JPET Fast Forward. Published on September 6, 2005 as DOI: 10.1124/jpet.105.090365

JPET #90365

Unequal neuroprotection afforded by the acetylcholinesterase inhibitors galantamine, donepezil and rivastigmine in SH-SY5Y neuroblastoma cells: role of nicotinic receptors

Esperanza Arias, Sonia Gallego-Sandín, Mercedes Villarroya, Antonio G. García and Manuela G. López

Instituto Teófilo Hernando, Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain (E.A., S.G-S., M.V., A.G.G., M.G.L.); Servicio de Farmacología Clínica. Hospital de la Princesa Madrid Spain (S.G-S., A.G.G.); and Instituto Universitario de Investigación Gerontológica y Metabólica, Hospital de la Princesa, 28006 Madrid, Spain (A.G.G., M.G.L.)

Running title: Neuroprotective effects of acetylcholinesterase inhibitors

Corresponding author: Manuela G. López

Departamento de Farmacología, Facultad de Medicina,

Universidad Autónoma de Madrid

C/ Arzobispo Morcillo 4

E-28029 Madrid. Spain

E-mail: manuela.garcia@uam.es

Phone: +34-914975386

Fax: +34-914975397

Number of text pages: 27

Number of tables: 1

Number of figures: 6

Number of references: 37

Number of words:

- Abstract: 248
- Introduction: 671
- Discussion: 1040

Abbreviations: AChE- Acetylcholinesterase; AChEI- Acetylcholinesterase inhibitor; Aβ – amyloid β; AD – Alzheimer´s disease; DMEM – Dulbecco´s Modified Eagle´s Medium; DMSO – Dimethylsulphoxide; FCS – Fetal calf serum; LDH – lactic dehydrogenase; nAChR - Nicotinic acetylcholine receptor; NEAAs – Non-essential amino-acids; PBS - Phosphate buffer solution; PI – propidium iodide; BuChE - Butyrylcholinesterase

Recommended section assignment: Neuropharmacology

Abstract

Donepezil, rivastigmine and galantamine are three drugs with acetylcholinesterase (AChE) inhibiting activity that are currently being used to treat patients suffering of Alzheimer's disease. We have studied the neuroprotective effects of these drugs, in comparison to nicotine, on cell death caused by β-amyloid (Aβ) and okadaic acid, two models that are relevant to Alzheimer´s pathology, in the human neuroblastoma cell line SH-SY5Y. Galantamine and donepezil showed a U-shaped neuroprotective curve against okadaic acid toxicity; maximum protection was achieved at 0.3 μM galantamine and at 1 μM donepezil; at higher concentrations, protection was diminished. Rivastigmine showed a concentration dependent effect; maximum protection was achieved at 3 μM. When apoptosis was induced by $A\beta_{25-35}$, galantamine, donepezil, and rivastigmine showed maximum protection at the same concentrations: 0.3 μM, 1 μM and 3 μM respectively. Nicotine also afforded protection against Aβ and okadaic acid-induced toxicity. The neuroprotective effects of galantamine, donepezil and nicotine, were reversed by the α7 nicotinic antagonist methyllycaconitine but not by the α4β2 nicotinic antagonist dihydro-β-erithroidine. The PI3K/AKt blocker LY294002 reversed the protective effects of galantamine, donepezil and nicotine but not that of rivastigmine. On the other hand, the bcl-2 antagonist HA 14-1 reversed the protective effects of the three AChE inhibitors and that of nicotine. Our results show that galantamine, donepezil and rivastigmine afford neuroprotection through a mechanism likely unrelated to AChE inhibition. Such neuroprotection seemed to be linked to α7 nicotinic receptors and the PI3K/Akt pathway in the case of galantamine and donepezil, but not for rivastigmine.

INTRODUCTION

Alzheimer´s disease (AD) is a progressive neurodegenerative disease and the most common form of dementia in the elderly population. Clinically, patients with AD show progressive deterioration of all cognitive functions resulting in their incapacitation. AD is characterized by the presence of two kinds of abnormal protein deposits, amyloid plaques and neurofibrillary tangles (NFT) in specific areas of the brain, and finally by the atrophy of the affected brain regions which results from extensive losses of synapses and neurons (Terry et al., 1981 and 1991; Price et al., 1991; Arriagada et al., 1992). Amyloid plaques are extracellular deposits containing β-amyloid peptide (Aβ) as the major core deposits. Aβ is a 39-43 amino acid peptide fragment derived through proteolysis from an integral membrane protein known as Aβ precursor protein (APP). The basis for the β-amyloid hypothesis arises from various studies showing that Aβ is toxic to neurons; for example, there is increased Aβ release and apoptotic cell death in cells that overexpress APP (Recuero et al., 2004). A correlation between memory deficits, Aβ elevation, and amyloid plaques on transgenic mice has also been reported (Billings et al., 2005).

The other major lesion in AD is the intracellular deposition of the microtubule binding protein tau in the form of NFTs, which are mainly constituted by hyperphosphorylated tau protein. It has been suggested that the load of this lesion is more closely linked to dementia characteristic of AD than Aβ plaque burden (Terry, 1998). According to the tau/tangle model, the formation of NFTs correlates positively with the disease severity (Tiraboschi et al., 2004). This is due to the observation that structural modifications of tau such as hyperphosphorylation and aggregation interferes with tau function, which is likely to lead to the neuronal dysfunction that causes AD.

The role of cholinergic neurotransmission in memory processing and storage is the basis of the widely accepted "cholinergic hypothesis". In AD there is a loss of cholinergic neurons in the basal forebrain and of the cholinergic innervation of the cerebral cortex (Perry et al., 1994). In addition, there is a severe loss of nicotinic acetylcholine receptors (nAChRs), which correlates with the severity of the disease at the time of death (Wilcock et al., 2000). During the past two decades, cholinesterase inhibition has become the most widely studied and effective clinical approach to treat the symptoms of AD (Soreq and Seidman, 2001; Lahiri and Farlow, 1996). Four cholinesterase inhibitors (ChEI), tacrine, donepezil, rivastigmine and galantamine have been approved by the FDA (USA) and the EMEA (EU) for treating the symptoms of AD. It is postulated that the most important therapeutic effect of ChEI for AD patients is to stabilize cognitive function, at least over 6 months of the clinical trial (Giacobini, 2003). Interestingly, although all have ChEI activity, they vary from one another. For instance, a recent comparative long-term clinical trial found significant advantages for galantamine in comparison to donepezil, in cognition improvement (Wilcock et al., 2003).

On the other hand, signalling through acetylcholine neuronal nicotinic receptors (nAChRs) is being increasingly recognized as playing an important role in different processes such as neurite outgrowth, synaptic transmission, control and synthesis of neurotrophic factors, and neuroprotection (Donnelly-Roberts and Brioni, 1998; Belluardo et al., 2000 and 2005; Hernandez and Terry, 2005). Recently, several preclinical studies have shown that some of the ChEI used to treat AD present neuroprotective properties; such are the cases of galantamine (Capsoni et al., 2002; Arias et al., 2004; Sobrado et al., 2004; Kihara et al., 2004) and donepezil (Akasofu et al., 2003; Takada et al., 2003). However, these are independent studies that use different cell models and toxic stimuli. Therefore,

we thought it would be interesting to study simultaneously, under the same experimental conditions, the potential neuroprotective effects of the main ChEI used in clinic to treat AD patients in comparison to nicotine, in "*in vitro*" models that could be relevant to the pathogenesis of AD such as Aβ and hyperphosphorylation induced toxicity in a a human neuroblastoma (SH-SY5Y) cell line. The results of such study are presented here.

METHODS

Materials

Galantamine was a kind gift of Janssen Pharmaceutica (Beerse, Belgium) and rivastigmine of Novartis (Switzerland); donepezil was purchased from A&A Pharmachem Inc. (Ottawa, Canada). Nicotine, tacrine, okadaic acid, dihydro-β-erythroidine and methyllycaconitine were purchased from Sigma (Madrid, Spain). LY-294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride) was from Tocris (Bristol, UK). HA 14-1 (Ethyl [2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)]-4H-chromene-3-carboxylate) was from Sigma (Madrid, Spain). Aβ protein was from Calbiochem (Germany).

Culture of SH-SY5Y cells

The neuroblastoma cell line SH-SY5Y was a kind gift of Dr F. Valdivieso from the Centro de Biología Molecular, Madrid, Spain. SH-SY5Y cells, at passages between 3 and 16 after de-freezing, were maintained in a DMEM containing 15 non-essential amino-acids (NEAAs) and supplemented with 10% foetal calf serum (FCS), 1 mM glutamine, 50

JPET Fast Forward. Published on September 6, 2005 as DOI: 10.1124/jpet.105.090365
This article has not been copyedited and formatted. The final version may differ from this version.

units/ml penicillin and 50 μg/ml streptomycin (reagents from GIBCO, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37ºC in 5% CO_2/humidified air. Stock cultures were passaged 1:4 twice weekly. For assays, SH-SY5Y cells were subcultured in 24 well plates at a seeding density of $2x10^5$ cells per well, or in 6 well plates at a seeding density of $5x10^5$ cells per well. Cells were treated with the drugs before confluence in DMEM free of serum. These cells, when undifferentiated, express functional nicotinic receptors (Dajas-Bailador et al., 2002)

Measurement of lactic dehydrogenase (LDH) activity

Extracellular and intracellular LDH activity was spectrophotometrically measured using a Cytotoxicity Cell Death kit (Roche-Boehringer, Mannheim, Germany) according to the manufacturer's indications. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; released LDH was defined as the percentage of extracellular compared to total LDH activity.

N**uclear staining of DNA**

For the detection of apoptotic nuclei, cells were incubated with the dye Hoechst 33342 (5 μg/ml, Vybrant Apoptosis kit, Molecular Probes) for 30 min at 37ºC, in the dark (Arias et al., 2004). Cells were viewed using a Nikon Diaphot inverted epifluorescence microscope with a 40x objective, using the appropriate filters for an excitation wavelength of 355 nm and an emission wavelength of 465 nm. Cells showing condensed or fragmented nuclei (apoptotic cells) were identified from an average of 300 cells per treatment and cell batch. Each individual experiment was repeated in 4 different cell batches; therefore, 1500

to 2100 cells were evaluated per treatment. The samples were examined by blinded counting; 4 samples from different fields were taken from each dish, fields were selected randomly. Data were expressed as percentage of apoptotic cells respect to the total amount of cells counted.

Measurement of apoptosis by flow cytometry

Apoptosis was determined by flow cytometry analysis of the cell cycle after DNA staining with propidium iodide (PI, Molecular Probes) (Robinson et al., 1997). Cells were grown in 6-well plates until they reached 50% confluence (typically after 24-48 h in culture). After treatment, cells that remained attached to plates were harvested in PBS/EDTA (5 mM EDTA in PBS) and collected together with those floating (detached) dead cells. Cells were then centrifuged, the supernatant discarded, and the cell pellet suspended in 0.5 ml PBS by pipetting thoroughly to avoid cell clumping. The cell suspension was transferred to 4.5 ml 70% cold ethanol and kept in this fixative for a minimum of 2 h. Ethanol–fixed cells were centrifuged and washed once with 10 ml PBS. Finally, the cell pellet was suspended in 1 ml of PI/Triton X-100 staining solution (0.1% Triton X-100, 20 μg/ml RNase in PBS) and incubated for 15 min at 37ºC. Samples were analyzed by flow cytometry (FACS-Calibur, Beckton Dickinson). The analysis of the samples included a first selection (gate 1) in which events with appropriate size (forward scatter) and complexity (side scatter) were selected. Then, selected events were analyzed to discard doublets using a PI intensity-width versus PI intensity-area dot plot (gate 2). Finally, events (cells) that were contained in gates 1 and 2 were plotted in a histogram representing the number of events (cells) containing a specific PI intensity-area (e.g.

specific amount of DNA). Apoptosis was measured as the percentage of cells with a sub G0/G1 DNA content in the PI intensity-area histogram plot.

Statistical analysis

Statistically significant differences between groups were determined by an ANOVA followed by a Newman-Keuls *post hoc* analysis. The level of statistical significance was taken at $p<0.05$.

RESULTS

Effect of increasing concentrations of galantamine, donepezil and rivastigmine on cell death induced by okadaic acid.

Okadaic acid is a toxin produced by marine algae that blocks protein phosphatases (PP) with an inhibitory potency of PP2A>PP1>PP2B; it induces hyperphosphorylation of tau and NFTs in different "*in vivo*" (Tian et al., 2004) and "*in vitro*" (Uberti et al., 1997) models. Thus, it is accepted that okadaic acid-induced toxicity is a good model for the neuronal death occurring in Alzheimer's disease and that is linked to tau hyperphosphorylation. Under our experimental conditions, 30 nM okadaic acid for 24 h increased cell death measured as LDH released to the extracellular medium, from 7.7±0.25% to 30.9±0.85% (n=4) in SH-SY5Y cells.

In parallel experiments, we evaluated the effects of the ChEIs galantamine, donepezil and rivastigmine at the concentrations of 0.1, 0.3, 1 and 3 μM on the okadaic acid-induced cell death. The drugs were preincubated for 24 h prior to and during the toxic stimuli (another 24 h). Galantamine afforded significant protection at 0.1, 0.3 and 1 μM,

although maximum protection was observed at 0.3 μM (Fig 1A). Donepezil afforded maximum protection at 1 μM although significant protection was also observed at 0.3 and 3 μM (Fig 1B). Curiously, both galantamine and donepezil presented a U-shape protective curve. In the case of rivastigmine, protection was milder, but it presented a concentration-response pattern, being significant at 1 and 3 μM (Fig. 1C).

Antiapoptotic effect of galantamine, donepezil, rivastigmine and nicotine on $A\beta_{25–35}$-induced toxicity

We used the toxic fragment of the Aβ which corresponds to the fragment 25-35 and we determined the fraction of cells suffering apoptosis by analysing the cell cycle in propidium-iodide stained cells by flow cytometry. Basal apoptotic cell death was 7.04±1.08% and rose to 17.94±2.40% in cells treated for 24 h with 10 μM $A\beta_{25-35}$ (n=5). For these studies we used two concentrations of each compound. Galantamine (0.3 μM) significantly reduced apoptosis to 10.26±1.64% ($p<0.001$; n=5), donepezil (1 μM) to 11.43±2.02% ($p<0.001$; n=5), rivastigmine (3 μM) to 13.23±1.12% ($p<0.01$; n=5) and nicotine (30 μM) to 9.88±2.12% ($p<0.001$; n=5) (see Fig. 2A). Tacrine at (0.3-3 μM) had no effect. For further studies we selected those concentrations that afforded maximum protection against okadaic acid and Aβ-induced toxicity i.e. 0.3 μM galantamine, 1 μM donepezil and 3 μM rivastigmine.

The antiapoptotic effect of these drugs was also confirmed by counting the number of apoptotic nuclei in cells stained with the fluorescent dye Hoechst 33342 (see Fig. 2B). Aβ25-35 increased the number of apoptotic nuclei to 22+2 from basal levels of 3+1.5; galantamine (0.3 μM) significantly reduced Aβ25-35-induced apoptosis to 11+2,3%,

donepezil (1 μM) to 9+1.7%, rivastigmine (3 μM) to 13+1.9% and nicotine (30 μM) to 9+1.3%.

Fig. 3 shows microphotographs of SH-SY5Y cells in basal conditions (panel A) and after 24 h exposure to $A\beta_{25-35}$ (10 μM) (panel B). Note the loss of birefringency of cells treated with Aβ, the loss of cells and the decrease of neuritis. Note also that galantamine (0.3 μM), donepezil (1 μM), rivastigmine (3 μM) and nicotine (30 μM) preserved the healthy appearance of the cells (panels C, D, E and F).

Implication of the nAChR in the protective effects of the different ChEIs and nicotine

To establish whether the neuroprotective actions of galantamine, donepezil and rivastigmine were mediated by nAChRs, we used dihydro-β-erythroidine (DHβE) to block α4β2 nAChRs and methyllycaconitine (MLA) to block the α7 nAChR. The protective effects of galantamine, donepezil and nicotine were reversed by MLA (10nM) but not by DHβE (Fig. 4). In the case of rivastigmine, protection remained unaffected in the presence of either of the nAChR antagonists used.

Also, the non specific nAChR antagonist mecamylamine reversed the protective effects of galantamine, donepezil and nicotine against $A\beta_{25-35}$-induced toxicity (data not shown).

Pre-treatment of the drugs and induction of protein synthesis is required for the neuroprotective effect of galantamine, donepezil and rivastigmine.

In order to determine whether the neuroprotective effect of the different AChEIs needs pre-incubation before the toxic agent is added to the cells, we performed experiments

where the protective drugs were co-applied with the toxic agent, or they were pre-incubated 24 h prior to the toxic agent. As shown in figure 5A, the different AChEIs, as nicotine, needed a pre-incubation period to afford neuroprotection; if they were co-applied with the toxic stimuli, protection was not observed.

Protein synthesis seems to be involved in the neuroprotective action of these compounds since cycloheximide, a protein synthesis inhibitor, prevented the neuroprotective action of the different AChEIs used in this study (Fig. 5B). A similar pattern was observed with nicotine.

Implication of the PI3K-Akt signalling pathway and bcl-2 on the neuroprotective actions of galantamine, donepezil, rivastigmine and nicotine

Nicotine´s neuroprotective mechanism has been related to the PI3K-Akt signaling pathway (Kihara et al., 2001). We have therefore used the antagonist of PI3K, LY294002 in order to determine if it can prevent the protective effects of the different AChEIs against $A\beta_{25-35}$-induced toxicity. The neuroprotective effect of galantamine, donepezil and nicotine was abolished by LY294002 but not that of rivastigmine (Fig. 6A).

Evidence indicating that the protective actions of all drugs tested in this study (galantamine, donepezil, rivastigmine and nicotine) are related to upregulation of bcl-2, are shown in the experiments of figure 6B, where HA 14-1, a drug that blocks the antiapoptotic action of bcl-2 (Wang et al., 2000), prevented the protective effect of these drugs. HA 14-1 alone did not significantly modify basal cell death.

DISCUSSION

We have found some analogies but also some differences in the extent of neuroprotection as well as in the mechanism involved, between galantamine, donepezil, rivastigmine and tacrine. In our model, donepezil afforded maximum protection, followed by galantamine and rivastigmine; tacrine had no cytoprotective effects.

Maximum protection afforded by galantamine, donepezil and rivastigmine was achieved at the concentrations of 0.3, 1 and 3 μM, respectively. Galantamine and donepezil showed a characteristic U-shape neuroprotective curve; the loss of protection at high concentrations could be explained as blockade of the nAChR (Di Angelantonio et al., 2004; Schattenholz et al., 1996), therefore, the survival signalling cascade can not be activated. The protective effect of these drugs was obtained at concentrations that differ from their IC_{50} to block AChE (see table 1). It therefore seems that the neuroprotective effects are not directly related to their capacity to block the enzyme. For example, tacrine, a potent blocker of AChE, did not afford protection. Also, physostigmine, a classical and potent AChE blocker, did not protect rat cortical neurons exposed to glutamate (Takada et al., 2003). During the last years, speculations have appeared on the link between the inhibition of AChE and neuroprotection. It seems that inhibition of a peripheral site of AChE may be related to neuroprotection (Dorronsoro et al., 2005); this is likely due to the fact that this peripheral site might be involved in the formation and deposit of β-amyloid in the brain. Considering this hypothesis, perhaps the interaction with the peripheral site correlates better with the neuroprotective effects of these drugs than with its interaction with the active site of the enzyme; however, this still remains to be proven.

Some relationship between nicotinic receptors and neuroprotection seems to be present at least for galantamine and donepezil, whose neuroprotective effects were reversed by MLA, an α7 selective nicotinic receptor blocker and by mecamylamine, a non-selective nicotinic receptor blocker. These findings agree with recent studies showing an α7 nicotinic receptor-mediated cytoprotective effect of galantamine against thapsigargin-induced cell death in chromaffin cells and SH-SY5Y cells (Arias et al., 2004) and an α7-mediated effect for donepezil against glutamate elicited toxicity in rat cortical neurons (Takada et al., 2003). In contrast to galantamine and donepezil, the cytoprotective action of rivastigmine did not seem to be linked to nicotinic receptors: neither MLA, dihydro-β-erythroidine nor mecamylamine reversed its effects.

Another interesting difference rests in the intracellular messenger pathways involved in the neuroprotective effects of the three agents. Activation of the PI3K/AKt pathway via α7 nAChRs and increase in bcl-2 expression has been described as the neuroprotective cascade for nicotine (Kihara et al., 2001); this cascade also seems to be targeted by galantamine and donepezil. Thus, galantamine induced the expression of the antiapoptotic protein bcl-2, corroborating a previous observation of our laboratory in bovine chromaffin cells and SH-SY5Y neuroblastoma cells (Arias et al., 2004). It has been documented that the induction of bcl-2, for instance in stably transfected PC12 cells, leads to enhanced cell resistance to toxic stimuli (Dispersyn et al., 1999). The cytoprotective effects of bcl-2 may be due to its ability to modify the cell's Ca^{2+} homeostasis, particularly by reducing the depletion of Ca^{2+} from its endoplasmic reticulum store (Pinton et al., 2000 and 2001). Whatever the ultimate mechanism is, what seems clear is that the induction of bcl-2 expression by galantamine is linked to the PI3K-AKt pathway; thus, interruption of

this pathway with the selective inhibitor LY294002 reversed the neuroprotective effects of galantamine. This agrees with a recent observation that galantamine induced phosphorylation of AKt at the time that it offered neuroprotection in rat cortical neurons (Kihara et al., 2004).

The neuroprotective mechanism of donepezil appears to be rather similar to that of galantamine. Neuroprotection afforded by donepezil was also prevented by MLA, HA 14-1 and LY294002 indicating that α7 nAChRs, activation of the PI3K-AKt pathway and overexpression of bcl-2 are implicated in its neuroprotective mechanism. This was not the case for rivastigmine; although it was able of inducing the antiapoptotic protein Bcl-2 like galantamine and donepezil, its protective effect was not reversed by nicotinic antagonists or the blocker of the PI3K/AKt.

There are several recent studies in the literature showing the neuroprotective actions of different AChEIs, although the results are rather controversial. Thus, in rat cortical neurons subjected to glutamate neurotoxcicity, galantamine and donepezil afforded neuroprotection (Takada et al., 2003); this agrees with our results. However, these authors found that tacrine had neuroprotective effects although we did not find this neuroprotection. On the other hand, donepezil afforded neuroprotection in rat cortical neurons exposed to oxygen and glucose deprivation, while galantamine did not (Akasofu et al., 2003). However, in a recent study from our group we have found that galantamine exhibited clear neuroprotective effects in rat hippocampal slices subjected to glucose and oxygen deprivation (Sobrado et al., 2004). The different cell types and/or cytotoxic models used in each study may explain the distinct results found in the various studies.

Differences in the extent of neuroprotection and/or in the mechanism involved may have clinical relevance. However, very few and not conclusive comparative clinical trials have been performed with the different AChEIs currently used to treat Alzheimer´s disease. The study by Aguglia et al. (2004) is the first to compare the effects of donepezil, rivastigmine and galantamine on the MMSE, ADAS-cog, IADL and ADL; however limitations of the study included its small population size, its open-label design, and the fact that patients were randomised only after the introduction of galantamine. The results of this study showed no statistical significant differences between the three drugs at 3 months, although numerical trends were observed suggesting the effect of rivastigmine > donepezil > galantamine. There is a long term clinical study published, but it compares galantamine and donepezil but not rivastigmine, in patients suffering AD; this study showed significant advantages for the treatment response to galantamine, versus donepezil, on cognition, measured by response rates on the MMSE and ADAS-cog (Wilcock et al., 2003). Therefore, there is still little information on the comparative effects of these drugs in AD patients.

In conclusion, the results of this study show that all the AChEI currently used in the clinic for AD can provide different degrees of neuroprotection in cytotoxic models that can be relevant to AD pathology.

References

Aguglia E, Onor ML, Saina M, Maso E. (2004) An open-label, comparative study of rivastigmine, donepezil and galantamine in a real-world setting. *Curr Med Res Opin.* 20: 1747-1752.

Akasofu S, Kosasa T, Kimura M and Kubota A (2003) Protective effect of donepezil in a primary culture of rat cortical neurons exposed to oxygen-glucose deprivation. *Eur J Pharmacol* 472:57-63.

Arias E, Ales E, Gabilan NH, Cano-Abad MF, Villarroya M, Garcia AG and Lopez MG (2004) Galantamine prevents apoptosis induced by beta-amyloid and thapsigargin: involvement of nicotinic acetylcholine receptors. *Neuropharmacology* 46:103-114.

Arriagada PV, Growdon HH, Hedley-White ET and Hyman BT (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology* 42:633-639.

Belluardo N, Mudo G, Blum M and Fuxe K (2000) Central nicotinic receptors, neurotrophic factor and neuroprotection. *Behavior Brain Research* 113:21-34.

Belluardo N, Olsson PA, Mudo´G, Sommer WH, Amato G and Fuxe K (2005) Transcription factor gene expression profiling after acute intermittent nicotine treatment in the rat cerebral cortex. *Neuroscience*. 133:787-796.

Billings LM, Oddo S, Green KN, McGaugh JL and Laferla FM (2005) Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron* 45:675-688.

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

Dajas-Bailador FA, Mogg AJ and Wonnacott S (2002) Intracellular Ca^{2+} signals evoked by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca^{2+} channels and Ca^{2+} stores. *J Neurochem.* 81:606-614.

Di Angelantonio S, Bernardi G and Mercuri NB (2004) Donepezil modulates nicotinic receptors of substantia nigra dopaminergic neurones. *Br J Pharmacol*. 141:644-652.

Dispersyn, G., Nuydens, R., Connors, R., Borgers, M., and Geerts, H (1999) Bcl-2 protects against FCCP-induced apoptosis and mitochondrial membrane potential depolarization in PC12 cells. *Biochim. Biophys. Acta* 1428:357-371.

Donnelly-Roberts DL and Brioni JD (1998) Preclinical evidence on the neuroprotective effects of nicotinic ligands. In: Arneric SP and Brioni JD Editors *Neuronal Nicotinic Receptors Pharmacology and Therapeutic Opportunities.* Wiley-Liss, Inc., pp. 337–348.

Dorronsoro I, Alonso D, Castro A, del Monte M, Garcia-Palomero E, Martinez A (2005) Synthesis and biological evaluation of tacrine-thiadiazolidinone hybrids as dual acetylcholinesterase inhibitors. *Arch Pharm (Weinheim)* 338:18-23.

Giacobini E (2003) Cholinesterases: new roles in brain function and in Alzheimer's disease. *Neurochem Res* 28:515-522.

Hernandez CM and Terry AV Jr (2005) Repeated nicotine exposure in rats: effects on memory function, cholinergic markers and nerve growth factor. *Neuroscience*. 130: 997-1012.

Kihara T, Shimohama S, Sawada H, Honda K, Nakamizo T, Shibasaki H, Kume T and Akaike A (2001) Alpha7 nicotinic receptor transduces signals PI3 kinase to block Aβ -induced neurotoxicity. *Journal of Biological Chemistry* 276:13541–13546.

Kihara T, Sawada H, Nakamizo T, Kanki R, Yamashita H, Maelicke A and Shimohama S (2004) Galantamine modulates nicotinic receptor and blocks Aβ-enhanced glutamate toxicity. *Biochem Biophys Res Commun* 325:976-982.

Lahiri DK and Farlow MR (1996) Differential effect of tacrine and physostigmine on the secretion of the beta-amyloid precursor protein in cell lines. *J Mol Neurosci* 7:41-49.

Perry EK, Haroutunian V, Davis KL, Levy R, Lantos P, Eagger S, Honavar M, Dean A, Griffiths M and McKeith I.G (1994) Neocortical cholinergic activities differentiate Lewy body dementia from classical Alzheimer's disease. *Neuroreport* 5:747-749.

Pinton P, Ferrari D, Magalhaes P, Schulze-Osthoff K, Di Vigilio F, Pozzan T, Rizzuto R (2000) Reduced loading of intracellular Ca^{2+} stores and downrelgulation of capacitative Ca^{2+} influx in Bcl-2 overexpressing cells. *J Cell Biol* 148:857-862.

Pinton P, Ferrari D, Rapizzi E, Di Vigilio F, Pozzan T, Rizzuto R (2001) The Ca^{2+} concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance of the molecular mechanism of Bcl-2 action. *EMBO J* 20:2690-2701.

Price JL, Davis PB, Morris JC and White DL (1991) The distribution of tangles, plaques and related immunohistochemical markers in healthy aging and Alzheimer's disease. *Neurobiol Aging* 12:295–312.

Recuero M, Serrano E, Bullido MJ and Valdivieso F (2004) Abeta production as consequence of cellular death of a human neuroblastoma overexpressing APP. *FEBS Lett* 570:114-118.

Robinson JP, Darzynkiewicz Z, Dean PN, Orfao A, Rabinovitch PS, Stewart CC, Tanke HJ and Weeless LL (1997) *Current Protocols in Cytometry* In: John Wiley & Sons, Inc., New York.

Schrattenholz A, Pereira EF, Roth U, Weber KH, Albuquerque EX and Maelicke A. (1996) Agonist responses of neuronal nicotinic acetylcholine receptors are potentiated by a novel class of allosterically acting ligands. *Mol Pharmacol.* 49:1-6.

Sobrado M, Roda JM, Lopez MG, Egea J and Garcia AG (2004) Galantamine and memantine produce different degrees of neuroprotection in rat hippocampal slices subjected to oxygen-glucose deprivation. *Neurosci Lett* 365:132-136.

Soreq H and Seidman S (2001) Acetylcholinesterase--new roles for an old actor. *Nat Rev Neurosci* 2:294-302. Review. Erratum in: *Nat Rev Neurosci* 2:670.

Takada Y, Yonezawa A, Kume T, Katsuki H, Kaneko S, Sugimoto H and Akaike A (2003) Nicotinic acetylcholine receptor-mediated neuroprotection by donepezil against glutamate neurotoxicity in rat cortical neurons. *J Pharmacol Exp Ther* 306:772-777.

Terry RD, Peck A, De Teresa R, Schechter R and Horowpian DS (1981) Some morphometric aspects of the brain on senile dementia of the Alzheimer type. *Ann Neurol* 10:184–192.

Terry RD, Masliah E, Salmon DP, Butters N, De Teresa R, Hill R, Hansen LA and Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol* 30:572–580.

Terry RD (1998) The cytoskeleton in Alzheimer disease. *J Neural Transm Suppl* 53:141-145.

Tian Q, Lin ZQ, Wang XC, Chen J, Wang Q, Gong CX and Wang JZ (2004) Injection of okadaic acid into the Meynert nucleus basalis of rat brain induces decreased acetylcholine level and spatial memory deficit. *Neuroscience* 126:277-284.

Tiraboschi P, Hansen LA, Thal LJ and Corey-Bloom J (2004) The importance of neuritic plaques and tangles to the development and evolution of AD. *Neurology* 62:1984-1989.

Uberti D, Rizzini C, Spano PF and Memo M (1997) Characterization of tau proteins in human neuroblastoma SH-SY5Y cell line. *Neurosci Lett* 235:149-153.

Wang JL, Liu D, Zhang ZJ, Shan S, Han X, Srinivasula SM, Croce CM, Alnemri ES and Huang Z (2000) Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc Natl Acad Sci USA* 97:7124-7129.

Wilcock GK, Lilienfeld S and Gaens E (2000) Efficacy and safety of galantamine in patients with mild to moderate Alzheimer's disease: multicenter randomised controlled trial. Galantamine International-1 Study Group. *BMJ* 321:1445–1449.

Wilcock G, Howe I, Coles H, Lilienfeld S, Truyen L, Zhu Y, Bullock R and Kershaw P (2003) A long-term comparison of galantamine and donepezil in the treatment of Alzheimer's disease. *Drugs Aging* 20:777-789.

Footnotes

a- **Financial support.** Ministry of Education and Science of Spain, grants No. BFI2003-02722 to AGG and No. SAF2003-04596 to MGL. Fundación La Caixa, CAM, Red CIEN, ISC III. Fundación Teófilo Hernando, Johnson and Johnson and Pfizer.

Meetings where part of the data have been presented. None

b- **Name and Address to whom reprint requests should be made.**

Manuela G. Lopez

Departamento de Farmacología, Facultad de Medicina,

Universidad Autónoma de Madrid

C/ Arzobispo Morcillo 4

28029 Madrid. Spain

e-mail: manuela.garcia@uam.es

Phone: +34-914975386

Fax: +34-914975397

Legends for Figures

Figure 1: Galantamine, donepezil and rivastigmine offer protection against okadaic acid-induced toxicity in the human neuroblastoma cell line SH-SY5Y. Neuroblastoma cells were treated with increasing concentrations (0.1, 0.3, 1 and 3 μM) of galantamine (A, Gal), donepezil (B, Dpz) or rivastigmine (C, Riv) 24 h before and during the 24 h incubation period with the toxic stimuli (30 nM okadaic acid). Cell death was quantified as the fraction of the total cell content of LDH, that was released into the incubation medium, after exposing the cells for 24 h to the okadaic acid treatment. Data are means ± SEM of triplicates of 4 different cell batches. **$p<0.01$ and ***$p<0.001$ in comparison to okadaic acid-lesioned cells in the absence of drug. ### $p<0.001$ comparing basal and okadaic acid-lesioned cells.

Figure 2: Galantamine, donepezil and rivastigmine offer protection against Aβ-induced toxicity in the human neuroblastoma cell line SH-SY5Y. (A) Neuroblastoma cells were treated with galantamine (0.3 and 3 μM), donepezil (0.3 and 1 μM), rivastigmine (1 and 3 μM) or nicotine (30 and 100 μM) for 24 h prior and during the 24 h period of exposure to 10 μM $A\beta_{25-35}$. Apoptosis was quantified by analysing the cell cycle in propidium iodine stained cells in a flow cytometer. **(B)** Neuroblastoma cells were treated with galantamine (0.3 μM), donepezil (1 μM), rivastigmine (3 μM) or nicotine (30 μM) for 24 h prior and during the 24 h period of exposure to 10 μM $A\beta_{25-35}$. Apoptosis was quantified by staining with Hoechst 33342 and counting the cells showing normal or apoptotic nuclei (250-300 cells per dish were counted); cells in apoptosis were expressed as

% of the total number of cells counted in each individual dish (ordinates). Data are means ± SEM of 5 different cell batches. **p<0.01 and ***p<0.001 in comparison to $A\beta_{25-35}$-induced apoptosis in the absence of drug. ### p<0.001 comparing basal and $A\beta_{25-35}$-lesioned cells.

Figure 3: Microphotographs of SH-SY5Y exposed to different treatments. Cells were visualized under a Nikon Eclipse TE300 microscope with an objective 20X. Basal cells were kept for 48 h with DMEM without any treatment and control lesion was obtained in cells treated with $A\beta_{25-35}$ (10 μM) for 24 h. AChEIs and nicotine were incubated at the concentrations were maximum protection was observed for each drug during 24 h; then, $A\beta_{25-35}$ (10 μM) was added for another 24 h in the presence of the neuroprotective drugs.

Figure 4: The neuroprotective effects of galantamine, donepezil and nicotine, but not of rivastigmine were reversed by the α7 nAChR antagonist methyllycaconitine. SH-SY5Y cells were incubated with the different drugs indicated by the horizontal bars at the bottom of the histograms in the absence or presence of 10 nM methyllycaconitine (MLA) or 1 μM dihydro-β-erythroidine (DHβE) for 24 h; then, the cells were maintained with the same treatments but $A\beta_{25-35}$ (10 μM) was added to induce cell toxicity for 24 h. Cell viability was quantified measuring the % of LDH released to the extracellular media. The data correspond to the mean ± SEM of triplicates of 5 different batches of cells. ***p<0.001, when comparing cells treated with or without the antagonist. NS= non significant.

Figure 5: The protective effects of the different AChEIs required pre-treatment and the *de novo* synthesis of proteins. (A) The neuroprotective effects of 0.3 μM galantamine (Gal), 1 μM donepezil (Dpz), 3 μM rivastigmine (Riv) and 30 μM nicotine (Nic) were time dependent; 24 h incubation with the drugs was required to observe protection. Data correspond to the mean ± SEM of triplicates of 5 different batches of cells *** $p<0.001$ when comparing pretreated for 24 h (Ptt) or co-application of the drugs with the toxic stimuli (Coap). (B) The presence of 1 μM of the protein synthesis inhibitor cycloheximide (CHX), throughout the experiment, reversed the protective effects of the compounds. Data correspond to the mean ± SEM of triplicates of 4 different batches of cells. *** $p < 0.001$ when comparing cells treated or untreated with CHX.

Figure 6: Implication of the PI3K/AKt pathway and bcl-2 in the protective effects of galantamine, donepezil, rivastigmine and nicotine. (A) The PI3K/AKt blocker LY 294002 (30 μM) reversed the protection of galantamine, donepezil and nicotine but not that of rivastigmine. Data correspond to the mean ± SEM of triplicates of 4 different batches of cells. **$p<0.01$ and ***$p<0.001$ when comparing cells treated with or without LY 294002. (B) HA 14-1 (30 μM) reversed the protection of galantamine, donepezil, rivastigmine and nicotine. Data correspond to the mean ± SEM of triplicates of 4 different batches of cells. **$p<0.01$ and ***$p<0.001$ comparing cells treated with or without the bcl-2 antagonist, HA 14-1.

JPET Fast Forward. Published on September 6, 2005 as DOI: 10.1124/jpet.105.090365
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: IC_{50} values to block AChase and BuChase in nM (taken from Greig et al., 2003)

Drug	**AChase**	**BuChase**	**Selectivity**
Tacrine	190 ± 40	47 ± 10	4 x BuChE
Donepezil	22 ± 38	4150 ± 1700	188 x AChE
Galantamine	800 ± 60	7300 ± 830	9 x AChE
Rivastigmine	4150 ± 160	37 ± 5	122 x BuChE

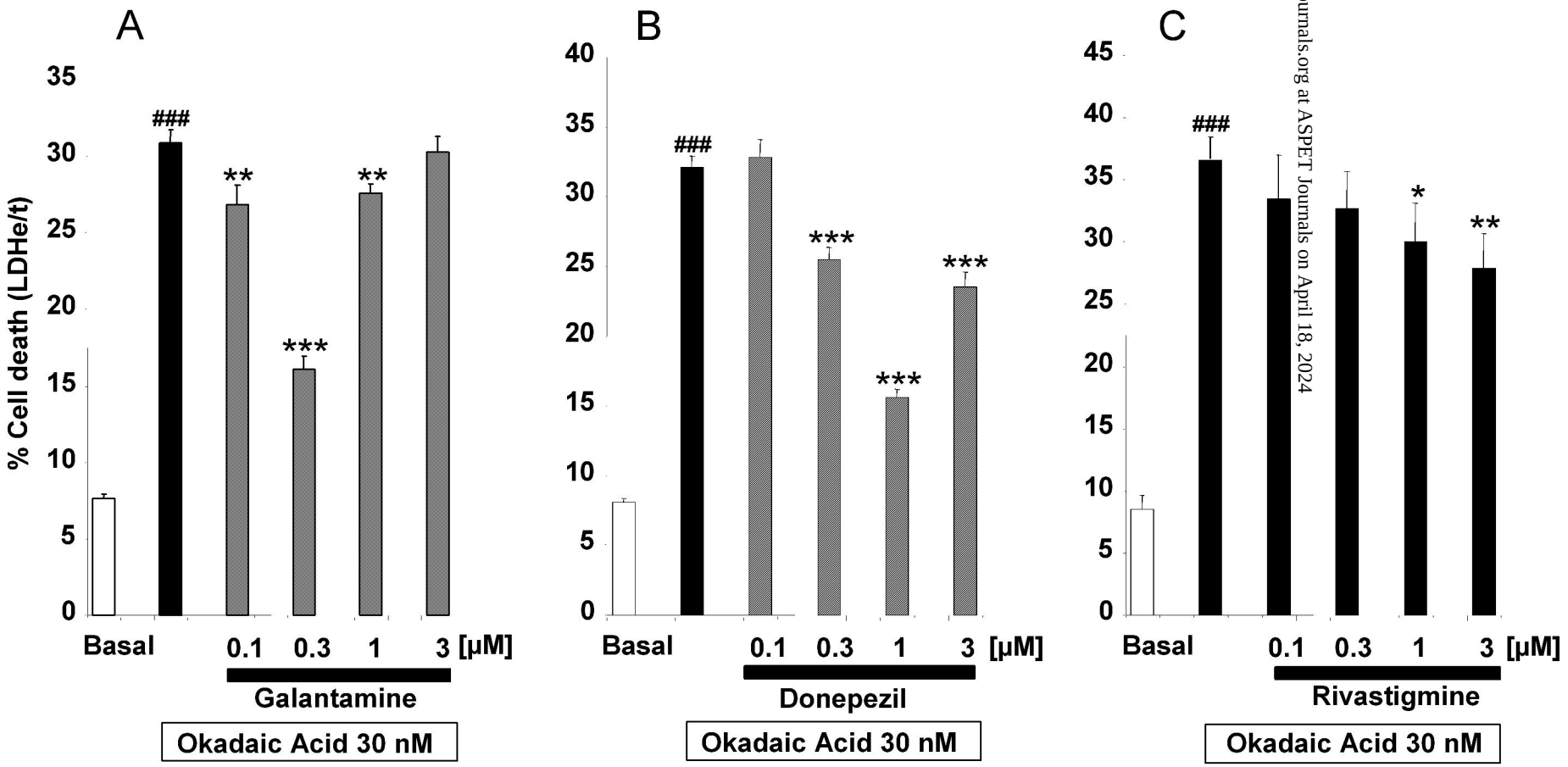


Figure 1

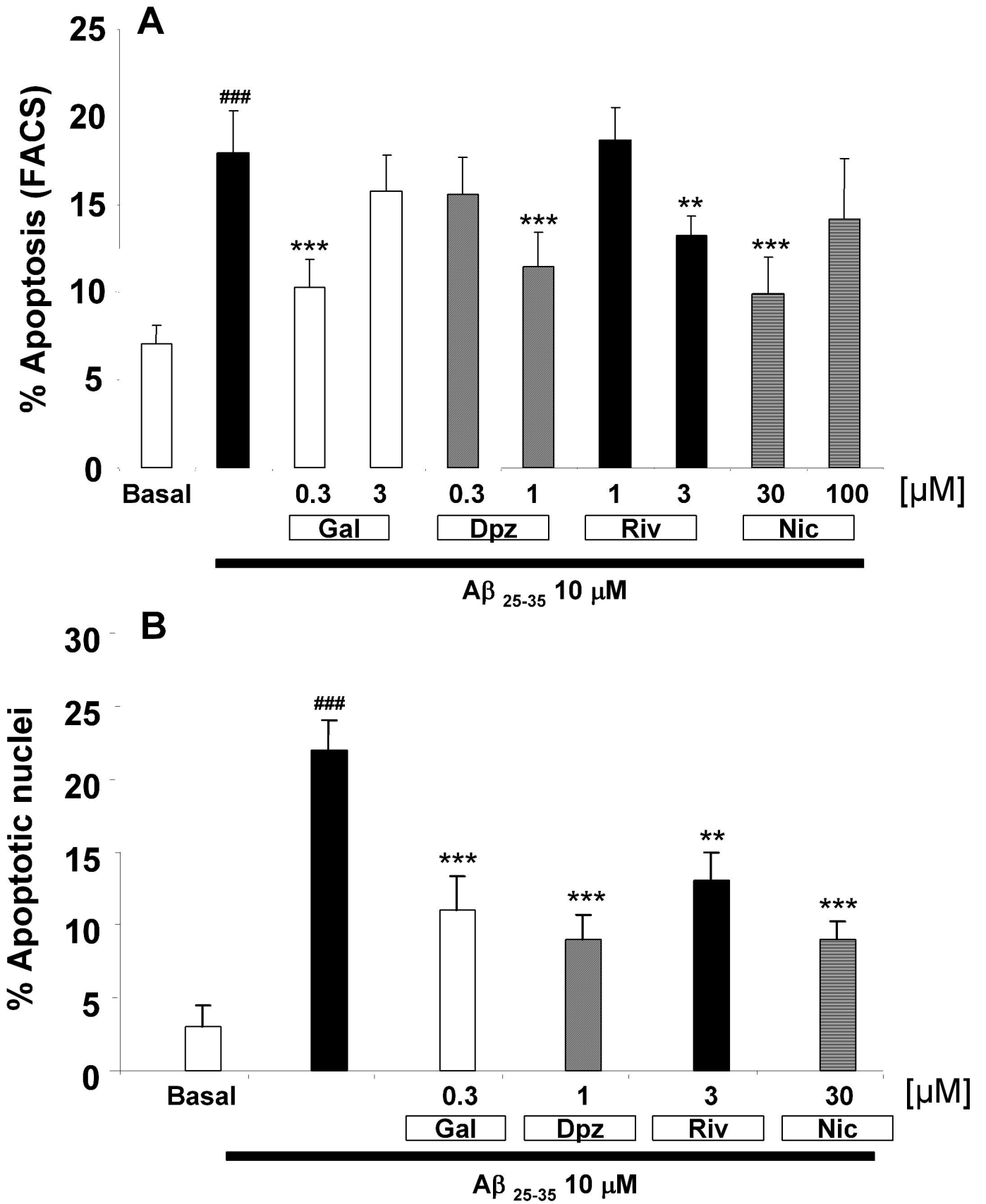

A
% Apoptosis (FACS)
25
20
15
10
5
0
###
***
***
**
***
Basal
0.3
3
0.3
1
1
3
30
100
[μM]
Gal
Dpz
Riv
Nic
Aβ 25-35 10 μM
B
% Apoptotic nuclei
30
25
20
15
10
5
0
###
***
***
**
***
Basal
0.3
1
3
30
[μM]
Gal
Dpz
Riv
Nic
Aβ 25-35 10 μM

Figure 2

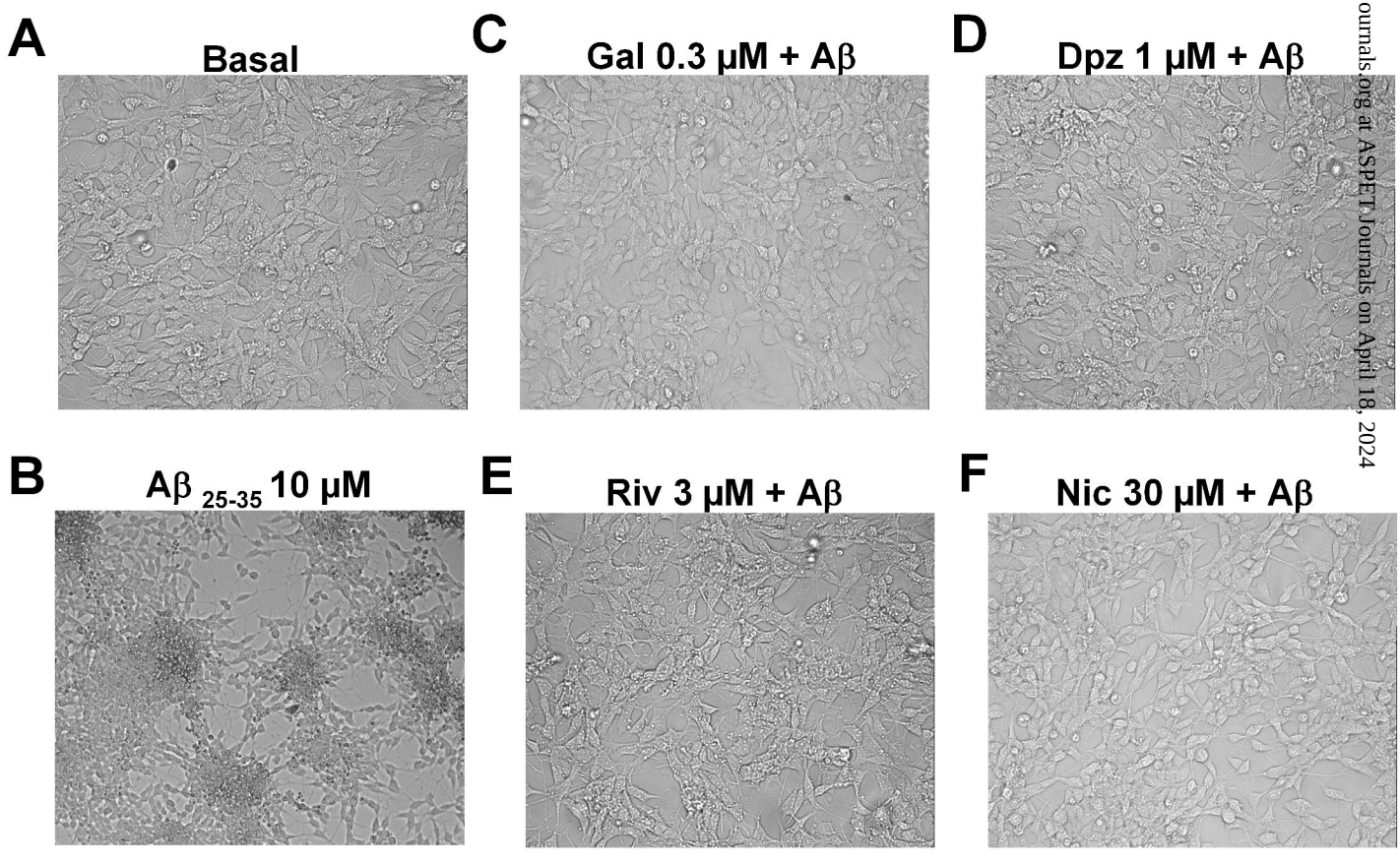


Figure 3

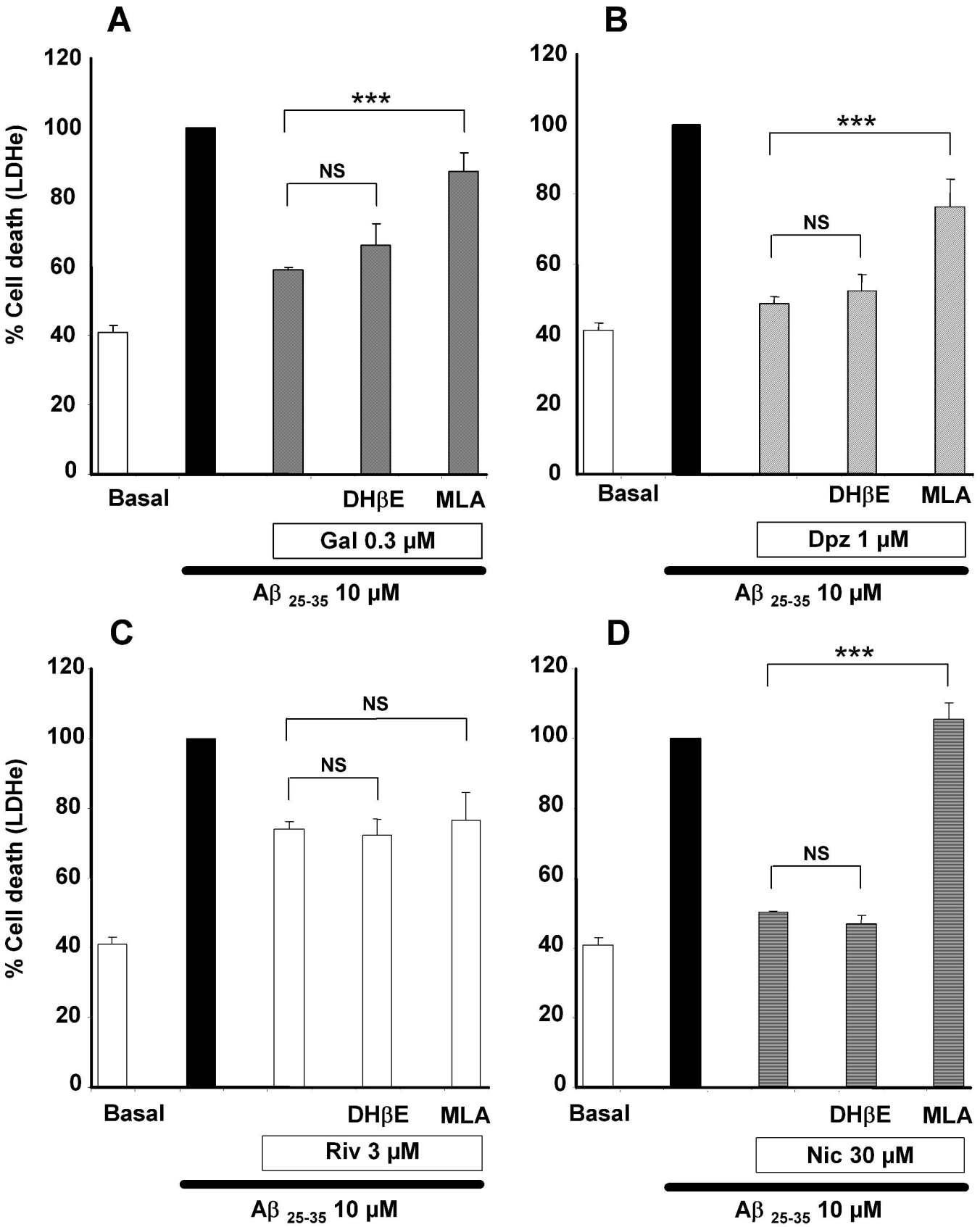

A
B
C
D
% Cell death (LDHe)
120
100
80
60
40
20
0
***
NS
Basal
DHβE
MLA
Gal 0.3 µM
Dpz 1 µM
Riv 3 µM
Nic 30 µM
Aβ 25-35 10 µM

Figure 4

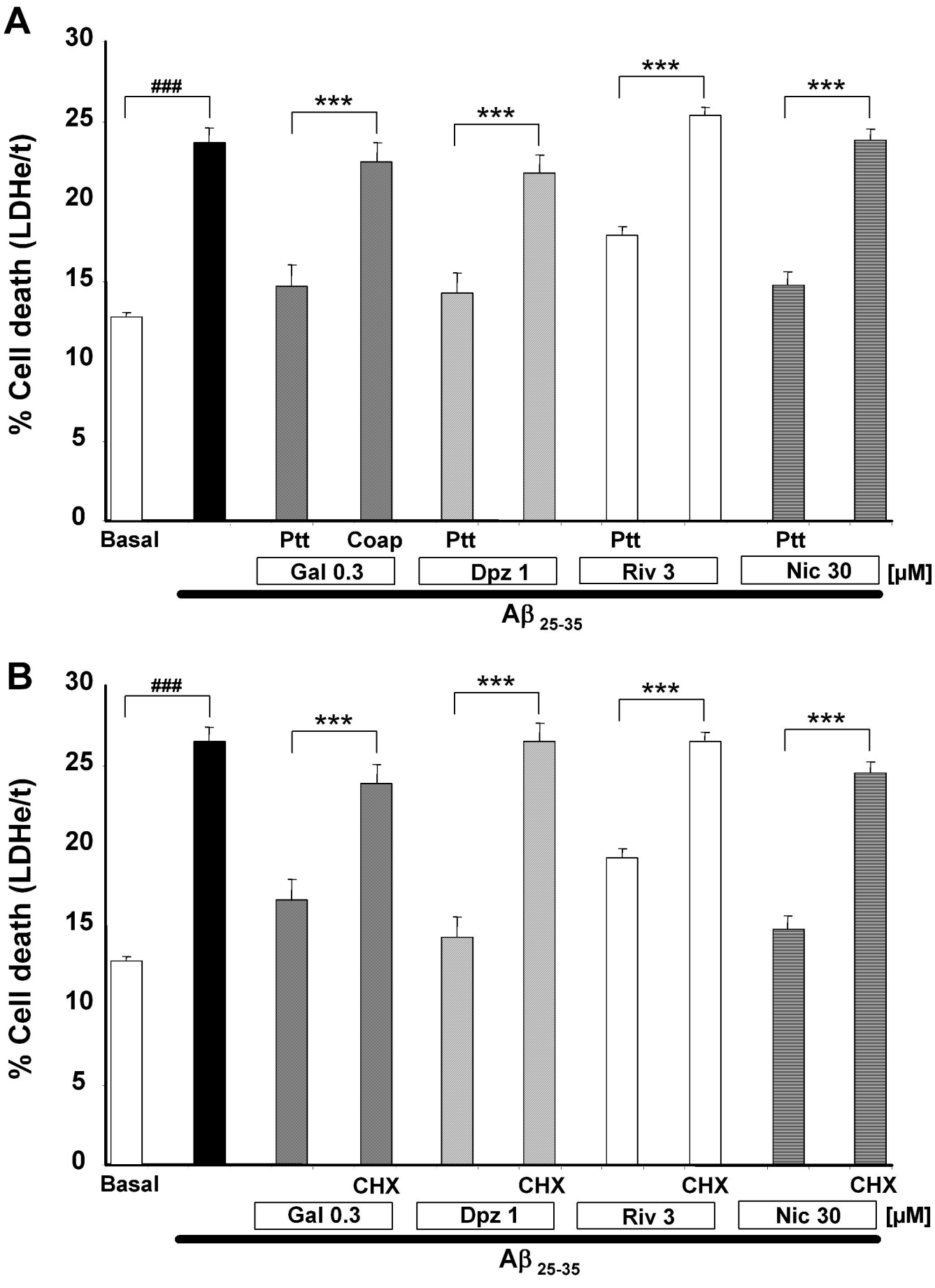
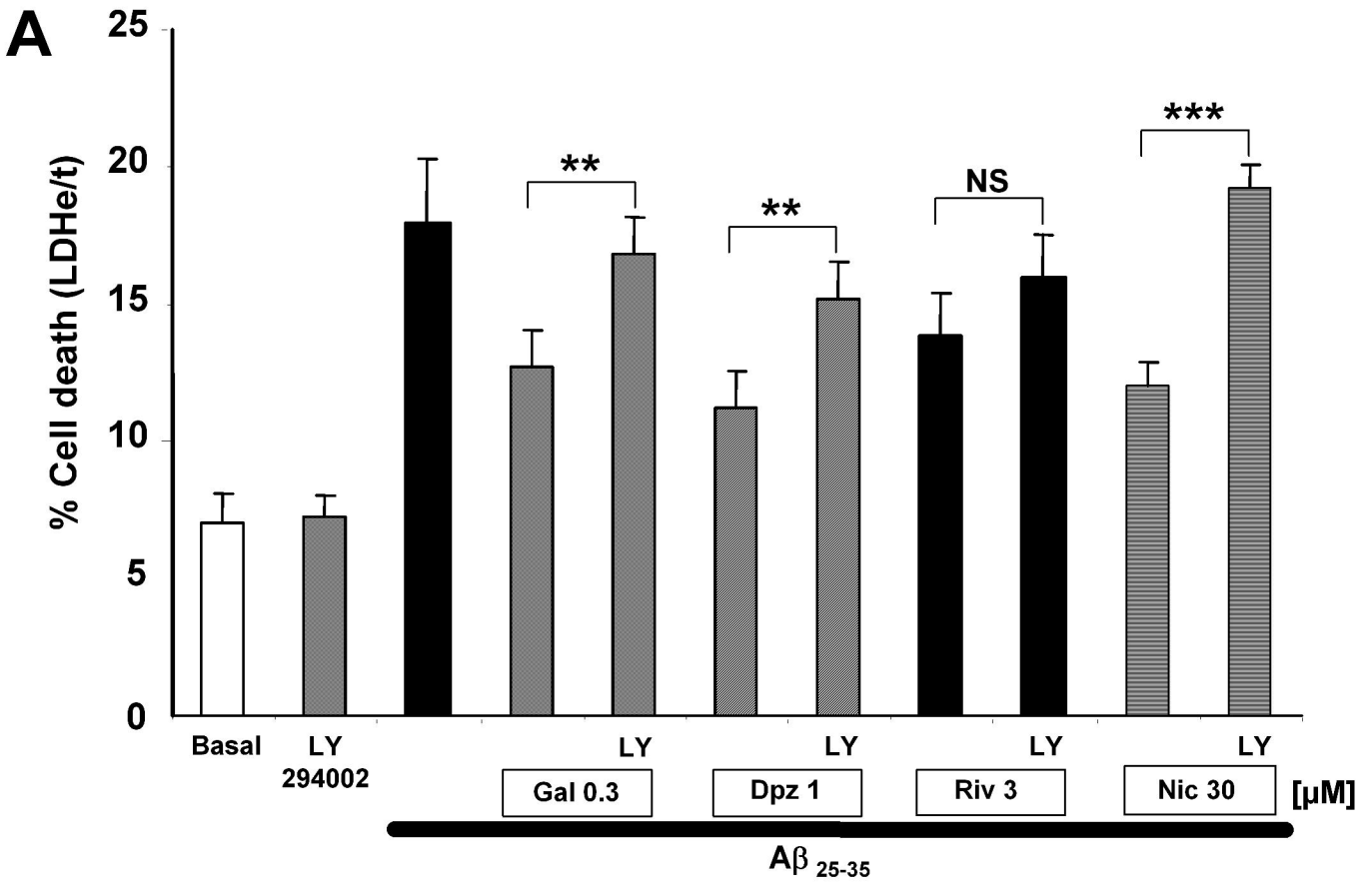

A
% Cell death (LDHe/t)
30
25
20
15
10
5
0
###
***
***
***
***
Basal
Ptt
Coap
Ptt
Ptt
Ptt
Gal 0.3
Dpz 1
Riv 3
Nic 30
[µM]
Aβ 25-35
B
% Cell death (LDHe/t)
30
25
20
15
10
5
0
###
***
***
***
***
Basal
CHX
CHX
CHX
CHX
Gal 0.3
Dpz 1
Riv 3
Nic 30
[µM]
Aβ 25-35

Figure 5


A
% Cell death (LDHe/t)
25
20
15
10
5
0
**
**
NS
***
Basal
LY 294002
LY
LY
LY
LY
Gal 0.3
Dpz 1
Riv 3
Nic 30
[µM]
Aβ 25-35

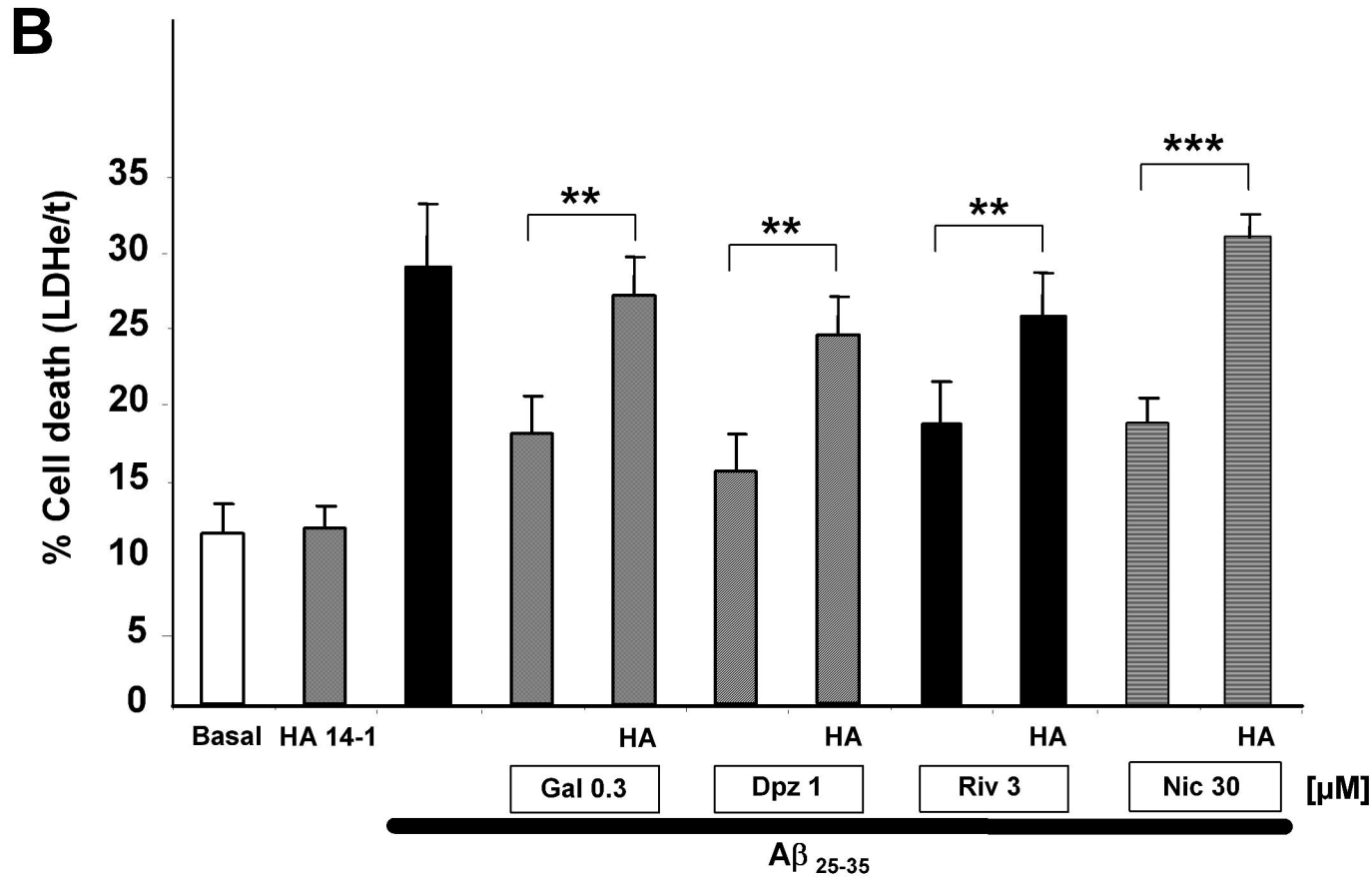

B
% Cell death (LDHe/t)
35
30
25
20
15
10
5
0
**
**
**
***
Basal
HA 14-1
HA
HA
HA
HA
Gal 0.3
Dpz 1
Riv 3
Nic 30
[µM]
Aβ 25-35

Figure 6