Ganstigmine and donepezil improve neurodegeneration in AD11 antinerve growth factor transgenic mice

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Abstract

Ganstigmine (CHF2819) is an acetylcholinesterase inhibitor that increases acetylcholine in rat hippocampus and ameliorates scopolamine-induced amnesia. In this article, we examined whether and how ganstigmine might prevent or rescue the neurodegenerative phenotype in AD11 antinerve growth factor (anti-NGF) mice, a transgenic model for Alzheimer's disease. The effects of ganstigmine were compared with those obtained after administration of donepezil. Results demonstrate that intraperitoneal and oral administration of ganstigmine and donepezil can reverse the cholinergic and behavioral deficit in AD11 mice but not the amyloid and phosphotau accumulation, uncovering different mechanisms leading to neurodegeneration in AD11 mice.

Key words: Alzheimer's disease, ganstigmine, donepezil, therapeutic agents, acetylcholinesterase inhibitors

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Introduction

Alzheimer's disease (AD), the most common dementing disorder of later life, is a major cause of disability and death in the elderly.[1] Although a number of theoretical causes exist, the etiology of the disease is still unknown. Consequently, the majority of therapeutic approaches are designed to ameliorate AD symptoms, leaving doubt as to whether amelioration might also have an effect on disease progression.

Among the different therapeutic agents, inhibitors of acetylcholinesterase (AChEI) are the most used due to the cholinergic hypothesis, whereby AD may be the result of degeneration of basal forebrain cholinergic neurons.[2] Encouraging symptomatic improvements have

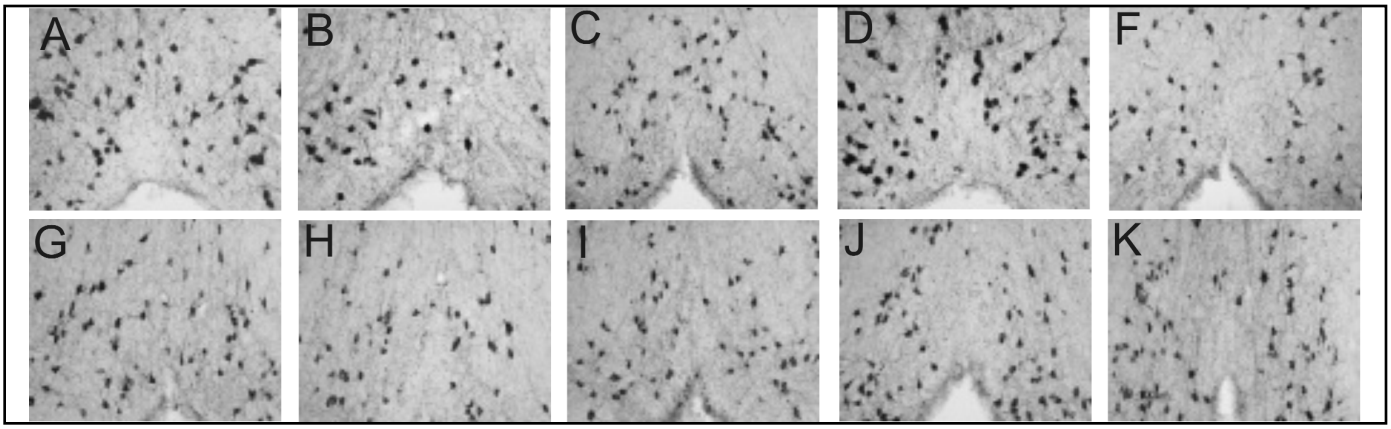

Figure 1. ChAT-positive neurons in two month-old (A) wild type mice, (B) AD11 untreated mice, (C) AD11 mice treated with 3 mg/kg/d of ganstigmine, (D) AD11 mice treated with 6 mg/kg/d of ganstigmine, (E) AD11 mice treated with donepezil; six month-old, (F) wild type mice, (G) AD11 untreated mice, (H) AD11 mice treated with 3 mg/kg/d of ganstigmine, (D) AD11 mice treated with 6 mg/kg/d of ganstigmine, (E) AD11 mice treated with donepezil.

been reported with AChEI use. However, acetylcholine is not the only neurotransmitter system affected in AD. Thus, drugs that have positive effects on different neurochemical systems are probably required to obtain greater therapeutic benefits.[3]

Among these compounds, the geneserine derivative ganstigmine is an AChEI with stimulatory effects on cholinergic functions.[4] The administration of ganstigmine to young and aged adult rats results in a marked increase in acetylcholine levels in the hippocampus and significantly improves scopolamine-induced amnesia measured using passive avoidance tasks.[5] Moreover, it protects chicken cortical neurons from ß-amyloid-induced toxicity.[6]

This study describes the effects of ganstigmine administration on AD11 antinerve growth factor (anti-NGF) mice, a comprehensive model for AD. These mice display a full range of progressive phenotypic alterations, including ß-amyloid plaques, tangles, neuronal loss, and cholinergic deficits linked to behavioral abnormalities.[7-10] In particular, the effects of ganstigmine were investigated in two- and six-month-old AD11 mice. At two months of age, neurodegeneration has begun and is characterized by a decrease in the number of cholinergic neurons in the basal forebrain and by the accumulation of phosphorylated tau in the entorhinal cortex.[8] In six-month-old AD11 mice, the neurodegeneration progresses; however, it is not full-blown. The degeneration linked to tau phosphorylation is spread to the entire cerebral cortex and hippocampus.[8] In this region, ß-amyloid accumulates intracellularly.[8] The cholinergic deficit increases and is related to impairments in behavior [unpublished data] and cortical synaptic plasticity.[11] Therefore, the aim of this study was to test the efficacy of ganstigmine in ameliorating the Alzheimer-like phenotype in AD11 mice at the early and moderate stages of neurodegeneration.

Materials and methods

Animals

AD11 anti-NGF mice were produced as described previously in another study.[10] Mice expressing the functional anti-NGF antibody were obtained by crossing mice expressing the light chain of the recombinant antibody with mice expressing the heavy chain of the recombinant antibody.[10] Mice were housed at constant room temperature (22 ± 1°C) and relative humidity (60 ± 1 percent) under a 12h light/dark cycle. Food and water were provided *ad libitum*. Effects of treatment on body weight and mortality were recorded. All experiments were conducted according to the guidelines of the European Animal Health and Welfare Act.

Pharmaceuticals

Ganstigmine was provided by Chiesi Farmaceutici S.p.A. (Parma, Italy). Donepezil was obtained from commercial sources.

Treatments and tissue collection

Ganstigmine (3 mg/kg/d and 6 mg/kg/d in saline) and donepezil (3.3 mg/kg/d) were administered intraperitoneally for three weeks to AD11 mice starting from 1.5 months of age until two months of age (n = 10). The same dose was used for a second group of animals, who received the drugs through oral gavage starting from two months of age until six months of age (n = 10). Control groups were constituted by age-matched wild type and AD11 mice (n = 10 for each group). Both control groups received placebo.

At the end of the treatment, animals were anesthetized

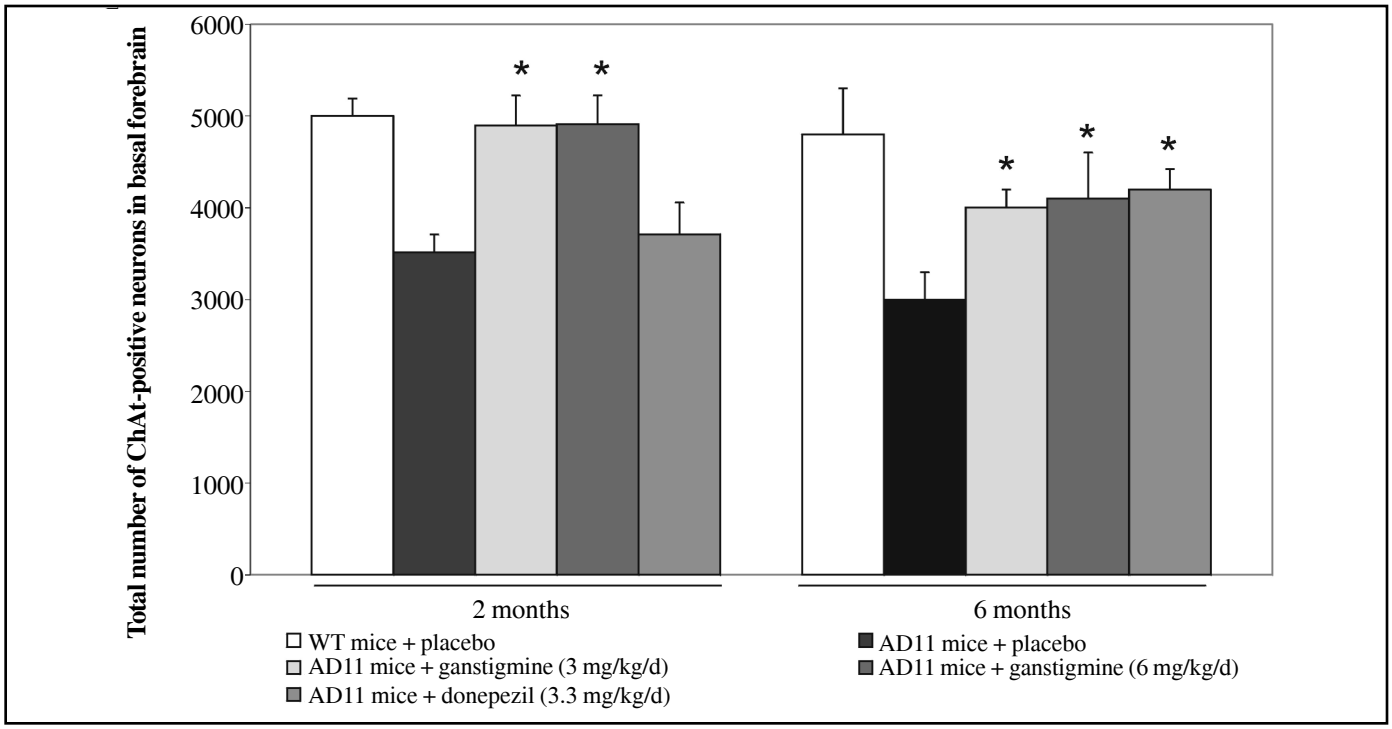

Figure 2. Total number of ChAT-positive neurons in wild type mice, AD11 mice, and AD11 mice treated with ganstigmine and donepezil (* $p < 0.05$ vs AD11 mice treated with placebo).

with 8 µl/gr of 10.5 percent chloral hydrate solution. Brains were removed, and the cranial portion containing the basal forebrain and one of the occipital poles were fixed in 4 percent paraformaldehyde/phosphate buffered saline. The second occipital pole, containing the entorhinal parietal cortices and the hippocampus,[12] was snap frozen on dry ice and stored at -80°C for biochemical analysis. The cerebellum was discarded from this set of experiments.

Immunohistochemistry

Analysis was performed as described previously.[9,10] The following primary antibodies were used: anticholine acetyltransferase (ChAT, 1:500) (Chemicon, Temecula, CA); antiphosphorylated tau (clone AT8, 1:10) (Innogenetics, Gent, Belgium); anti-ß-amyloid (polyclonal R3660), kindly provided by Gennaro Schettini and Claudio Russo, University of Genoa, Italy;[13] anti-ß secretase (BACE1, 1:100) (also from Chemicon); and antipresenilin 1 (PS1, 1:100) (Sigma, St. Louis, MO).

Quantitative neurostereology

The number of ChAT-positive neurons in the basal forebrain was determined as described previously.[10] The same stereological analysis was applied to count ß-amyloid, PS1, and BACE1 positive clusters of cells in the hippocampus and to determine the number of phosphotau-positive neurons in layer II of the lateral entorhinal cortex.[14] Statistical analysis was performed using an ANOVA one way t-test.

Western blot test

The posterior portion of the right occipital pole was frozen and stored at -80°C until processing. The brain was homogenized as described before.[9] Supernatant was collected and stored at -80°C until it was used. A 10 percent polyacrylamide gel was prepared, and SDS-PAGE (polyacrylamide gel electrophoresis) was performed. Samples were blotted on a nitrocellulose membrane and incubated with the following primary antibodies: antiphosphotau (clone AT8) and antitubulin (clone YOL-1), kindly provided by Cesar Milstein of the MRC Laboratory of Molecular Biology, Cambridge, United Kingdom. The intensities of the immunoreactive bands were quantified and analyzed using the image analysis program LUCIA (Laboratory Imaging Ltd., Hostivar, Czech Republic) after normalizing for protein content, evaluated by the intensity of the tubulin band. Statistical analysis was performed using the ANOVA test.

Object recognition test

Object recognition tasks are widely used in humans to test aspects of working memory and to characterize amnesic

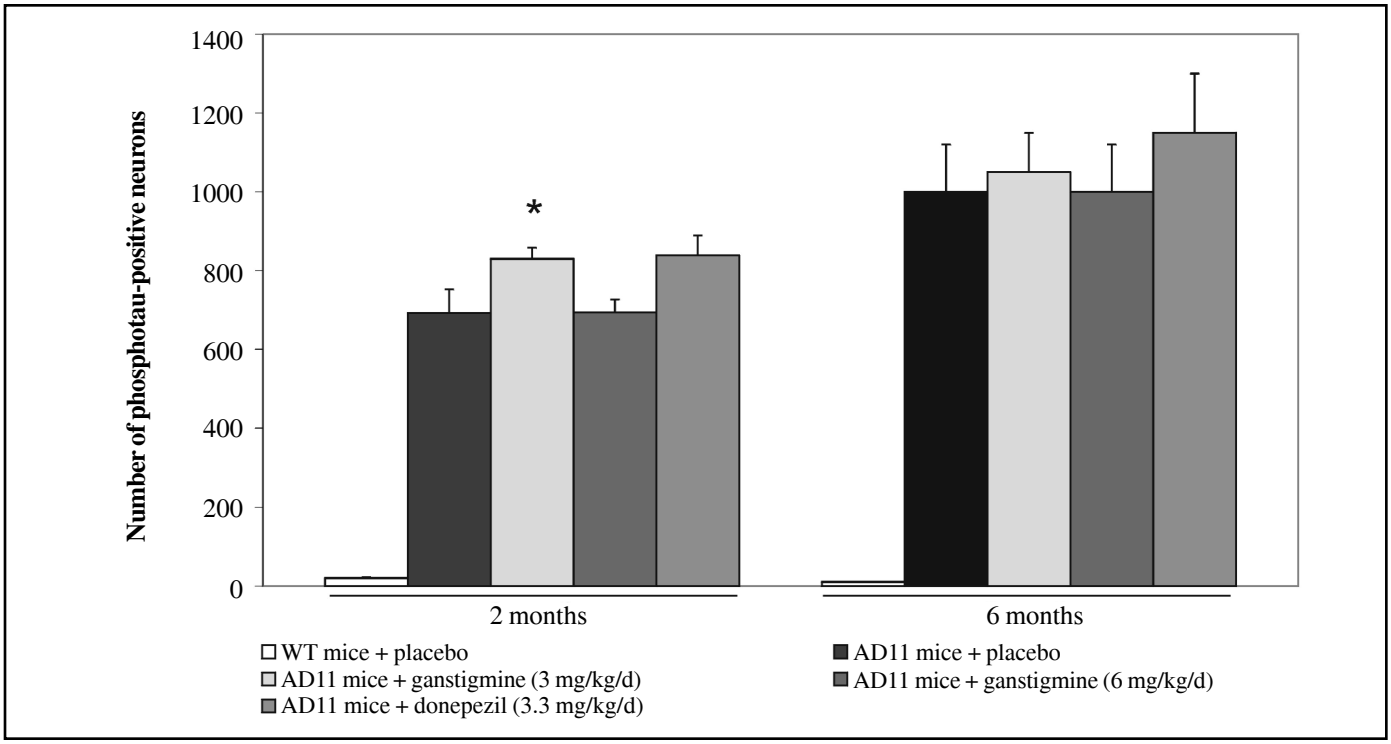

Figure 3. Total number of phosphotau-positive neurons in layer II of the lateral entorhinal cortex in wild type mice, AD11 mice, and AD11 mice treated with ganstigmine and donepezil (* $p < 0.05$ vs AD11 mice treated with placebo).

syndromes. Similar tasks have been developed in nonhuman primates and rats using delayed nonmatching-to-sample or delayed matching-to-sample paradigms.[15] However, these tasks require: 1) the introduction of constraints, such as food deprivation, to motivate subjects to perform the task; and 2) long learning sessions that do not allow analyzing impairments related to recent memory, which are the predominant memory deficits in AD.[16] To overcome these constraints, Ennaceur and Delacour[17] developed an object recognition test based on the spontaneous tendency of rodents to explore a new object more than a familiar one.[17] The task is divided in two trials in which, in the first trial, rodents are placed in an open field in the presence of two identical objects, while in the second trial, they are allowed to explore a familiar object and a new one. Recognition memory is assessed by comparing the time spent in exploring each object during this second trial.

The object recognition test was performed in six-month-old AD11 mice only, since the behavioral deficit is not present in AD11 mice at earlier ages (Berardi et al., unpublished data). The apparatus consisted of a square arena (60 cm × 60 cm × 30 cm) constructed of PVC with black walls and white floor. The objects were cubes (12 cm wide) made of transparent Plexiglas® that contained the visual patterns to be discriminated. In this way, the experimenter could clean the cubes' surface to avoid olfactory cues.

The pattern recognition test was composed of three phases. In the first (habituation phase), the mice were placed in the empty arena for five minutes to become familiar with the apparatus. After two minutes, the sample phase started: two cubes with white visual patterns were presented in two opposite corners of the arena. The mice were left to explore the cubes in the arena for five minutes.

The choice phase was executed at 1 hour and 24 hours. In this phase, one of the patterns was replaced by a new visual pattern (for example black rows, black circles), and the mice were allowed to explore the cubes for an additional five minutes.

The standard measure for the statistic analysis was the time spent exploring the two objects. The exploration of an object was defined as directing the nose to the object at a distance of less than 2 cm and touching it with the nose. Turning around, climbing over, or sitting on the object was not included. In the sample phase, if the exploration time was less than three seconds, the mouse was left out. Trials were excluded if a mouse spends less than one second exploring both new and familiar objects in the test phase. Measurements were based on exploration and discrimination. In the sample phase, the total time spent exploring each object was recorded. The discrimination index was calculated as the difference between the time spent exploring new and old objects divided by the total time spend exploring the object (N-F/F+N). Analysis of variance (ANOVA) was used for statistical analysis.

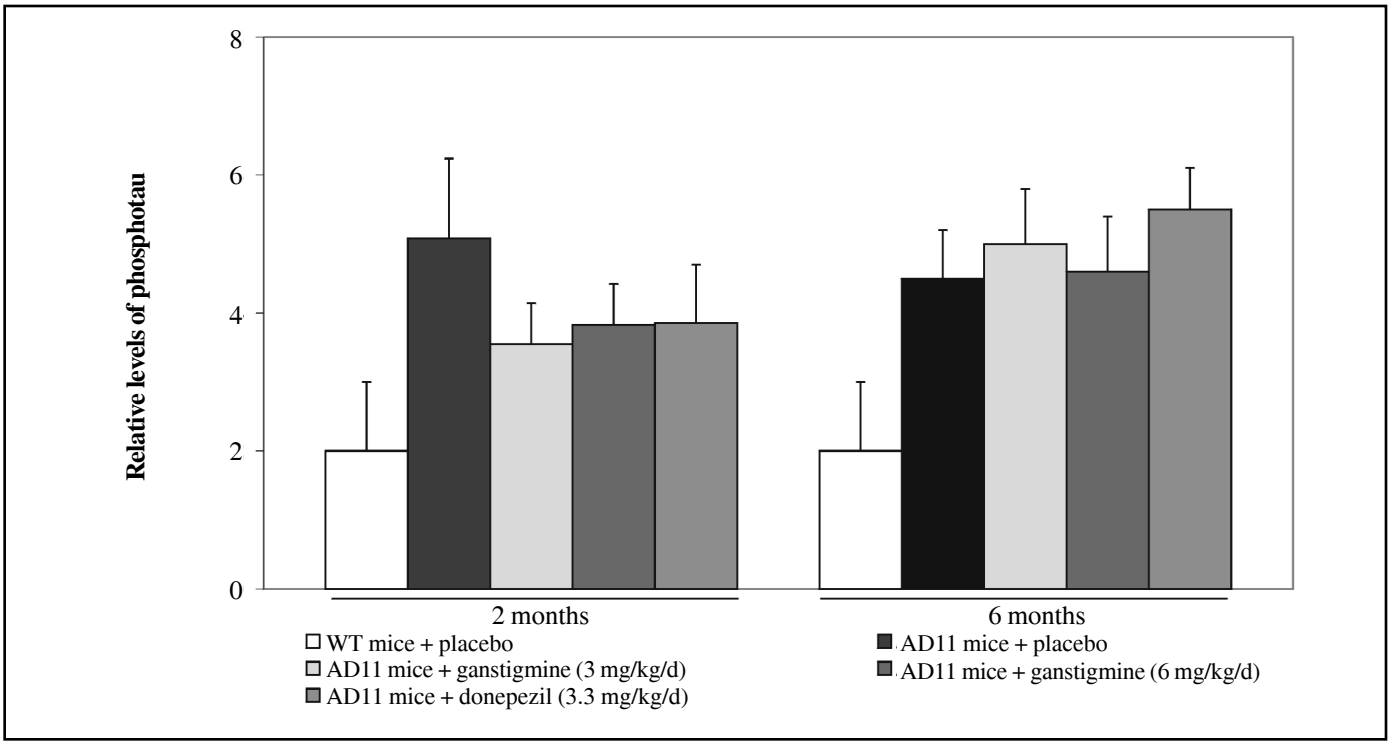

Figure 4. Relative levels of phosphorylated tau in the entorhinal cortex in wild type mice, AD11 mice and AD11 mice treated with ganstigmine and donepezil (* $p < 0.05$ vs AD11 mice treated with placebo).

Results

Mortality and effect on body weight

The administration of ganstigmine to AD11 mice did not result in increased lethality in two-month-old mice, while two mice from the group treated with 6 mg/kg/d of ganstigmine from two to six months of age died during treatment. The administration of donepezil resulted in the death of two AD11 mice in the group treated between 1.5 to 2 months of age. No differences in body weight were recorded in mice that completed the treatment.

Rescue of cholinergic loss in the basal forebrain by administration of ganstigmine and donepezil

The rescue of cholinergic loss in the basal forebrain was assessed by neurostereologic methods after immunohistochemistry using antibodies against ChAT. At two months of age, ganstigmine induced the rescue of the loss of cholinergic neurons at both dosages used during treatment ($p < 0.05$). At the same age, donepezil failed to increase the number of cholinergic neurons ($p > 0.05$). In six-month-old AD11 mice, both ganstigmine and donepezil ameliorated the cholinergic deficit in the basal forebrain (Figures 1 and 2).

Lack of effect on phosphorylated tau after ganstigmine and donepezil administration

In two-month-old AD11 mice, ganstigmine (3 mg/kg/d) increased the number of cells labeled by antiphosphotau antibodies in the entorhinal cortex ($p < 0.05$) (Figure 3). No statistical difference was shown between AD11 mice treated with placebo and those treated

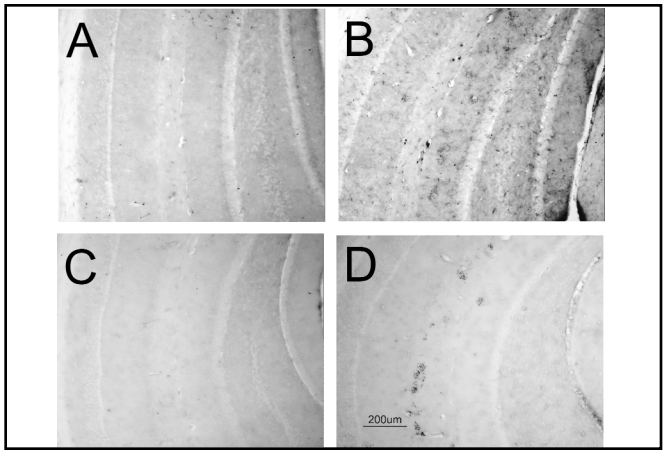

Figure 5. Expression of PS1 in six-month-old (A) wild type and (B) AD11 mice. The clusters express also BACE1, (C) six-month-old wild type and (D) AD11 mice.

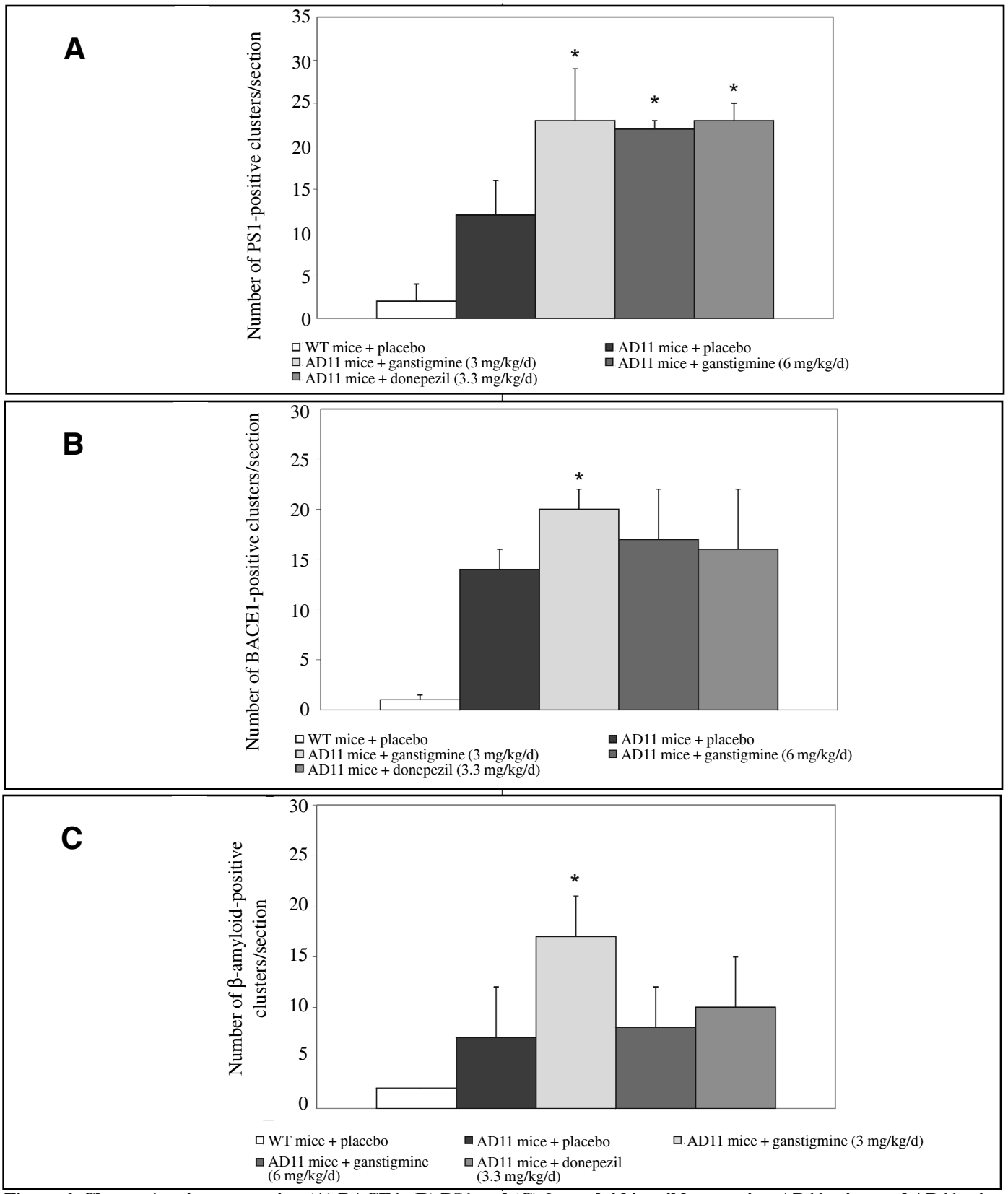

Figure 6. Clusters/section expressing (A) BACE1, (B) PS1 and (C) ß-amyloid in wild type mice, AD11 mice, and AD11 mice treated with ganstigmine and donepezil (* $p < 0.05$ vs AD11 mice treated with placebo).

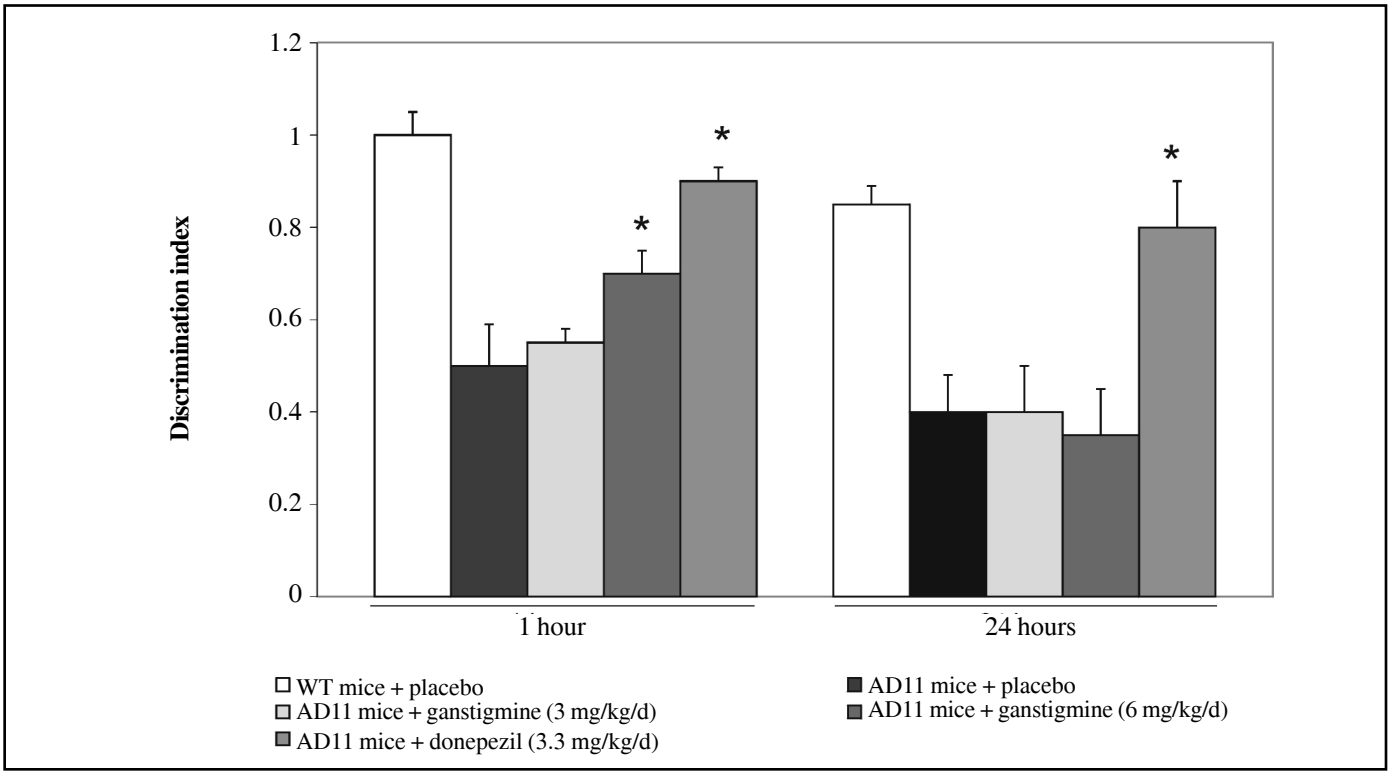

Figure 7. Object recognition test after treatment with ganstigmine and donepezil (* p < 0.05 vs AD11 mice treated with placebo).

with ganstigmine (6 mg/kg/d) or donepezil ($p > 0.05$). However, the increase in the number of phosphotau-positive neurons did not correspond to an increase in the levels of phosphotau as determined by western blot (Figure 4).

Immunohistochemistry and neurostereologic analysis performed on brain sections from mice treated from two to six months of age showed that ganstigmine and donepezil had no effect on the number of cells expressing phosphorylated tau in the entorhinal cortex ($p > 0.05$) (Figure 3). Biochemical analysis confirmed these data ($p > 0.05$) (Figure 4).

Lack of effect on BACE1, PS1, and ß-amyloid expression

At six months of age, expression of ß-amyloid is absent in wild type mice but starts appearing in AD11 mice in the form of clusters located primarily in the hippocampus.[8,14] The expression of ß-amyloid is associated with an increased expression of PS1 (Figures 5A and B) and BACE1 (Figures 5C and D). The administration of 3 mg/kg/d ganstigmine determined an increase in the number of clusters expressing PS1, BACE1, and ß-amyloid (Figures 6A-C). Ganstigmine (6 mg/kg/d) and donepezil increased the number of PS1-positive clusters only; it did not affect the number of clusters expressing BACE1 and ß-amyloid (Figures 6A-C).

Oral administration of ganstigmine and donepezil rescue behavioral deficit in AD11 mice

At the behavioral level, AD11 mice showed an impairment of their ability to discriminate new objects from familiar ones (Figure 7). The deficit was assessed at 1 hour and 24 hours (choice phases) after the exposure to two identical objects (sample phase). This deficit was prevented by the administration of 6 mg/kg/d of ganstigmine when the choice phase was performed 1 hour after the sample phase. However, no treatment effects were observed 24 hours after the end of the sample phase. Treatment with ganstigmine 3 mg/kg/d did not affect the behavioral deficit. The administration of 3.3 mg/kg/d of donepezil prevented the deficit both at 1 and 24 hours after the end of the sample phase (Figure 7).

Discussion

A cascade of pathophysiological events generated by AD ultimately leads to cellular and organization dysfunction, failure of neurotransmission, altered protein processing, and cell death. Each stage of this cascade affords the possibility for therapeutic intervention. Cholinesterase inhibitors are the most widely used available treatments for patients with mild to moderate AD, helping to maintain functional abilities and delaying decline in cognitive function.[18] Whether or not

these agents also affect the endpoints of the disease, in addition to ameliorating symptoms, remains to be determined.

For this reason, the use of animal models is of particular value in revealing a comprehensive pathological phenotype and testing the effects of therapeutics on the different hallmarks of the disease. In this study, it is particularly significant that AD11 mice develop a progressive neurodegeneration that is highly reminiscent of AD,[7-10] which allowed testing of the effects of ganstigmine and donepezil on different AD endpoints.

In this study, we assessed the ability of pharmacological treatments to reverse the early phases of the progressive neurodegenerative phenotype in AD11 mice prior to the onset of advanced neurodegeneration observed in aged mice. Ganstigmine exerts a neuroprotective effect against loss of cholinergic neurons in the basal forebrain and against deficits of recognition memory as assessed using the object recognition test. Similar results were obtained, although with some differences, with donepezil. Indeed, donepezil was able to rescue the cholinergic deficit only after a prolonged administration, while ganstigmine increased the number of cholinergic neurons after only a short period of administration. Conversely, the prolonged administration of donepezil improved memory retention longer than ganstigmine as assessed using the object recognition test.

The rescue of cholinergic neurons was observed before in AD11 mice using another AChEI, galantamine, which has a dual mechanism of action.[14] The analysis of the data obtained on ß-amyloid intracellular accumulation showed different mechanisms of action between the three AChEI. Galantamine was able to completely rescue the cholinergic deficit as well as the intracellular deposition of ß-amyloid.[14] On the contrary, both ganstigmine and donepezil were ineffective in reducing ß-amyloid accumulation. Indeed, while ganstigmine and donepezil act mainly as AChEI, galantamine also increases the activity of nicotinic receptors. The greater potency of ganstigmine versus donepezil in rescuing ChAT expression in basal forebrain neurons could be the result of the additional pharmacological activity (besides that of AChEI) shown by ganstigmine, as has been suggested by other studies.[5] Indeed, ChAT expression read-out is not necessarily directly related to AChE inhibition but more to an effect on the cholinergic neuron per se.

Thus, results of this study helped illustrate the mechanisms behind anti-NGF induced neurodegeneration, confirming that the accumulation of phosphotau and ß-amyloid do not derive from the same mechanisms, as has been suggested. Moreover, the use of AChEIs with different mechanisms of action highlighted that the cascade of events leading to ß-amyloid deposition is different from that preceding neuronal loss in the basal forebrain.

In conclusion, our results show that the complex neurodegeneration in AD11 mice might be due to multiple, concomitant processes. These processes can be partially reversed by the administration of AChEI and, therefore, a combined treatment should be designed to rescue all the aspects of AD neurodegeneration. AD11 mice represent an invaluable experimental tool for studying new potential therapies that target multiple aspects of the illness cascade.

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