg/kg galantamine increased the number of living pyramidal neurons, reduced TUNEL, active caspase-3 and SOD-2 immunoreactivity, and preserved spatial memory after ischemia-reperfusion injury; however, the effects of the combination were not statistically different from those observed in animals treated with galantamine alone. We believe these results are of interest from a clinical point of view because the association of both drugs is being used in clinical practice and in clinical trials to treat Alzheimer's disease and vascular dementia.

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Abbreviations: CCA, common carotid artery; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling; SOD, superoxide dismutase; DI, discrimination index

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1. Introduction

Cerebrovascular disease is a common cause of dementia, contributing to cognitive loss in Alzheimer's disease. Galantamine and memantine are both drugs used in the treatment of Alzheimer's disease. Galantamine, as well as memantine, have been shown to reduce the progression of vascular dementia improving cognition, behavior and activities of daily living (Erkinjuntti et al., 2003; Orgogozo et al., 2002).

Galantamine and memantine act on several targets. Galantamine is an acetylcholinesterase inhibitor (Thomsen and Kewitz, 1990) 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; Schilstrom et al., 2007). Neuroprotective effects have been described *in vitro* (Arias et al., 2004, 2005; Kihara et al., 2004; Sobrado et al., 2004), as well as in anti-nerve growth factor mice (Capsoni et al., 2002) and after global cerebral ischemia (Ji et al., 2007; Lorrio et al., 2007).

Memantine is a reversible blocker of NMDA receptors (Bormann 1989), which are related to learning and memory, as well as to excitotoxic neuronal death. This drug blocks NMDA receptors in pathological situations, allowing neurotransmission in physiological conditions (Parsons et al., 1999). Memantine has shown neuroprotection *in vitro* (Culmsee et al., 2004; Seif el Nasr et al., 1990; Sobrado et al., 2004) and *in vivo* including focal (Backhauss and Krieglstein, 1992; Chen et al., 1998; Culmsee et al., 2004; Lapchak, 2006) and global cerebral ischemia models (Block and Schwarz, 1996; Duszczyk et al., 2005; Gao et al., 2006; Seif el Nasr et al., 1990).

Galantamine is indicated in mild to moderate, and memantine in moderate to severe stages of Alzheimer's disease. Both drugs are actually being co-administered to patients in moderate to severe stages due to the worsening derived from galantamine's withdrawal. Therefore, it is of great interest to assess the effects of associating these drugs in experimental models involving neuronal death. The first study comparing the neuroprotective effects of galantamine and memantine in the same model was performed in hippocampal slices subjected to oxygen and glucose deprivation and it demonstrated that both agents were neuroprotective, although memantine exerted less neuroprotection (Sobrado et al., 2004). The next step has been to test whether galantamine or memantine, given separately or in association, protect hippocampal neurons from ischemia *in vivo* in a transient global cerebral ischemia model. The results related to galantamine's neuroprotection were previously published (Lorrio et al., 2007). Outcome was evaluated 3 days after ischemia because it is not until that time that a stabilized and reproducible lesion is obtained (Kirino 1982), and neuroprotection as well as some of the mechanisms involved in such protection can be studied. The present work shows that memantine did not exert a clear neuroprotective effect, corroborated by the loss of spatial memory. However, the association of galantamine and memantine exerted neuroprotection and preserved spatial memory as much as galantamine alone. This study suggests that memantine does not interfere with the neuroprotective and behavioral effects of galantamine. This outcome is relevant in a clinical context because the association of both drugs is being used in clinical practice; and clinical trials are being performed with the association of both drugs (Porsteinsson et al., 2008).

2. Results

2.1. *Physiological parameters*

Physiological parameters (weight, brain and body temperature, arterial blood pressure, heart rate and glucose concentration) remained within normal levels, and basal values did not show statistical differences among groups (Table 1). Morbidity was evaluated at 30, 60, and 120 min after the recovery of the animals, using the method of McGraw (McGraw 1977); no statistical differences were found among groups.

2.2. *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) (Figs. 1A, B, and I) that was much lower 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) (Figs. 1C, D, and I). Animals treated with memantine did not show the neuroprotective effect (78±19.9 neurons/mm at 10 mg/kg and 43±15.7 neurons/mm at 20 mg/kg) (Figs. 1E, F, and I). The combination of 1 mg/kg galantamine and 10 mg/kg memantine was not neuroprotective either (25±7.1 neurons/mm) (Figs. 1G and I). However, the combination of 10 mg/kg galantamine and 10 mg/kg memantine increased the number of viable neurons/mm to values which were similar to those of sham animals (227±20 neurons/mm) (Figs. 1H and I). From the results described above, the following assays were performed to investigate the mechanisms through which the neuroprotective effect was exerted.

2.3. *TUNEL-stained pyramidal neurons in CA1*

After ischemia-reperfusion injury, numerous TUNEL-positive neurons appeared (252±42.4 neurons/mm) (Fig. 2 and Fig. 3A). Galantamine decreased this number in a dose-dependent manner (60±22.9 neurons/mm at the dose of 1 mg/kg and 7±4.9 neurons/mm at the dose of 10 mg/kg). Animals treated with memantine also showed reduced DNA fragmentation (44±14.9 neurons/mm at the dose of 10 mg/kg and 107±35.4 neurons/mm at the dose of 20 mg/kg). Treatment with both galantamine and memantine reduced TUNEL-positive neurons to values which were similar to those of sham animals (0±0.1 neurons/mm).

2.4. *Active caspase-3-stained pyramidal neurons in CA1*

DNA fragmentation is often related to apoptosis. However, the TUNEL method can also stain necrotic cells. Therefore, we analyzed an additional parameter related to apoptosis: active caspase-3 (Fig. 2 and Fig. 3B). Sham animals showed few active caspase-3-positive neurons (6±2.2 neurons/mm). Transient global ischemia induced a marked increase (67±23.9

Table 1 – The drugs did not induce changes in physiological variable data

Parameters	Groups	Basal	Occlusion	Reperfusion
Mean arterial blood pressure (mm Hg)	Sham	74.1±2.9	72.1±2.7	70.0±2.8
	Ischemia	78.0±2.6	111.0±3.9	75.4±2.8
	Galantamine 1 mg/kg	73.4±5.3	99.1±3.6	72.8±2.1
	Galantamine 10 mg/kg	74.9±1.6	99.4±2.1	71.0±2.4
	Memantine 10 mg/kg	73.4±3.9	99.0±5.3	71.9±1.8
	Memantine 20 mg/kg	75.1±3.2	101.9±4.1	69.1±3.6
	Gal 1+Mem 10	73.4±1.8	105.0±1.7	74.4±3.3
	Gal 10+Mem 10	70.1±4.1	102.9±4.5	64.3±3.0
Heart rate (bpm)	Sham	337.5±24.4	315.0±12.0	281.3±10.0
	Ischemia	368.6±20.9	360.0±18.5	336.4±19.9
	Galantamine 1 mg/kg	306.4±22.6	335.6±22.8	313.1±15.6
	Galantamine 10 mg/kg	303.8±10.0	361.9±24.8	291.4±16.7
	Memantine 10 mg/kg	300.0±16.0	302.1±25.0	317.1±21.3
	Memantine 20 mg/kg	301.9±24.3	288.8±16.2	296.3±10.5
	Gal 1+Mem 10	298.1±10.4	307.5±13.6	320.6±19.6
	Gal 10+Mem 10	266.3±16.6	267.0±27.8	240.0±10.1
Arterial blood glucose (mg/dl)	Sham	99.9±7.0	103.0±5.1	104.4±5.5
	Ischemia	79.9±4.4	110.5±8.4	107.4±5.5
	Galantamine 1 mg/kg	108.4±6.6	124.3±9.4	110.0±7.8
	Galantamine 10 mg/kg	114.0±9.4	126.4±7.1	122.7±8.2
	Memantine 10 mg/kg	86.3±4.4	110.1±5.5	91.0±3.2
	Memantine 20 mg/kg	92.0±1.9	112.9±4.0	98.0±5.6
	Gal 1+Mem 10	82.1±8.5	107.6±7.9	107.1±7.9
	Gal 10+Mem 10	105.0±9.8	114.0±9.3	109.5±7.7

There were no significant differences in the physiological variables in the drug-treated groups compared with the vehicle-treated ischemic group.

neurons/mm). Galantamine treatment reduced active caspase-3-positive neurons in a dose-dependent manner (16±4.8 neurons/mm at 1 mg/kg and 14±3.7 neurons/mm at 10 mg/kg). Animals treated with memantine also showed reduced number of active caspase-3-positive neurons (26±6.9 neurons/mm at 10 mg/kg and 25±7.7 neurons/mm at 20 mg/kg). Treatment with both galantamine and memantine reduced the number of active caspase-3-positive neurons to values which were similar to those of sham animals (13±3.4 neurons/mm).

2.5. *SOD-2-stained pyramidal neurons in CA1*

After ischemia, the inducible antioxidant enzyme SOD-2 markedly increased over sham animals (95±28.6 neurons/mm); this enzyme was markedly reduced in galantamine treated groups (5±2.5 neurons/mm at 1 mg/kg and 1±0.6 neurons/mm at 10 mg/kg). Animals treated with memantine also showed reduced number of SOD-2-positive neurons (8±3.7 neurons/mm at 10 mg/kg and 19±6.0 neurons/mm at 20 mg/kg). Once more, treatment with both galantamine and memantine significantly reduced the number of SOD-2-positive neurons (3±1.4 neurons/mm) (Fig. 2 and Fig. 3C).

2.6. *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. 4A.

Clear differences in the performance of animals were observed. Sham animals discriminated between the old and new location, whereas animals subjected to ischemia did not (Fig. 4B). Animals treated with galantamine preserved their ability to discriminate old and new location, but this ability was impaired in animals treated with memantine. The failure of this drug to preserve spatial memory ability corroborates its lack of neuroprotective effect. Treatment with the low dose of galantamine (1 mg/kg) plus memantine did not prevent spatial memory loss, but the high dose of galantamine (10 mg/kg) plus memantine (10 mg/kg) significantly preserved spatial memory.

3. Discussion

The results of the present study show that the association of galantamine and memantine exerted neuroprotection and preserved spatial memory, as much as galantamine alone, in a model of transient global cerebral ischemia in the gerbil, and that memantine did not interfere with the neuroprotective and behavioral effects of galantamine.

Most studies with galantamine report neuroprotective and cognition enhancing effects in several experimental conditions, and specifically against cerebral ischemia (Iliev et al., 2000; Ji et al., 2007; Lorrio et al., 2007), although lack of neuroprotection has been once reported (Zhao et al., 2005). This discrepancy may be attributed either to the different experimental models used or to the different administration profiles. Galantamine is believed to exert neuroprotection mainly by inhibiting apoptosis (Arias et al., 2004; Lorrio et al., 2007) and oxidative damage (Ezoulin et al., 2008; Traykova et al., 2003).

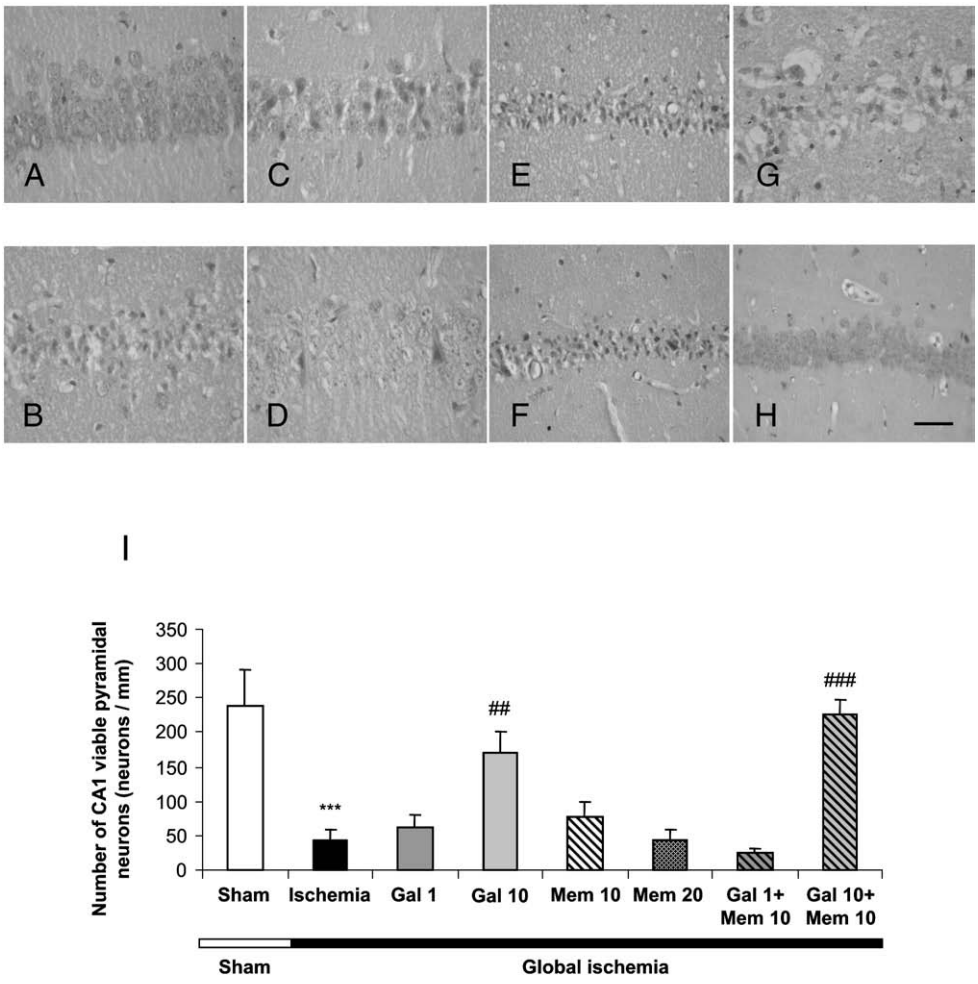

Fig. 1 – Galantamine alone and combined with memantine increased the number of viable pyramidal neurons in CA1 after transient global ischemia. Photomicrographs of CA1 pyramidal layer stained with hematoxylin-eosin of the experimental groups: sham (A), ischemia (B), 1 mg/kg galantamine (C), 10 mg/kg galantamine (D), 10 mg/kg memantine (E), 20 mg/kg memantine (F), 1 mg/kg galantamine plus 10 mg/kg memantine (G), and 10 mg/kg galantamine plus 10 mg/kg memantine (H). Scale bar, 40 μm. I, histogram representing the data for each treatment group. Statistical differences were determined with Kruskal–Wallis and ANOVA tests followed by Bonferroni post hoc. ***, $p<0.001$ compared with sham; ## $p<0.01$; ### $p<0.001$ compared with ischemia.

The neuroprotective effects of memantine are more controversial. Thus, on one hand it has been shown to be neuroprotective and to enhance cognition in different experimental models including focal cerebral ischemia (Backhauss and Krieglstein, 1992; Chen et al., 1998; Culmsee et al., 2004; Lapchak, 2006) and global cerebral ischemia (Block and Schwarz, 1996; Duszczyk et al., 2005; Gao et al., 2006; Seif el Nasr et al., 1990). On the other hand, lack of neuroprotection has also been reported (Back et al., 2007; Danilczuk et al., 2005; Rimpilainen et al., 2001). The global ischemia studies reporting neuroprotection were performed in rats. Plasma levels of memantine in the therapeutic range can be achieved in rats either by acute i.p. injection of 2.5–5 mg/kg, or after infusion of 10 to 30 mg/kg/day using osmotic pumps (Hesselink et al., 1999; Misztal et al., 1996). In gerbils, neuroprotection has been reported after 30 mg/kg/day infusion of memantine, so the gerbil brain must have been exposed to effective drug concentrations. This points to a similar therapeutic range of concentrations achieved both in rats and gerbils after administration of equal doses of memantine. we have used a protocol extensively described in the literature to exert neuroprotection in brain ischemia, that is, single i.p. injections of 10 and 20 mg/kg 1 h before, some minutes before or some minutes after ischemia (Backhauss and Krieglstein, 1992; Block and Schwarz, 1996; Culmsee et al., 2004; Dogan et al., 1999; Gao et al., 2006; Gorgulu et al., 2000; Seif el Nasr et al., 1990; Stieg et al., 1999). It is possible that lower doses (2.5–5 mg/kg) may be more appropriate to obtain plasma levels in the therapeutic range (0.4–1 μM) 15 to 30 min after i.p. administration (Danysz et al., 1994; Wenk et al., 2006). However, we decided to stick to the literature on memantine's neuroprotective action in brain ischemia in order to try to reproduce in our experimental model the positive results found in earlier works. In this case, the most frequently reported doses are 10 and 20 mg/kg i.p.

An acute dosing regime has been the choice in previous literature. Our reason to follow this procedure is the fact that the target of memantine is the NMDA receptor. Since extracellular glutamate levels increased by global ischemia

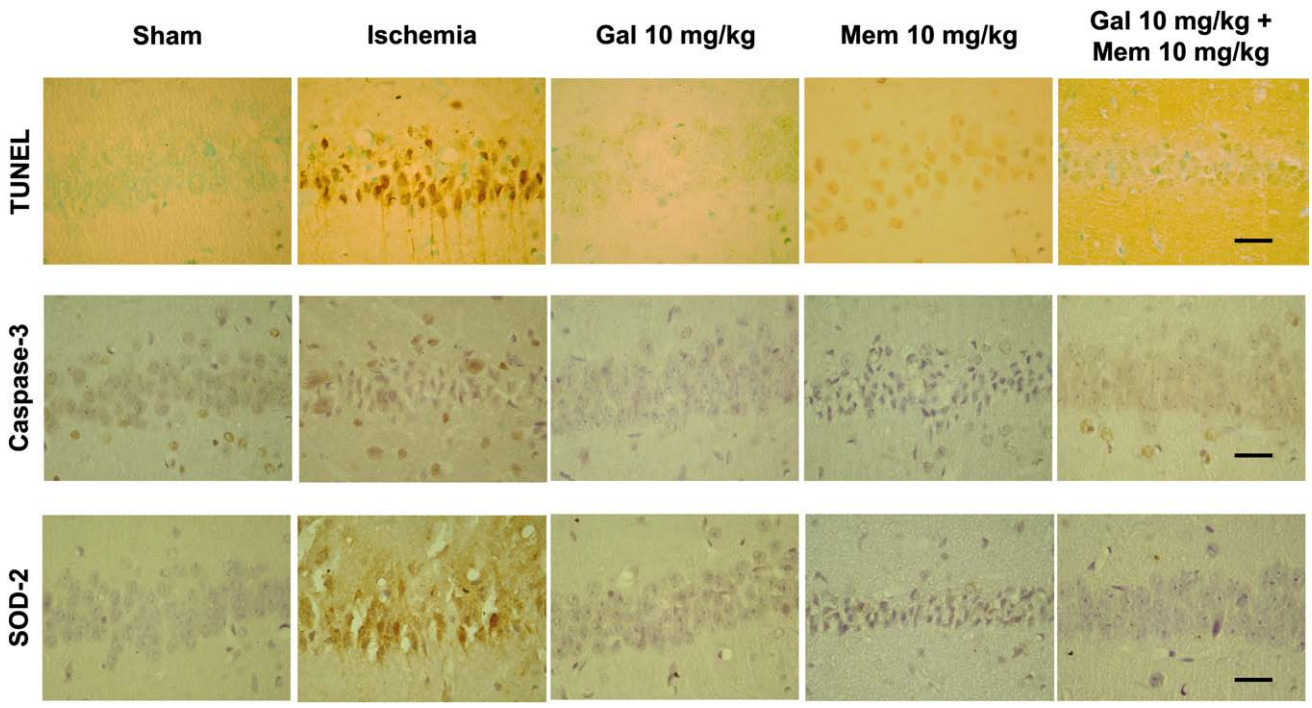

Fig. 2 – Galantamine alone and its combination with memantine reduced DNA fragmentation, active caspase-3 and SOD-2. Photomicrographs of CA1 pyramidal neurons stained with diaminobenzidine (brown) to detect TUNEL, active caspase-3, and SOD-2 in sections corresponding to sham, ischemia, 10 mg/kg galantamine, 10 mg/kg memantine, and 10 mg/kg galantamine plus 10 mg/kg memantine treated animals. Sections are counterstained with methyl green (TUNEL) or hematoxylin (active caspase-3 and SOD-2). Scale bars, 40 μm.

return to basal levels 20 min after the insult in rats and cats (Dohmen et al., 2005; Nakayama et al., 2002), and 40 min after the insult in gerbils (Yu and Cai, 2003), we can therefore presume that the effective time window of memantine would be precisely this 40 min of elevated glutamate levels. Taking these arguments into account, we administered memantine 30 min prior to the insult so that it had time to reach its target (Danysz et al., 1994; Wenk et al., 2006) and it was present while glutamate-induced excitotoxicity was taking place. Based on these grounds, we think that giving memantine chronically would not have provided further neuroprotection.

In the case of galantamine, we decided to use a chronic dosing regime because the drug has a multimodal mechanism of action, i.e. acetylcholinesterase inhibition, nicotinic receptor activity potentiation, facilitation of synaptic transmission, antiapoptotic protein Bcl-2 induction, and free radical scavenging. These actions are most probably interfering in various steps of the damaging processes, occurring at different time lapses from the ischemic insult and until sacrifice. Therefore, a chronic dosing regime was chosen for the different actions of galantamine to be present along the experiment to avoid the ischemic cascade and the delayed cell death of CA1 neurons. We decided to use a pre-treatment protocol because it had been previously described by our group that pre-treatment with the drug prevented neuronal death *in vitro* and induced the expression of the antiapoptotic protein Bcl-2 (Arias et al., 2004).

In rats, 1 h after oral administration of 10 mg/kg galantamine, plasma concentration reaches 1.49 μM, and 8 h after, the concentration is in the therapeutic range, 0.34 μM (Bores et al., 1996). We designed a protocol with subcutaneous administration every 12 h in order to obtain moderate and sustained plasma levels of the drug. A suboptimal dose of galantamine (1 mg/kg) was included to allow the possible observation of synergic neuroprotection when combined with memantine.

Neuronal death following focal cerebral ischemia models begins in the early reperfusion period. In contrast, cell death after transient global cerebral ischemia is delayed 48 h in hippocampal CA1 pyramidal neurons (Kirino 1982). This difference could be responsible for the neuroprotection observed with memantine in focal brain ischemia models and the lack of neuroprotection we are describing here in a global cerebral ischemia model. In fact, it has been reported that slowly progressing neuronal death is enhanced by memantine and other NMDA antagonists, whereas rapidly progressing neuronal death is decreased by them (Ikonomidou et al., 2000). The mechanisms that cause this discrepancy are not fully understood. Low-intensity stimulation of NMDA receptors increases intracellular Ca^{2+} concentration and protects cells from caspase-mediated death (Yano et al., 1998). Thus, decrease of intracellular Ca^{2+} concentration caused by blockade of Ca^{2+}-permeable NMDA channels may be a mechanism contributing to the lack of neuroprotection in slowly progressing neuronal death by NMDA antagonists. However, our results show that memantine reduced DNA fragmentation and caspase-3 cleavage, indicators of apoptotic cell death, as well as SOD-2, an enzyme related to oxidative damage which is induced by ischemia. Therefore, a hypothetical inhibition of low protecting intracellular Ca^{2+} concentration by memantine would not explain the lack of neuroprotection we

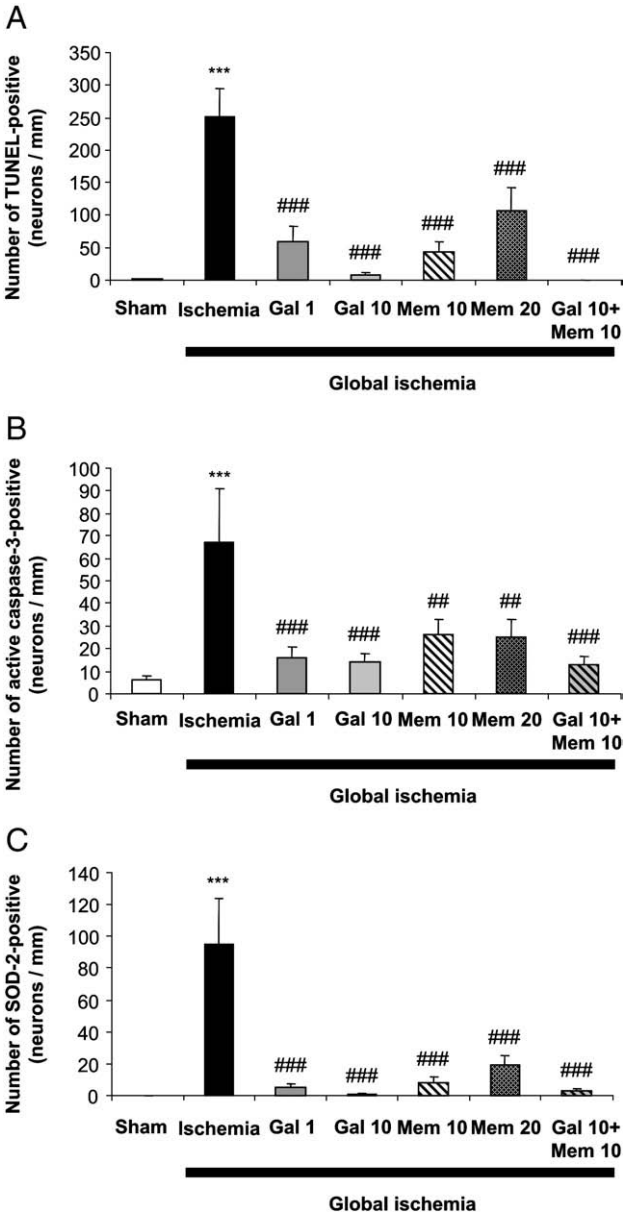

Fig. 3 – Galantamine, memantine and the combination of both, reduced the number of TUNEL-active caspase-3-, and SOD-2-positive pyramidal neurons in CA1. (A) histogram representing the TUNEL data for each treatment group. (B) histogram representing the active caspase-3 data for each treatment group. (C) histogram representing the SOD-2 data for each treatment group. Statistical differences were determined with Kruskal–Wallis and ANOVA tests followed by Bonferroni post hoc. ***, $p<0.001$ compared with sham; ##, $p<0.01$; ###, $p<0.001$ compared with ischemia.

have observed. Rather than this, we think that the beneficial actions exerted by memantine were insufficient to reach neuroprotection in our experimental conditions; however, they may be part of the mechanisms contributing to the neuroprotection observed by others.

The use of combined galantamine and memantine was encouraged by *in vitro* experiments (Zhao et al., 2006). However, no studies report the effects of the association of both drugs on neuroprotection. Comparison of the neuroprotective effects of galantamine and memantine were first evaluated in the same experimental model in our laboratory (Sobrado et al., 2004); surprisingly, it was reported that memantine was less potent than galantamine. Likewise, it has been published that the combination of galantamine and memantine did not improve learning over galantamine alone in older rabbits with delayed eyeblink classical conditioning, although neuroprotection was not evaluated (Woodruff-Pak et al., 2007). Furthermore, it has been recently reported that memantine did not show an advantage over placebo in Alzheimer's disease patients on stable cholinesterase inhibitor treatment (Porsteinsson et al., 2008). These results are in line with the ones we are describing in the present paper: memantine was less potent than galantamine, and the beneficial effects of the association of both drugs could probably be attributed to those of galantamine. It is worth noting that the co-administration of galantamine with memantine did not interfere with the neuroprotective or the behavioral beneficial effects of galantamine. Certainly, this is of great interest because the two drugs are being co-administered to patients in clinical practice.

In conclusion, this study shows that the association of galantamine plus memantine prevented neuronal death and preserved spatial memory that was impaired after transient global cerebral ischemia. This proves that memantine does not interfere with the neuroprotective and behavioral effects of galantamine.

4. Experimental procedures

4.1. *Induction of global ischemia*

One hundred and thirty-four 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 European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the Spanish Real Decreto of 10 October 2005 (RD 1201/2005). All efforts were made to minimize animal suffering and to reduce the number of animals used. 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, Madrid, Spain). A catheter was inserted into the right femoral artery to continuously monitor arterial blood pressure, heart rate 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 (Wild-M650; Leica, Wetzlar, Germany), and the incision was closed.

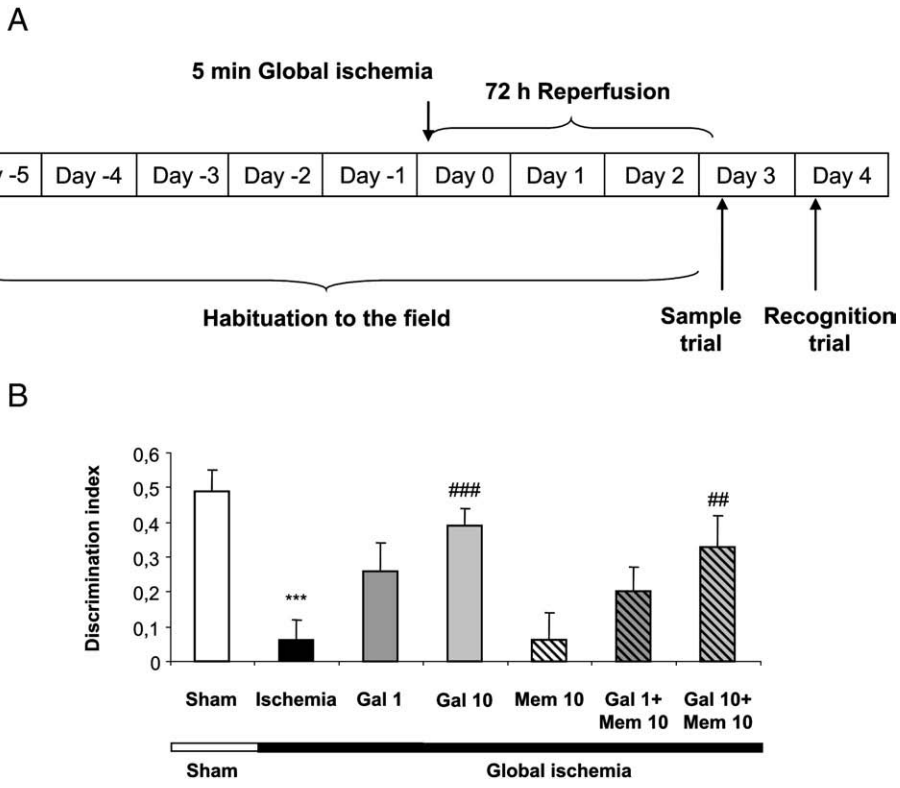

Fig. 4 – Spatial memory recovery after treatment with galantamine, but not with memantine. (A) schematic representation showing the protocol used in the object placement test. (B) histogram representing the memory discrimination index. Statistical differences were determined with Kruskal–Wallis and ANOVA tests followed by Bonferroni post hoc. ***, $p<0.001$ compared with sham; ## $p<0.01$; ### $p<0.001$ compared with ischemia.

The sham operation group was treated in the same way, but without CCA occlusion. It is not until 3 days after reperfusion that a stabilized and reproducible lesion is obtained (Kirino 1982), and neuroprotection as well as some of the mechanisms involved in such protection 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).

4.2. *Drug administration protocol*

Gerbils were randomly divided into eight groups ($n=8$): the sham operation group (sham), the ischemia placebo-treated group (ischemia), the ischemia 1 mg/kg galantamine group (galantamine 1), the ischemia 10 mg/kg galantamine group (galantamine 10), the ischemia 10 mg/kg memantine group (memantine 10), the ischemia 20 mg/kg memantine group (memantine 20), the ischemia 1 mg/kg galantamine plus 10 mg/kg memantine group (galantamine 1 plus memantine 10), and the ischemia 10 mg/kg galantamine plus 10 mg/kg memantine group (galantamine 10 plus memantine 10). Galantamine (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. Memantine (Sigma-Aldrich, St. Louis, MO) was administered by i.p. injection 30 min before the onset of ischemia. Doses were chosen according to previous literature (Block and Schwarz, 1996; Ji et al., 2007; Lorrio et al., 2007; Seif el Nasr et al., 1990) and administration protocols were adapted to the different mechanisms of action of the drugs. A vehicle (0.9% NaCl saline solution) was substituted for the drugs when necessary.

4.3. *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/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.

4.4. *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/1 mm length at dorsal CA1 *stratum pyramidale* of both hemispheres were counted at 1000×.

4.5. *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 [anti-active 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/1 mm length at dorsal CA1 *stratum pyramidale* in both hemispheres were counted at 1000×.

4.6. *Object placement test*

Object placement is a hippocampal-dependent spatial memory task. The current method of object placement was adapted for gerbils (Lorrio et al., 2007) from methods used in rats (Ennaceur et al., 1997). Animals ($n=10$) 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. 4A). 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 previously reported (Blalock et al., 2003): $DI=(N-F)/(N+F)$, 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. 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.

4.7. *Statistical analysis*

Results were expressed as mean ± S.E.M. Statistical differences were determined with Kruskal–Wallis and ANOVA tests followed by Bonferroni post hoc. Statistical significance was set at $p<0.05$.

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REFERENCES

Arias, E., Ales, E., Gabilan, N.H., Cano-Abad, M.F., Villarroya, M., Garcia, A.G., Lopez, M.G., 2004. Galantamine prevents apoptosis induced by beta-amyloid and thapsigargin: involvement of nicotinic acetylcholine receptors. Neuropharmacology 46 (1), 103–114.

Arias, E., Gallego-Sandin, S., Villarroya, M., Garcia, A.G., Lopez, M.G., 2005. Unequal neuroprotection afforded by the acetylcholinesterase inhibitors galantamine, donepezil, and rivastigmine in SH-SY5Y neuroblastoma cells: role of nicotinic receptors. J. Pharmacol. Exp. Ther. 315 (3), 1346–1353.

Back, T., Otto, D., Kittner, D., Schuler, O.G., Hennerici, M.G., Mennel, H.D., 2007. Failure to improve the effect of thrombolysis by memantine in a rat embolic stroke model. Neurol. Res. 29 (3), 264–269.

Backhauss, C., Krieglstein, J., 1992. Extract of kava (Piper methysticum) and its methysticin constituents protect brain tissue against ischemic damage in rodents. Eur. J. Pharmacol. 215 (2–3), 265–269.

Blalock, E.M., Chen, K.C., Sharrow, K., Herman, J.P., Porter, N.M., Foster, T.C., Landfield, P.W., 2003. Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. J. Neurosci. 23 (9), 3807–3819.

Block, F., Schwarz, M., 1996. Memantine reduces functional and morphological consequences induced by global ischemia in rats. Neurosci. Lett. 208 (1), 41–44.

Bores, G.M., Huger, F.P., Petko, W., Mutlib, A.E., Camacho, F., Rush, D.K., Selk, D.E., Wolf, V., Kosley Jr., R.W., Davis, L., Vargas, H.M., 1996. Pharmacological evaluation of novel Alzheimer's disease therapeutics: acetylcholinesterase inhibitors related to galanthamine. J. Pharmacol. Exp. Ther. 277 (2), 728–738.

Bormann, J., 1989. Memantine is a potent blocker of N-methyl-D-aspartate (NMDA) receptor channels. Eur. J. Pharmacol. 166 (3), 591–592.

Capsoni, S., Giannotta, S., Cattaneo, A., 2002. Nerve growth factor and galantamine ameliorate early signs of neurodegeneration in anti-nerve growth factor mice. Proc. Natl. Acad. Sci. U. S. A. 99 (19), 12432–12437.

Chen, H.S., Wang, Y.F., Rayudu, P.V., Edgecomb, P., Neill, J.C., Segal, M.M., Lipton, S.A., Jensen, F.E., 1998. Neuroprotective concentrations of the N-methyl-D-aspartate open-channel blocker memantine are effective without cytoplasmic

vacuolation following post-ischemic administration and do not block maze learning or long-term potentiation. Neuroscience 86 (4), 1121–1132.
Culmsee, C., Junker, V., Kremers, W., Thal, S., Plesnila, N., Krieglstein, J., 2004. Combination therapy in ischemic stroke: synergistic neuroprotective effects of memantine and clenbuterol. Stroke 35 (5), 1197–1202.
Danilczuk, Z., Ossowska, G., Lupina, T., Cieslik, K., Zebrowska-Lupina, I., 2005. Effect of NMDA receptor antagonists on behavioral impairment induced by chronic treatment with dexamethasone. Pharmacol. Rep. 57 (1), 47–54.
Danysz, W., Gossel, M., Zajaczkowski, W., Dill, D., Quack, G., 1994. Are NMDA antagonistic properties relevant for anti-Parkinsonian-like activity in rats?—case of amantadine and memantine. J. Neural. Transm. Parkinson's Dis. Dement. Sect. 7 (3), 155–166.
Dogan, A., Eras, M.A., Rao, V.L., Dempsey, R.J., 1999. Protective effects of memantine against ischemia-reperfusion injury in spontaneously hypertensive rats. Acta Neurochir. (Wien) 141 (10), 1107–1113.
Dohmen, C., Kumura, E., Rosner, G., Heiss, W.D., Graf, R., 2005. Extracellular correlates of glutamate toxicity in short-term cerebral ischemia and reperfusion: a direct in vivo comparison between white and gray matter. Brain Res. 1037 (1–2), 43–51.
Duszczyk, M., Gadamski, R., Ziembowicz, A., Danysz, W., Lazarewicz, J.W., 2005. NMDA receptor antagonism does not inhibit induction of ischemic tolerance in gerbil brain in vivo. Neurotox. Res. 7 (4), 283–292.
Ennaceur, A., Neave, N., Aggleton, J.P., 1997. Spontaneous object recognition and object location memory in rats: the effects of lesions in the cingulate cortices, the medial prefrontal cortex, the cingulum bundle and the fornix. Exp. Brain Res. 113 (3), 509–519.
Erkinjuntti, T., Kurz, A., Small, G.W., Bullock, R., Lilienfeld, S., Damaraju, C.V., 2003. An open-label extension trial of galantamine in patients with probable vascular dementia and mixed dementia. Clin. Ther. 25 (6), 1765–1782.
Ezoulin, M.J., Ombetta, J.E., Dutertre-Catella, H., Warnet, J.M., Massicot, F., 2008. Antioxidative properties of galantamine on neuronal damage induced by hydrogen peroxide in SK-N-SH cells. Neurotoxicology 29 (2), 270–277.
Gao, Y., Chen, H.J., Qian, L.H., Chen, G.Y., 2006. Long-term effects of memantine therapy on neonatal rats with hypoxic-ischemic brain damage. Zhongguo Dang Dai Er Ke Za Zhi 8 (1), 38–40.
Gorgulu, A., Kins, T., Cobanoglu, S., Unal, F., Izgi, N.I., Yanik, B., Kucuk, M., 2000. Reduction of edema and infarction by Memantine and MK-801 after focal cerebral ischaemia and reperfusion in rat. Acta Neurochir. (Wien) 142 (11), 1287–1292.
Hesselink, M.B., De Boer, B.G., Breimer, D.D., Danysz, W., 1999. Brain penetration and in vivo recovery of NMDA receptor antagonists amantadine and memantine: a quantitative microdialysis study. Pharm. Res. 16 (5), 637–642.
Ikonomidou, C., Stefovska, V., Turski, L., 2000. Neuronal death enhanced by N-methyl-D-aspartate antagonists. Proc. Natl. Acad. Sci. U. S. A. 97 (23), 12885–12890.
Iliev, A.I., Traykov, V.B., Mantchev, G.T., Stoykov, I., Prodanov, D., Yakimova, K.S., Krushkov, I.M., 2000. A post-ischaemic single administration of galantamine, a cholinesterase inhibitor, improves learning ability in rats. J. Pharm. Pharmacol. 52 (9), 1151–1156.
Ji, X., Li, C., Lu, Y., Chen, Y., Guo, L., 2007. Post-ischemic continuous administration of galantamine attenuates cognitive deficits and hippocampal neurons loss after transient global ischemia in gerbils. Neurosci. Lett. 416 (1), 92–95.
Kihara, T., Sawada, H., Nakamizo, T., Kanki, R., Yamashita, H., Maelicke, A., Shimohama, S., 2004. Galantamine modulates nicotinic receptor and blocks Abeta-enhanced glutamate toxicity. Biochem. Biophys. Res. Commun. 325 (3), 976–982.
Kirino, T., 1982. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res. 239 (1), 57–69.
Lapchak, P.A., 2006. Memantine, an uncompetitive low affinity NMDA open-channel antagonist improves clinical rating scores in a multiple infarct embolic stroke model in rabbits. Brain Res. 1088 (1), 141–147.
Lorrio, S., Sobrado, M., Arias, E., Roda, J.M., Garcia, A.G., Lopez, M.G., 2007. Galantamine post ischemia provides neuroprotection and memory recovery against transient global cerebral ischemia in gerbils. J. Pharmacol. Exp. Ther. 322 (2), 591–599.
McGraw, C.P., 1977. Experimental cerebral infarction effects of pentobarbital in Mongolian gerbils. Arch. Neurol. 34 (6), 334–336.
Misztal, M., Frankiewicz, T., Parsons, C.G., Danysz, W., 1996. Learning deficits induced by chronic intraventricular infusion of quinolinic acid-protection by MK-801 and memantine. Eur. J. Pharmacol. 296 (1), 1–8.
Moriguchi, S., Marszalec, W., Zhao, X., Yeh, J.Z., Narahashi, T., 2004. Mechanism of action of galantamine on N-methyl-D-aspartate receptors in rat cortical neurons. J. Pharmacol. Exp. Ther. 310 (3), 933–942.
Nakayama, R., Yano, T., Ushijima, K., Abe, E., Terasaki, H., 2002. Effects of dantrolene on extracellular glutamate concentration and neuronal death in the rat hippocampal CA1 region subjected to transient ischemia. Anesthesiology 96 (3), 705–710.
Orgogozo, J.M., Rigaud, A.S., Stoffler, A., Mobius, H.J., Forette, F., 2002. Efficacy and safety of memantine in patients with mild to moderate vascular dementia: a randomized, placebo-controlled trial (MMM 300). Stroke 33 (7), 1834–1839.
Parsons, C.G., Danysz, W., Quack, G., 1999. Memantine is a clinically well tolerated N-methyl-D-aspartate (NMDA) receptor antagonist—a review of preclinical data. Neuropharmacology 38 (6), 735–767.
Porsteinsson, A.P., Grossberg, G.T., Mintzer, J., Olin, J.T., 2008. Memantine treatment in patients with mild to moderate Alzheimer's disease already receiving a cholinesterase inhibitor: a randomized, double-blind, placebo-controlled trial. Curr. Alzheimer Res. 5 (1), 83–89.
Rimpilainen, J., Pokela, M., Kiviluoma, K., Vainionpaa, V., Hirvonen, J., Ohtonen, P., Jantti, V., Anttila, V., Heinonen, H., Juvonen, T., 2001. The N-methyl-D-aspartate antagonist memantine has no neuroprotective effect during hypothermic circulatory arrest: a study in the chronic porcine model. J. Thorac. Cardiovasc. Surg. 121 (5), 957–968 discussion 968–970.
Santos, M.D., Alkondon, M., Pereira, E.F., Aracava, Y., Eisenberg, H.M., Maelicke, A., Albuquerque, E.X., 2002. The nicotinic allosteric potentiating ligand galantamine facilitates synaptic transmission in the mammalian central nervous system. Mol. Pharmacol. 61 (5), 1222–1234.
Schilstrom, B., Ivanov, V.B., Wiker, C., Svensson, T.H., 2007. Galantamine enhances dopaminergic neurotransmission in vivo via allosteric potentiation of nicotinic acetylcholine receptors. Neuropsychopharmacology 32 (1), 43–53.
Schrattenholz, A., Pereira, E.F., Roth, U., Weber, K.H., Albuquerque, E.X., Maelicke, A., 1996. Agonist responses of neuronal nicotinic acetylcholine receptors are potentiated by a novel class of allosterically acting ligands. Mol. Pharmacol. 49 (1), 1–6.
Seif el Nasr, M., Peruche, B., Rossberg, C., Mennel, H.D., Krieglstein, J., 1990. Neuroprotective effect of memantine demonstrated in vivo and in vitro. Eur. J. Pharmacol. 185 (1), 19–24.
Sobrado, M., Roda, J.M., Lopez, M.G., Egea, J., Garcia, A.G., 2004. Galantamine and memantine produce different degrees of neuroprotection in rat hippocampal slices subjected to oxygen-glucose deprivation. Neurosci. Lett. 365 (2), 132–136.
Stieg, P.E., Sathi, S., Warach, S., Le, D.A., Lipton, S.A., 1999. Neuroprotection by the NMDA receptor-associated open-channel blocker memantine in a photothrombotic

model of cerebral focal ischemia in neonatal rat. Eur. J. Pharmacol. 375 (1–3), 115–120.
Thomsen, T., Kewitz, H., 1990. Selective inhibition of human acetylcholinesterase by galanthamine in vitro and in vivo. Life Sci. 46 (21), 1553–1558.
Traykova, M., Traykov, T., Hadjimitova, V., Krikorian, K., Bojadgieva, N., 2003. Antioxidant properties of galantamine hydrobromide. Z. Naturforsch., C 58 (5–6), 361–365.
Wenk, G.L., Parsons, C.G., Danysz, W., 2006. Potential role of N-methyl-D-aspartate receptors as executors of neurodegeneration resulting from diverse insults: focus on memantine. Behav. Pharmacol. 17 (5–6), 411–424.
Woodruff-Pak, D.S., Tobia, M.J., Jiao, X., Beck, K.D., Servatius, R.J., 2007. Preclinical investigation of the functional effects of memantine and memantine combined with galantamine or donepezil. Neuropsychopharmacology 32 (6), 1284–1294.
Yano, S., Tokumitsu, H., Soderling, T.R., 1998. Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature 396 (6711), 584–587.
Yu, S., Cai, J., 2003. Effects of aniracetam on extracellular levels of transmitter amino acids in the hippocampus of the conscious gerbils: an intracranial microdialysis study Neurosci. Lett. 339 (3), 187–190.
Zhao, C.S., Puurunen, K., Schallert, T., Sivenius, J., Jolkkonen, J., 2005. Effect of cholinergic medication, before and after focal photothrombotic ischemic cortical injury, on histological and functional outcome in aged and young adult rats. Behav. Brain Res. 156 (1), 85–94.
Zhao, X., Marszalec, W., Toth, P.T., Huang, J., Yeh, J.Z., Narahashi, T., 2006. In vitro galantamine–memantine co-application: mechanism of beneficial action. Neuropharmacology 51 (7–8), 1181–1191.