Neurotoxicity Induced by Okadaic Acid in the Human Neuroblastoma SH-SY5Y Line Can Be Differentially Prevented by α7 and β2* Nicotinic Stimulation

Laura del Barrio,*,†,‡,[1] María Dolores Martín-de-Saavedra,*,†,§,[1] Alejandro Romero,*,†,¶ Esther Parada,*,† Javier Egea,*,†,§ Jesús Avila,‖ John Michael McIntosh,‖‖‖ Susan Wonnacott,‡ and Manuela G. López*,†,§,[2]

*Instituto Teófilo Hernando and †Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain; ‡Department of Biology and Biochemistry, University of Bath, BA27AY Bath, UK; §Hospital La Paz Research Institute-IdiPaz, 28029 Madrid, Spain; ¶Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, 28040 Madrid, Spain; ‖Centro de Biología Molecular ''Severo Ochoa,'' Universidad Autónoma de Madrid, 28049 Madrid, Spain; and ‖‖‖Department of Biology and Psychiatry, University of Utah, 84112 Salt Lake City, Utah 84112

[1]These authors contributed equally.

[2]To whom correspondence should be addressed at Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, C/Arzobispo Morcillo 4, E-28029 Madrid, Spain. Fax: +34-914973120. E-mail: manuela.garcia@uam.es.

A good model of neuronal death that reproduces the characteristic tau (τ) hyperphosphorylation of Alzheimer's disease is the use of okadaic acid (OA). The aim of this study was to determine the contribution of α7 and β2* nicotinic acetylcholine receptor (nAChR) subtypes to neuroprotection against OA in the SH-SY5Y cell line by using the selective α7 and β2* nAChR agonists PNU 282987 and 5-Iodo-A85380, respectively. The results of this study show that both α7 and β2* nAChR can afford neuroprotection against OA-induced neurotoxicity. Protection mediated by α7 nAChRs was independent of Ca²⁺ and involved the intracellular signaling pathway Janus Kinase-2/Phosphatidylinositol-3-kinase/Akt. When Ca²⁺ entry was promoted through the α7 nAChR by using the α7-selective positive allosteric modulator PNU 120596, protection was lost. By contrast, protection mediated by β2* nAChRs was Ca²⁺ dependent and implicated the signaling pathways PI3K/Akt and extracellular regulated kinase 1/2. Both α7 and β2* nAChR activation converged on downregulation of GSK-3β and reduction of τ phosphorylation in cells undergoing cell death induced by OA. Therefore, targeting nAChR could offer a strategy for reducing neurodegeneration secondary to hyperphosphorylation of protein τ.

Key Words: okadaic acid; SH-SY5Y neuroblastoma; nicotinic receptors; hyperphosphorylation of tau; PNU 282987; 5IA 85380.

Alzheimer disease (AD) is characterized by a progressive loss of memory, often with deterioration of language as well as deficits in visual and motor coordination. At the cellular level, it has been shown that cell death (Cummings, 2004) occurs especially in cholinergic neurons (Whitehouse et al., 1982) and that this neuronal death is related to the loss of memory in AD patients (Haass and Selkoe, 2007). At the molecular level, AD is characterized by the presence of tangles rich in the intracellular microtubule-associated protein tau (τ) (Lee and Trojanowski, 1992) and extracellular deposits of amyloid peptides that form senile plaques (Price et al., 1991). A link between these two proteins has been demonstrated because beta-amyloid can trigger τ phosphorylation and the subsequent degeneration of the affected neurons (Alvarez et al., 2002; Busciglio et al., 1995; Liu et al., 2004). It has also been shown that beta-amyloid fibrils can induce neurotoxicity and τ hyperphosphorylation without plaque formation; it therefore seems that beta-amyloid pathology could precede hyperphosphorylation of τ and the formation of neurofibrillary tangles and neurodegeneration (Busciglio et al., 1995)

In this study, we have focused on τ hyperphosphorylation as a mechanism for neuronal death (Irizarry et al., 1997; Pizzi et al., 1995). In the brains of AD patients, there is a high percentage of hyperphosphorylated τ protein (Avila, 2000) and an accumulation, in certain neurons, of paired helical filaments (PHF) in which hyperphosphorylated τ is the major protein present (Grundke-Iqbal et al., 1986, 1988). Destabilization of microtubules and the consequent decrease in axonal transport is believed to be responsible for retrograde degeneration of neurons in AD (Alonso et al., 1994, 1997; De Vos et al., 2008) and also for cell death in "in vitro" models of τ hyperphosphorylation (Cowan et al., 2010; Perez et al., 2002; Tanaka et al., 1998). Inhibition of phosphatases that act on some abnormally phosphorylated sites characteristic of AD (Gong et al., 1994) may also lead to τ protein hyperphosphorylation (Tanaka et al., 1998). These phosphatases include the Ser/Thr PP1, PP2A, and PP2B (Gong et al., 1994; Wang et al., 1995), and their activity

has been shown to be diminished in brain tissue from AD patients (Gong *et al.*, 1995, 1993).

Okadaic acid (OA), a seaweed toxin, inhibits phosphatases with the preference PP2A > PP1 > PP2B. A consequence of its action is τ hyperphosphorylation "*in vivo*" (Tian *et al.*, 2004) and *in vitro* (Tanaka *et al.*, 1998; Uberti *et al.*, 1997). It therefore provides a good model that reproduces τ hyper-phosphorylation and cell death. In this study, we have used OA in the human neuroblastoma cell line SH-SY5Y to model aspects of cell damage relevant to AD (Arias *et al.*, 2005; Cowan *et al.*, 2010.; Perez *et al.*, 2002; Tanaka *et al.*, 1998), in order to investigate neuroprotective strategies.

There is a body of evidence for nicotinic acetylcholine receptors (nAChRs) exerting neuroprotective effects against a variety of insults (Dajas-Bailador and Wonnacott, 2004; Picciotto and Zoli, 2008). With respect to AD, nicotinic agonists have been reported to be effective against Aβ-induced toxicity *in vivo* and *in vitro* (Arias *et al.*, 2005; Kihara *et al.*, 1998). Their efficacy in τ hyperphosphorylation models is less well documented.

The majority of studies have highlighted the α7 nAChR as the main nAChR subtype responsible for neuroprotective effects mediated by nicotinic agonists (Arias *et al.*, 2005; Bitner *et al.*, 2009; Haydar and Dunlop, 2010). However, non-α7 nAChR subtypes have also been found to contribute to neuroprotection (Tizabi *et al.*, 2004), depending on the cell type and toxic stimulus. Because the roles of different nAChR subtypes are not fully understood, we have exploited subtype-selective ligands, namely, the α7 nAChR agonist PNU 282987 (Bodnar *et al.*, 2005), the β*-selective agonist 5-Iodo-A-85380 (5IA 85380) (Mukhin *et al.*, 2000), and the α7 nAChR-positive allosteric modulator PNU 120596 (Hurst *et al.*, 2005) in this study

The results demonstrated nAChR-mediated neuroprotection against OA. Protection mediated by α7 nAChRs was in-dependent of Ca^{2+} and involved the intracellular signaling pathway JAK2/PI3K/Akt. Sustained activation of α7 nAChR to promote Ca^{2+} entry was achieved with the positive allosteric modulator, but under these conditions, protection was lost. In contrast, protection mediated by β2* nAChRs was Ca^{2+} dependent and implicated the signaling pathways PI3K/Akt and extracellular regulated kinase 1/2 (ERK 1/2). Both α7 and β2* nAChR activation converged on the inhibition of GSK-3β and consequently τ phosphorylation to afford neuroprotection.

MATERIALS AND METHODS

Materials. PNU 282987, 5IA 85380, PNU 120596, AG-490, LY 294002, and PD 98059 were purchased from Tocris Cookson Inc. (Bristol, UK). OA, α-bungarotoxin (BGT), dihydro-β-erythroidine (DHβE), methyllycaconitine (MLA), mecamylamine, and AR-A01448 were purchased from Sigma-Aldrich (St Louis, MO). α-Conotoxin MII and α-conotoxin AuIB were synthesized as previously described (Luo *et al.*, 1998). Alpha-conotoxin AuIB selectively blocks α3β4 nAChRs and nicotine-evoked norepinephrine release (Luo *et al.*, 1998).

Culture of SH-SY5Y cells. SH-SY5Y cells were maintained in a 1:1 mixture of F-12 Nutrient Mixture (Ham12) (Sigma-Aldrich, Madrid, Spain) and

Eagle's minimum essential medium (EMEM) supplemented with 15 non-essential amino acids, 1mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (reagents from Invitrogen, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For assays, SH-SY5Y cells were subcultured in 48-well plates at a seeding density of 1×10^5 cells per well. Cells were treated with the drugs before confluence in F-12/EMEM with 1% FBS. These cells, when undifferentiated, express functional nicotinic receptors (Dajas-Bailador *et al.*, 2002a). All cells used in this study were used at a low passage number (< 13).

Measurement of lactate dehydrogenase activity. Extracellular and in-tracellular lactate dehydrogenase (LDH) activity was measured spectrophoto-metrically using a cytotoxicity cell death kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; released LDH was defined as the percentage of extracellular compared with total LDH activity.

Calcium fluorimetry. Increases in intracellular Ca^{2+} in confluent cultures of SH-SY5Y cells grown in 96-well plates were monitored as described by Dajas-Bailador *et al.* (2002a). In brief, SH-SY5Y cells were washed twice with Tyrode's salt solution (TSS: in mM 137 NaCl, 2.7 KCl, 1.0 $MgCl_2$, 2.5 $CaCl_2$, 0.2 NaH_2PO_4, 12 $NaHCO_3$, and 5.5 glucose; pH 7.4) and incubated with the membrane-permeable Ca^{2+}-sensitive dye fluo-3 acetoxymethyl ester (10μM) and 0.02% pluronic F127 for 1 h at room temperature in the dark. Cells were then washed twice with TSS before adding 80 μl buffer, with or without nAChR antagonists or PNU 120596, per well. After 10 min, basal fluorescence (excitation 485 nm and emission 520 nm) was recorded for 4 s using a Fluoroskan Ascent fluorescence plate reader (Labsystems, Helsinki, Finland). nAChR agonists (20 μl) were added using an automatic dispenser, and changes in fluorescence were monitored for a further 10 s. To normalize fluo-3 AM signals, responses from each well were calibrated by determination of the maximum and minimum fluorescence values by addition of 0.2% Triton-X100 (F_{max}) followed by 40mM $MnCl_2$ (F_{min}). Maximum fluorescence responses were calculated as a percentage of $F_{max} - F_{min}$.

Immunoblotting. SH-SY5Y cells were washed once with cold PBS and lysed in 100 μl ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mmol/l NaCl, 20 mmol/l Tris-HCl, pH 7.5, 1 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, 20 mmol/l NaF, 1 mmol/l sodium pyrophosphate, and 1 mmol/l Na_3VO_4). Protein (30 μg) from the cell lysates was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Amersham). Membranes were incubated with the following anti-bodies: anti-ERK 1/2 and anti-phospho-ERK 1/2 at 1:1000 (Santa Cruz Biotechnology Inc., Heidelberg, Germany); anti-JAK2, anti-phospho-Tyr-211-JAK2, anti-phospho-Thr-308-Akt, anti-Akt, anti-phospho-Ser-9-GSK3-β, anti-phospho-Tyr-216-GSK3-β, and anti-GSK-3β at 1:1000 (Cell Signaling, Izasa S.A., Barcelona, Spain); and anti-β-actin at 1:100,000 (Sigma). Membranes were also incubated with anti-phospho-Ser-396/404-τ and anti-τ at 1:1000, which were previously used by Lucas *et al.* (2001). Appropriate peroxidase-conjugated secondary antibodies at 1:10,000 were used to detect proteins by enhanced chemiluminescence.

Cytoskeleton staining. SH-SY5Y cells were plated on coverslips: 24–48 h after plating the cells, they were separated into four groups: two were treated with EMEM, one with 10μM PNU 282987, and another one with 100μM 5IA 85380 for 24 h. Then, the control group was maintained in EMEM, and the rest of the groups were treated with OA for 5 h. After exposure of the cells to the different treatments, they were rinsed three times with PBS (9g/l NaC1, 10mM NaH_2PO_4, 10mM K_2HPO_4). Then, they were fixed in 2% paraformaldehyde in PBS for 15 min and washed three more times with PBS. Cells were permeabilized by exposure to 0.1% triton in PBS for 1 min. Preparations were washed several times with PBS and then incubated at room temperature with rhodamine-labeled 1:1000 phalloidin (Sigma-Aldrich) in PBS. Coverslips were then thoroughly washed with PBS. In the second rinsing, Hoechst (Invitrogen)

was added (1 μl/ml) in order to mark nuclei, and after the third rinsing, the coverslips were mounted in glycerol-PBS (1:1; vol/vol). Images were taken with a confocal microscope (TCS SPE; Leica, Wetzlar, Germany).

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

RESULTS

Effect of Different Nicotinic Agonists and the α7 Allosteric Modulator PNU 120596 on OA-Induced Toxicity

Exposure of SH-SY5Y cells to 30nM OA for 16 h increased LDH release from 7–10% (basal conditions) to 36–42% (OA treatment), consistent with the induction of modest levels of cell death (Fig. 1). To determine if nAChRs are able to protect against OA-induced toxicity, we incubated SH-SY5Y cells with either the α7- or β2*-selective agonist PNU 282987 (Parada *et al.*, 2010) or 5IA 85380, respectively, or with the α7 allosteric modulator PNU 120596. With preincubation increasing concentrations of PNU 282987, for 24 h before the addition of OA (see protocol at the top of Fig. 1), we observed a gradual reduction in cell death measured as LDH released into the extracellular medium. PNU 282987 exerted a signifi-

cant but partial protection against OA-induced toxicity at 10 and 30μM (Fig. 1A). 5IA 85380 also produced significant protection at 100μM (Fig. 1B). The α7-positive allosteric modulator PNU 120596 alone showed no protection at any of the concentration assayed (0.3–10μM) (Fig. 1C).

Intracellular Ca²⁺ Increases Mediated by PNU 282987, 5IA 85380, and PNU 120596 Plus PNU 282987

SH-SY5Y cells express α3, α5, α7, β2, and β4 nAChR subunits (Gentry and Lukas, 2002; Peng *et al.*, 1994). In order to determine the effects of PNU 282987 and 5IA 85380 on intracellular Ca^{2+} levels and their selectivity on nAChR subtypes, we measured Ca^{2+} signals in SH-SY5Y cells loaded with the dye fluo-3. For these experiments, we employed concentrations of the agonists that showed protection in the previous section in order to gather information about the possible neuroprotective mechanisms of action.

PNU 282987 alone did not induce a significant increase in $[Ca^{2+}]_c$ at the neuroprotective concentration of 10μM. However, when the agonist was added to cells preincubated with the allosteric modulator PNU 120596, a significant increase in fluorescence was observed, consistent with increased cytosolic Ca^{2+}. These $[Ca^{2+}]_c$ increases were mediated
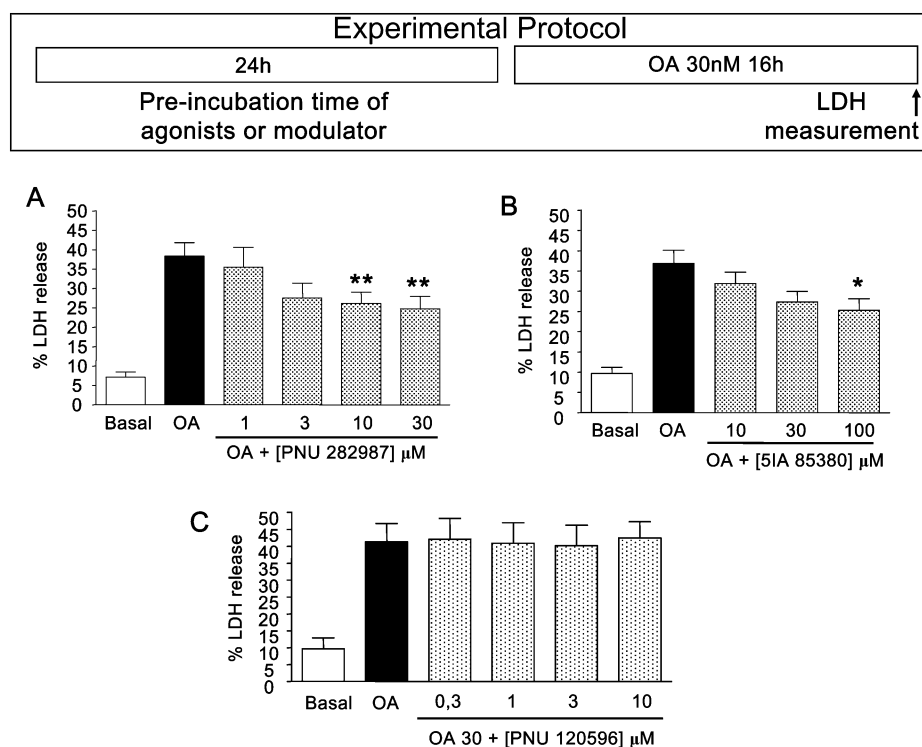
FIG. 1. Protective effects mediated by the nicotinic agonists PNU 282987 and 5IA 85380 and the α7 allosteric modulator PNU 120596 against OA-induced toxicity. Cells were pretreated for 24 h with increasing concentrations of the α7 nicotinic agonist PNU 282987, the β2* nicotinic agonist 5IA 85380, or the α7-positive allosteric modulator PNU 120596. Thereafter, SH-SY5Y were incubated for 16 h with 30nM OA, and at the end of the experiment, cell death was evaluated by measuring the release of LDH to the extracellular medium (see top of the figure). PNU 282987 (A) and 5IA 85380 (B) offered protection against OA-induced toxicity; however, the α7 allosteric modulator PNU 120596 did not (C). The data represent means ± SEM of triplicates of at least four different batches of cells. * $p < 0.05$ and **$p < 0.01$ with respect to OA alone.

by activation of nAChR because the signal was significantly attenuated by the nonselective nAChR antagonist mecamylamine. In particular, these Ca^{2+} signals could be attributed to stimulation of the $\alpha 7$ subtype nAChR because the selective $\alpha 7$ nAChR antagonists αBGT and MLA completely inhibited the increases in fluorescence (Fig. 2A).

5IA 85380, at the protective concentration of 100μM, produced a significant intracellular Ca^{2+} signal by itself (Fig. 2B). Selective $\beta 2^*$ antagonists such as DHβE and α-conotoxin MII or mecamylamine were able to significantly block Ca^{2+} signals induced by 5IA 85380. However, $\alpha 3 \beta 4$ (α-conotoxin AuIB) or $\alpha 7$ (BGT) antagonists did not modify the $[Ca^{2+}]_c$ responses mediated by 5IA 85380.

Therefore, under these experimental conditions, 5IA 85380 appears to be activating heteromeric $\beta 2$-containing nAChRs, whereas PNU 282987 is activating $\alpha 7$ nAChRs.

Ca^{2+} Dependence in the Protective Effects of 5IA 85380 and PNU 282987

To determine the influence of Ca^{2+} entry mediated by $\alpha 7$ and $\beta 2^*$ nAChRs in the neuroprotective effects afforded by PNU 282987 and 5IA 85380, we performed experiments in the absence or presence of extracellular Ca^{2+}. Because prolonged lack of extracellular Ca^{2+} can be a toxic stimulus per se, we first performed a time course to determine the minimum time of agonist preincubation required to achieve the maximal protective effect (see protocol on top of Fig. 3).

As Figure 3 illustrates, PNU 282987 reduced LDH release in cells injured with OA in a time-dependent manner; cell death was significantly reduced following only 5 min incubation with the agonist, 24 h before exposing the cells to 16 h to OA (Fig. 3A). In the case of 5IA 85380, protection was significant following 1 h preincubation (Fig. 3B). For comparability, we

selected a 4-h preincubation period for both agonists because this length of exposure produced a level of protection similar to that seen following 24 h preincubation. The presence or absence of extracellular Ca^{2+} was maintained only during the 4-h exposure to the nicotinic agonist; thereafter, the medium was replaced with fresh EMEM containing Ca^{2+} for 20 h, and OA was then added for 16 h. The results show that protection mediated by 5IA 85380 was dependent on extracellular Ca^{2+} (Fig. 3D), whereas that induced by PNU 282987 was not (Fig. 3C). Moreover, when cells were pre- and coincubated with the $\alpha 7$ allosteric modulator PNU 120596 in the presence of Ca^{2+}, the neuroprotective effect of PNU 282987 was lost (Fig. 3C). Finally, in the absence of extracellular Ca^{2+}, PNU 120596 did not reverse the neuroprotection mediated by PNU 282987 (Fig. 3C). These results suggest that Ca^{2+} entry via $\beta 2^*$ nAChR activation is required to afford a neuroprotective effect. However, in the case of the $\alpha 7$ nAChR, PNU 282987 exerted a Ca^{2+}-independent neuroprotective effect. Furthermore, as shown by the experiments with the $\alpha 7$ agonist in the presence of the $\alpha 7$ nAChR allosteric modulator that promotes Ca^{2+} entry through the $\alpha 7$ nAChR subtype (Fig. 2A), protection was lost in a Ca^{2+}-dependent way (Fig. 3C).

JAK2 Is Involved in Protection Mediated by $\alpha 7$ but Not by $\beta 2^*$ nAChRs

JAK2 is a protein that can be physically associated with $\alpha 7$ nAChRs and activated when nicotine binds to this receptor subtype (Shaw et al., 2002). To determine if $\alpha 7$ and/or $\beta 2^*$ nAChR subtype stimulation could activate this kinase under our experimental conditions, SH-SY5Y cells were incubated with 10μM PNU 282987, 10μM PNU 282987, plus 10μM PNU 120596 or 100μM 5IA 85380 for different times (5, 15, and 30 min and 1 or 24 h) and JAK2 phosphorylation was then analyzed
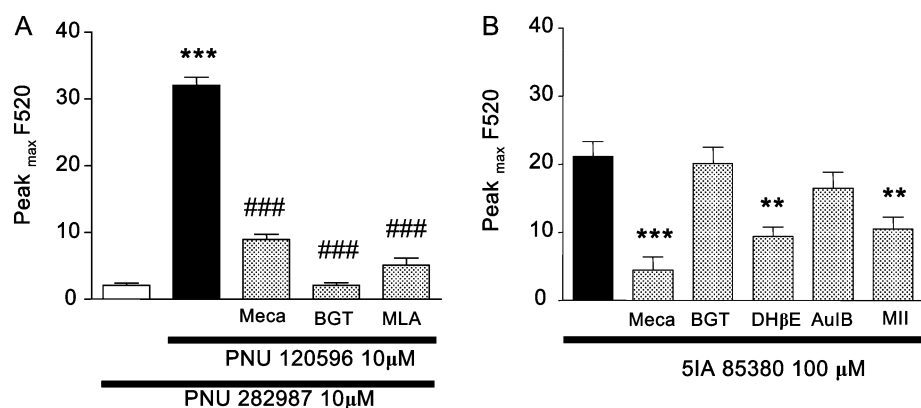
FIG. 2. Intracellular Ca^{2+} increases mediated by the nicotinic agonists PNU 282987 and 5IA 85380 in SH-SY5Y cells loaded with fluo-3. (A) SH-SY5Y cells were stimulated with 10μM PNU 282987 alone or in cells preincubated during 15 min with the $\alpha 7$ allosteric modulator PNU 120596 (10μM); PNU 120596 was also coincubated with various nicotinic antagonists such as mecamylamine (30μM ''Meca''), ''BGT'' (100nM), or ''MLA'' (100nM). (B) Cytosolic Ca^{2+} increases induced by 5IA 85380 at 100μM alone or in the presence of the nicotinic antagonist 30μM Meca, 100nM BGT, α-conotoxin AuIB (10μM ''AuIB''), dihydro-β-eritroidine (10μM ''DHβE''), or α-conotoxin MII (200nM ''MII''). The data represent the average of maximum fluorescence increases during 10 s after the implementation of 10μM PNU 282987 (A) or 100μM 5IA 85380 (B). Experiments were conducted in triplicate on at least six different batches of cells. ***$p < 0.001$ with respect to PNU 282987 alone (A) or with respect to 5IA 85380 (B); ###$p < 0.001$ with respect to PNU 282987 + PNU 120596 (B); **$p < 0.01$ with respect to 5IA 85380 (B).
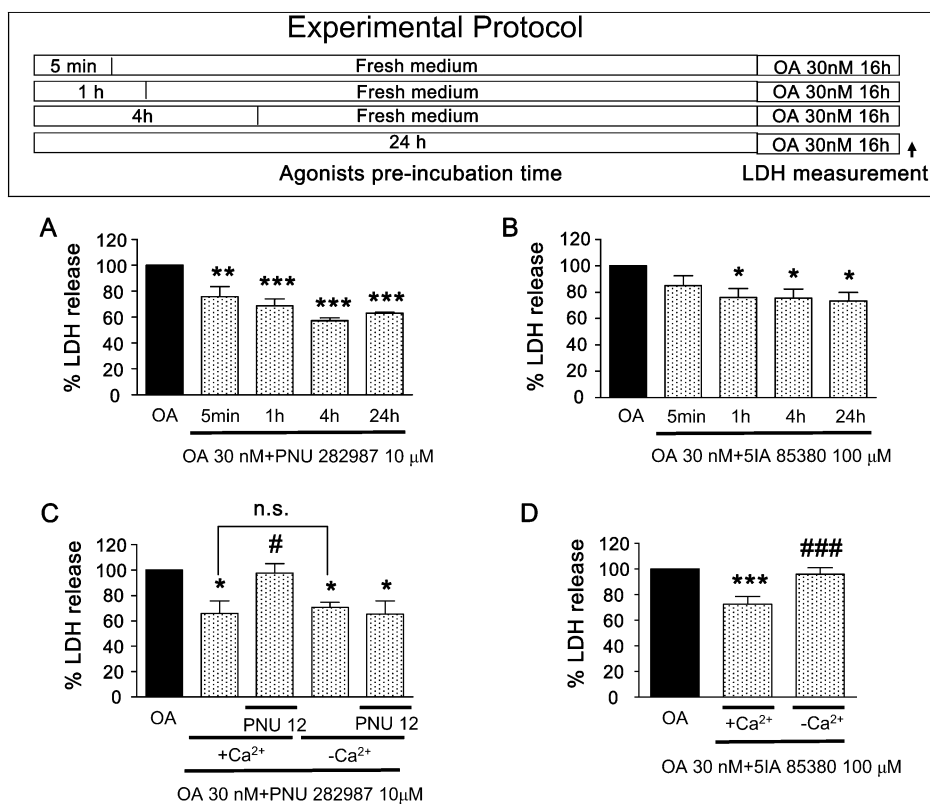
FIG. 3. Time and Ca^{2+} dependence of cytoprotection mediated by PNU 282987 and 5IA 85380. As shown in the upper panel, cells were incubated with PNU 282987 (10μM) (A) or 5IA 85380 (100μM) (B) during 5 min, 1 h, 4 h, or 24 h, and then the agonist was removed, and fresh EMEM was added until to complete 24 h; after this period, EMEM was replaced by OA (30nM) for 16 h. At the end of the experiment, cell death was evaluated by LDH. Figures (C) and (D) show the mean of LDH values when agonists were preincubated for 4 h in the presence or absence of extracellular Ca^{2+} using the same protocol described above. PNU 120596 (10μM ''PNU 12'') was preincubated for 15 min and coincubated during the application of PNU 282987 (C). Data represent the mean of LDH values normalized with respect to OA alone ± SEM of triplicates of at least six different cultures. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ with respect to OA; #$p < 0.05$ and ###$p < 0.001$ with respect to the agonists plus OA.

by Western blotting. Cells treated with 10μM PNU 282987 showed maximally phosphorylated JAK2 after 15 min incubation; thereafter, phospho-JAK2 decreased to basal levels (Fig. 4A). In contrast, 5IA 85380 failed to promote the activation of JAK2 at any of the times tested (Fig. 4B). Also, no alteration in phospho-JAK2 levels was observed when the α7 agonist was incubated in the presence of the α7 allosteric modulator (Fig. 4C).

JAK2 has been previously shown to be involved in neuro-protection induced by nicotine via α7 nAChR stimulation in neurons injured with $A\beta_{1-42}$ (Shaw et al., 2002). For this reason and because the α7-selective agonist increased JAK2 phosphory-lation, we evaluated if the protective effect of PNU 282987 against OA would be lost in the presence of a JAK2 inhibitor, AG-490. To corroborate that JAK2 activation was linked with its action on the α7 and not with the β2* nAChRs, we also included 5IA 85380 in these experiments. As shown in Figure 4D, the protective effect of PNU 282987 was completely prevented by pre- (15 min before) and coincubation with AG-490, but this compound did not affect protection afforded by 5IA 85380. Therefore, these results indicate that JAK2 is linked to α7 nAChR-mediated survival. Moreover, the presence of the α7 allosteric modulator prevented PNU 282987–

induced JAK phosphorylation (Fig. 4C), and this result correlates with the loss of protection under the same drug treatment conditions (see Fig. 1C).

Implication of PI3K/Akt in the Neuroprotective Effect Mediated by α7 and β2* nAChR Stimulation

Multiple nicotinic agonists cause activation of PI3K/Akt (Kihara et al., 2001). PI3K/Akt is known to be one of the targets of JAK2 (Shaw et al., 2002). To investigate if exposure to α7 and β2* nAChR agonists led to Akt activation, we measured Akt phosphorylation by Western blot analysis. Incubation with PNU 282987 or 5IA 85380 produced a statistically significant activation of Akt at 5 and 15 min for both agonists (Figs. 5A and 5B). To determine if protection afforded by α7 and β2* nAChR was dependent on the PI3K/Akt pathway, cells were pre- (15 min before) and coincubated with a PI3K inhibitor, LY 294002 (3μM). Protection elicited by PNU 282987 and 5IA 85380 against OA toxicity was prevented by the inhibitor LY 294002 (Fig. 5C). Taken together, these results indicate that protection obtained with both nicotinic agonists is dependent on activation of PI3K/Akt.
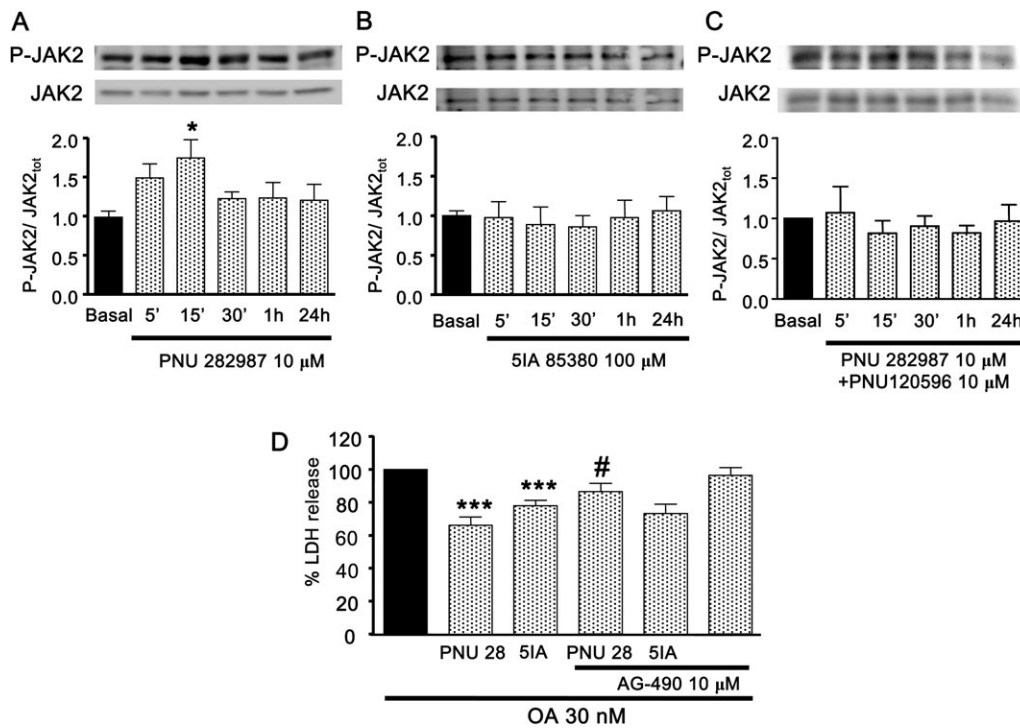
FIG. 4. Differential phosphorylation and implication of JAK2 in neuroprotection mediated by PNU 282987 and 5IA 85380. PNU 282987 (A), 5IA 85380 (B), and PNU 282987 plus the allosteric modulator PNU 120596 were incubated during different time periods (5, 15, and 30 min and 1 and 24 h); at the end, P-JAK2 (immunoblots on the top) and JAK2 total (immunoblots at the bottom) were quantified by Western blot. (D) Represents the effect of the JAK2 inhibitor, AG-490, on the neuroprotective action of $10\mu M$ PNU 282987 (''PNU 28'') and $100\mu M$ 5IA 85380 (''5IA''). Data are the mean ± SEM of at least four different cultures; *$p < 0.05$ with respect to the basal (A); ***$p < 0.001$ with respect to OA alone and #$p < 0.05$ with respect to PNU 282987 (B).

ERK 1/2 Is Involved in the Neuroprotective Effect Mediated by 5IA 85380 but Not by PNU 282987

The mitogen-activated protein kinase (MAPK) intracellular pathway has also been implicated in neuroprotection processes mediated by nAChR activation (Egea *et al.*, 2007). To assess if PNU 282987 and/or 5IA 85380 were able to activate ERK 1/2, we measured the phosphorylation of these kinases by Western blot. As shown in Figure 6A, $10\mu M$ PNU 282987 by itself did not produce ERK 1/2 phosphorylation at any of the time intervals studied. However, 5IA 85380 significantly increased the level of P-ERK 1/2 after 15 min (Fig. 6B). To corroborate the involvement of MAPK activation in the neuroprotective effect mediated by 5IA 85380 and not by PNU 282987, we employed an MEK inhibitor, PD 98059. As shown in Figure 6C, the presence of PD 98059 completely inhibited the protection induced by 5IA 85380 but had no effect on that of PNU 282987. From these results, it can be concluded that MEK participates in the neuroprotective effect mediated by β2* but not by α7 nAChR stimulation.

Involvement of GSK-3β in Neuroprotection Induced by α7 and β2* nAChR

GSK-3β is a substrate of Akt. Akt phosphorylates GSK-3β at position Ser-9, and this phosphorylation inactivates GSK-3β (Stambolic and Woodgett, 1994). On the other hand, phosphorylation at Tyr-216 has the opposite effect and activates GSK-3β; however, the kinases responsible for this phosphorylation are not well characterized (Cole *et al.*, 2004; Hughes *et al.* 1993; Wang *et al.* 1994). We therefore measured nAChR agonist-induced GSK-3β phosphorylation by Western blot. As shown in Figure 7, both PNU 282987 and 5IA 85380 were able to increase Ser-9 phosphorylation after 15 and 5 min incubation, respectively (Figs. 7A and 7B). Subsequently, this phosphorylation decreased over time, and after 24 h, it was not statistically significantly different from OA control. P-Tyr 216-GSK-3β and total GSK-3β levels were not altered at any of the incubation times (data not shown).

To determinate if GSK-3β inhibition could be involved in the protection mediated by PNU 282987 and 5IA 85380, we conducted a neuroprotection experiment in the presence or absence of a GSK-3β inhibitor, AR-A014418. Figure 7C shows how AR-A014418 alone produced protection against OA and such protection was not statistically different from that obtained with the nicotinic agonists alone. Because the combination of nAChR agonists plus AR-A014418 did not afford a greater neuroprotective effect, we deduce that both agonists were acting through a common mechanism, which involves a transient inactivation of GSK-3β.

To further investigate how nicotinic agonists and the GSK-3β inhibitor, preincubated for 24 h before adding OA, induced neuroprotection, we evaluated the activation and the expression
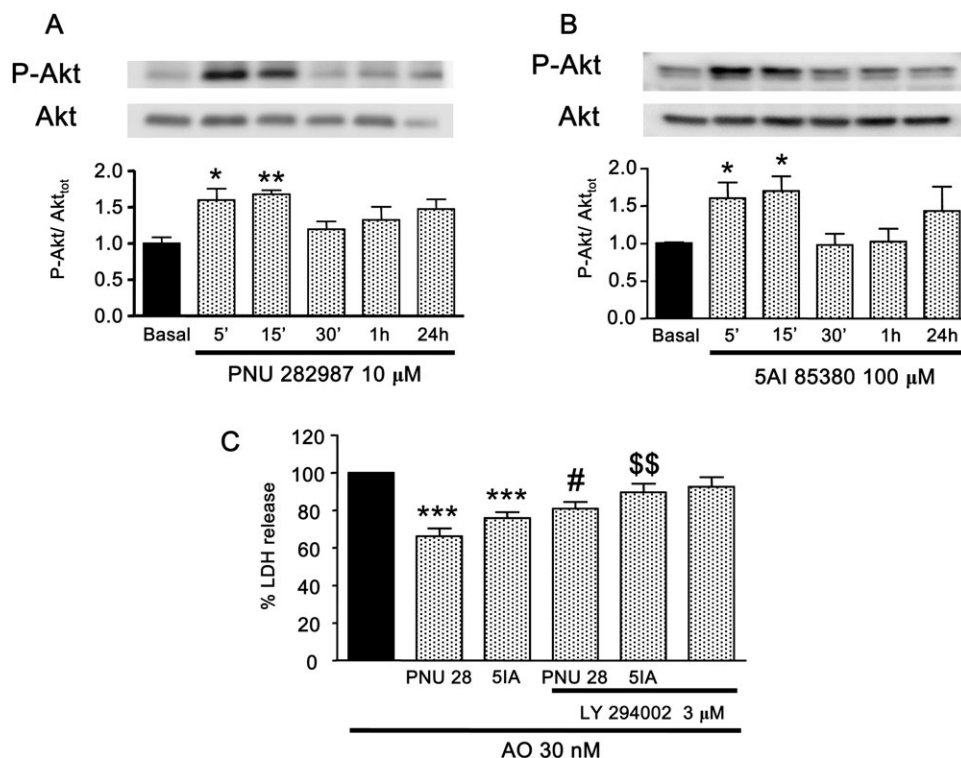
FIG. 5. Phosphorylation of Akt and implication of PI3K in neuroprotection mediated by PNU 282987 and 5IA 85380. Akt phosphorylation by $10 \mu M$ PNU 282987 (A) and $100 \mu M$ 5IA 85380 (B) at different incubation periods. (C) Shows the effect of the PI3K inhibitor $3 \mu M$ LY 294002 on the neuroprotection mediated by $10 \mu M$ PNU 282987 (''PNU 28'') and $100 \mu M$ 5IA 85380 (''5IA'') against OA-induced toxicity. Each value is a mean of at least four different cell cultures $\pm$ SEM. $*p < 0.05$ and $**p < 0.01$ with respect to the basal in figures (A) and (B); $***p < 0.001$ with respect to OA alone, $^{\#}p < 0.05$ with respect to PNU 282987, and $^{\$\$}p < 0.01$ with respect to 5IA 85380 (C).

of GSK-3β at the end of the experiment, i.e., after OA treatment. Western blot analysis of GSK-3β was carried out after OA exposure for 16 h alone or in the presence of the neuroprotective compounds. As shown in Figure 8A, the expression of total GSK-3β increased after OA treatment, and nicotinic agonists prevented this increased expression to almost basal levels (Fig. 8A); a similar effect was obtained with the GSK-3β antagonist AR-A014418. Interestingly, OA produced a significant increase of total P-Tyr 216 that was also significantly reduced by the nicotinic agonists and AR-A014418 (Fig. 8B).

### $\alpha7$ and $\beta2^*$ Nicotinic Agonists Reduced τ Hyperphosphorylation

As previously mentioned, OA is an inhibitor of phosphatases that augments τ hyperphosphorylation. In addition, one of the kinases involved in τ phosphorylation is GSK-3β, whose target in the τ substrate is Ser-396/404 (Cho and Johnson, 2004). The question of how GSK-3β activity affected the phosphorylation on τ Ser-396/404 residue was raised. After OA treatment for 16 h in the presence or absence of PNU 282987, 5IA 85380, and AR-A014418, we measured Ser-396/404 τ phosphorylation by Western blot. As shown in Figure 9, PNU 282987, 5IA 85380, and AR-A014418 significantly reduced τ phosphorylation induced by OA on its Ser-396/404 residue.

Effect of $\alpha7$ and $\beta2^*$ Nicotinic Agonists on the Cytoskeleton Alterations Caused by OA

OA, besides causing hyperphosphorylation of τ, induces changes in the cytoskeleton that are identified with strong retraction and rounding in cell morphology (Cabado et al., 2004; Vale and Botana, 2008; Yoon et al., 2008). Therefore, we also analyzed how treatment of the cells with nicotinic agonists could modify the changes in the cytoskeleton induced by OA treatment.

As shown in Figure 10, control cells exposed only to EMEM (Fig. 10A) showed a fibrous cytoarchitecture with a well extended and branched morphology. OA-treated cells showed a retracted morphology with almost no neurites (Fig. 10B). When cells were preincubated for 24 h with PNU 282987 ($10 \mu M$) (Fig. 10C) or 5IA 85380 ($100 \mu M$) (Fig. 10D) before adding OA, we observed a partial recovery of the fibrous and branched cytoarchitecture as observed in intact cells. This partial recovery of the cytoarchitecture correlates with the partial neuroprotective effects observed when measured as LDH release (Figs. 1A and 1B).
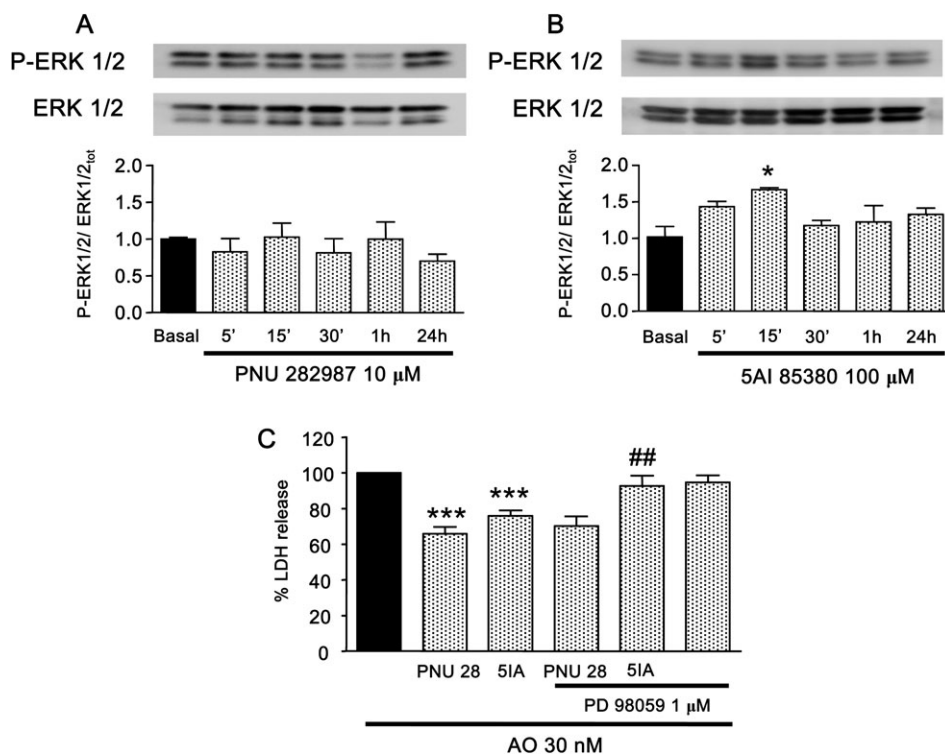
FIG. 6. Phosphorylation of ERK 1/2 and implication of MEK in neuroprotection mediated by PNU 282987 and 5IA 85380. ERK 1/2 phosphorylation by 10μM PNU 282987 (A) and 100μM 5IA 85380 (B) at different incubation periods. (C) Shows the effect of the MEK inhibitor 1μM PD 98059 on neuroprotection mediated by 10μM PNU 282987 (''PNU 28'') and 100μM 5IA 85380 (''5IA'') against OA-induced toxicity. Each value is a mean of at least four different cell cultures ± SEM. *$p < 0.05$ with respect to the basal in (B); ***$p < 0.001$ with respect to OA alone, and ##$p < 0.01$ with respect to 5IA 85380 (C).

DISCUSSION

Central to this study is the observation that both α7 and β2* nAChR can transduce signals through different mechanisms to provide protection of cells that undergo hyperphosphorylation of τ. Ultimately, these distinct signaling pathways converge on the regulation of GSK-3β and reduction of τ phosphorylation.

To analyze how Ca^{2+} could be involved in the protective effects of PNU 282987 and 5IA 85380, we first measured cytosolic Ca^{2+} levels in fluo-3–loaded SH-SY5Y cells. Interestingly, PNU 282987 at the protective concentration of 10μM did not induce a measurable intracellular Ca^{2+} increase, but in the presence of the allosteric modulator PNU 120596, it gave a robust intracellular Ca^{2+} rise, which was blocked by the α7 nAChR-selective antagonists BGT and MLA. These results are in agreement with those previously reported in bovine chromaffin cells (del Barrio et al., 2011) or PC12 cells (Dickinson et al., 2007). Unlike PNU 282987, 5IA 85380 alone, at a protective concentration, was able to increase intracellular Ca^{2+} levels; this response was blocked by selective β2* antagonists such as DHβE and α-conotoxin MII but not by α7 (Dickinson et al., 2007) or α3β4-selective antagonists, which corroborates the selectivity of 5IA 85380 for β2* nAChR subtype (Dajas-Bailador et al., 2002a; Mukhin et al., 2000).

The observation that the α7 agonist exerted its protective effect without apparently inducing intracellular Ca^{2+} increases is against the belief that Ca^{2+} is the second messenger responsible for kinases' intracellular activation involved in neuroprotection mediated by α7 nAChR (Dajas-Bailador et al., 2002b; Donnelly-Roberts et al., 1996; Ferchmin et al., 2003). However, examination of the protective efficacy of PNU 282987 in a medium without Ca^{2+} corroborated the Ca^{2+} independence of the protective effect. In fact, when Ca^{2+} was forced to increase via α7 nAChR activation, with the aid of the positive allosteric modulator, protection was lost in a Ca^{2+}-dependent way. In contrast, the protective effect of 5IA 85380 was dependent on extracellular Ca^{2+}, which corroborates a previous study in which this agonist offered Ca^{2+}-dependent neuroprotection against glutamate-induced toxicity (Ueda et al., 2008). Taken together, these results indicate that α7 nAChR protection is Ca^{2+} independent, whereas β2* nAChR protection is Ca^{2+} dependent.

Regarding the intracellular signaling pathways involved in the neuroprotective mechanism associated with the nAChR activation, there are several intracellular kinases involved in this process (Buckingham et al., 2009). In the case of the α7 nAChR, JAK2 kinase is able to bind to this nAChR subtype under nicotinic stimulation to transduce signals to PI3 kinase and Akt (Shaw et al., 2002), which results in neuroprotection. Our results show that only stimulation of the α7 and not the β2* nAChR can
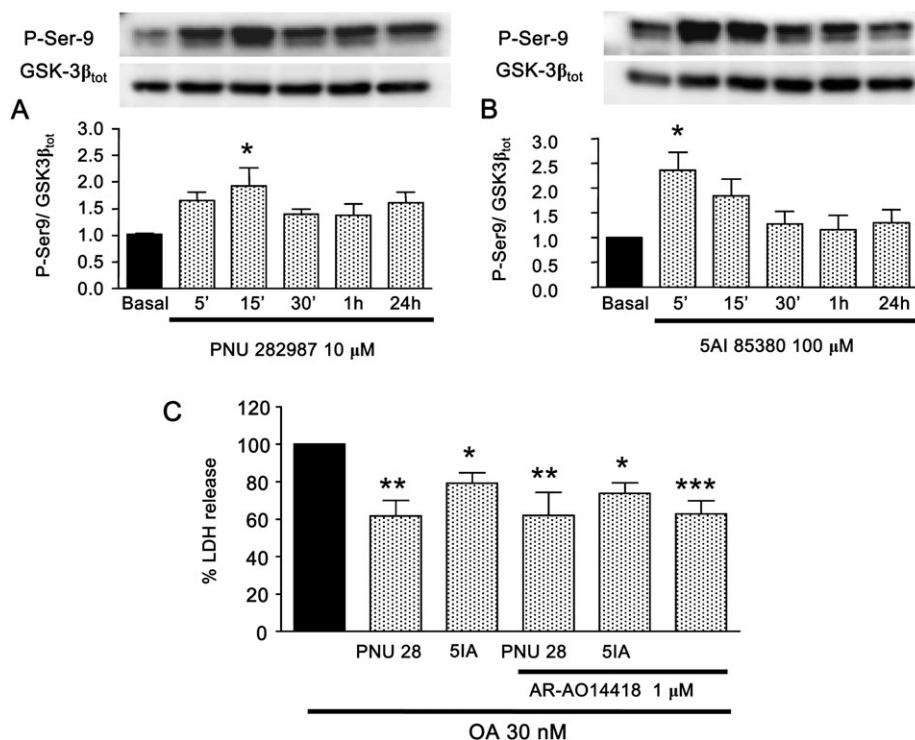
FIG. 7. GSK-3β phosphorylation and implication in neuroprotection mediated by PNU 282987 and 5IA 85380. Time course of Ser-9 were analyzed by Western blot in protein extracts obtained from cells treated with 10μM PNU 282987 (A) and 100μM 5IA 85380 (B) during different time periods. (C) Shows the effect of the GSK-3β inhibitor 1μM AR-A014418 on the neuroprotection mediated by 10μM PNU 282987 (''PNU 28'') and 100μM 5IA 85380 (''5IA'') against OA-induced toxicity. Each value is a mean of at least four different cultures ± SEM. *$p < 0.05$ with respect to the basal in (A and B); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ with respect to OA alone (C).

induce JAK2 phosphorylation. Activation of the JAK2/PI3K/Akt route can be independent of Ca^{2+} (Chernyavsky et al., 2009), which agrees with the results of this study. The implication of this kinase in protection mediated by α7 nAChR was confirmed when protection induced by PNU 282987, but not by 5IA 85380, was lost in cells treated with the JAK2 inhibitor AG-490. Furthermore, the loss of protection by the α7 agonist in the presence of the allosteric modulator seems to be related to the loss of phosphorylation of JAK2 under similar conditions, as determined by the Western blot experiments (Fig. 4C).

Continuing with the intracellular signaling pathways, data from other groups (Buckingham et al., 2009; Quesada et al., 2008) as well as our own (Arias et al., 2005; Canas et al., 2007) have shown that activation of PI3K/Akt is related to cell survival. In line with these observations, we have seen that both PNU 282987 and 5IA 85380 increased Akt phosphorylation. Furthermore, the PI3K inhibitor LY 294002 blocked the protective effects of the α7 and β2* agonist. Therefore, this signaling pathway is shared by both nAChR subtypes. In the case of α7 nAChR, it has been described that one of the targets of JAK2 is precisely PI3K/Akt (Shaw et al., 2002); these kinases can be activated in a Ca^{2+}-dependent (Soletti et al., 2010) or independent manner (Chernyavsky et al., 2009).

Based on a previous study that showed that non-α7 nAChRs are able to activate MAPK/ERK in a Ca^{2+}-dependent way

(Nakayama et al., 2001), we evaluated if 5IA 85380 could induce ERK 1/2 phosphorylation. In our experimental conditions, ERK 1/2 activation was only induced by 5IA 85380. Participation of MAPK in β2* nAChR-mediated protection was supported with the experiments with the inhibitor PD 98059 (Fig. 6C).

Because both agonists activated Akt, we tried to identify Akt targets that could be related to the mechanism of action of OA, which induces τ hyperphosphorylation. One of the kinases that phosphorylates τ is GSK-3β (Grimes and Jope, 2001), and it is known that P-Akt inactivates GSK-3β by phosphorylation of its Ser-9 (Cross et al., 1995; Srivastava and Pandey, 1998). We observed that α7 and β2* nAChR stimulation increased phosphorylation of Ser-9-GSK-3β after 15 min, but it decreased after 24 h exposure to the agonist. This temporal pattern excludes sustained inactivation of GSK-3β as a prerequisite for the observed neuroprotection. However, protection mediated by both nAChR subtypes does seem to be related to inhibition of GSK-3β because the combination of AR-A014418 (a GSK-3β inhibitor) with the α7 or β2* nAChR agonist did not afford additional protective effects (Fig. 7G).
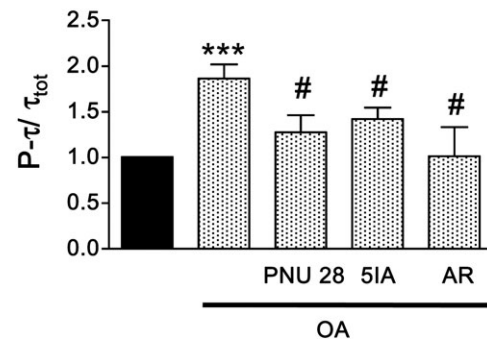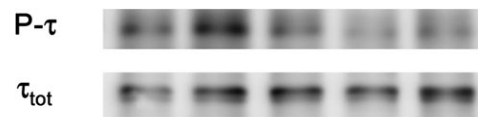
After exposing the cells for 16 h to OA, total GSK-3β expression was increased by almost twofold. Increases in the expression of GSK-3β have been reported in glial progenitor cells of AD patients treated with Aβ (He and Shen, 2009) as well as in postmortem brain of Alzheimer patients (Lau et al.,
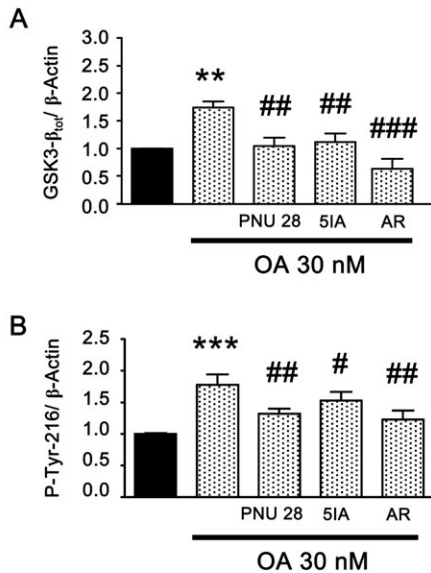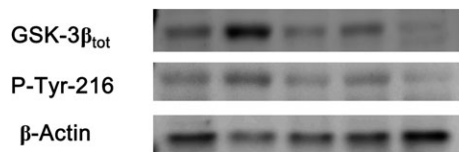
FIG. 8. GSK-3β expression/phosphorylation under the treatment of OA. Protein samples were collected from cells that were preincubated for 24 h with 10μM PNU 282987, 100μM 5IA 85380, or 1μM AR-A014418, and subsequently, this pretreatment was replaced by medium with 30nM OA for 16 h. Histograms represent the densitometric quantification of total GSK-3β (A) and total Tyr-216 (B) phosphorylation, using β-actin for normalization. Each value is a mean of four different cultures ± SEM. **$p < 0.01$, ***$p < 0.001$ with respect to basal levels and #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ with respect to OA alone.

FIG. 9. Effect of nicotinic agonists and AR-A014418 on Ser-396/404 τ phosphorylation in cells treated with OA. Protein samples were collected from cells that were preincubated for 24 h with 10μM PNU 282987, 100μM 5IA 85380, or 1μM AR-A014418, and subsequently, medium was replaced by 30nM OA for 16 h. Histograms represent the densitometric quantification of P-Ser-396/404-τ using τ total for normalization. Each value is a mean of five different cultures ± SEM. ***$p < 0.001$ with respect to basal levels and #$p < 0.05$ with respect to OA alone.

1999). In fact, there are several transgenic models that overexpress GSK-3β and reproduce many pathophysiological features of AD (Engel et al., 2006). Taken together, OA seems to increase the expression of GSK-3β as observed in AD patients. Therefore, reduction of total GSK-3β expression induced by nicotinic agonists and the GSK-3β inhibitor could be an interesting therapeutic approach. Recent studies have shown that lithium, a GSK-3β inhibitor, and tobacco extracts are able to reduce messenger RNA and GSK-3β protein levels, respectively (Mendes et al., 2009; Tian et al., 2009). Moreover, total Tyr-216-GSK-3β phosphorylation (active levels) was also increased by OA alone, and this increase was significantly reduced by nAChR agonists and AR-A014418.

GSK-3β specifically phosphorylates Ser-396/404 of τ (PHF-1, paired helicoidal filament-1) both in cell cultures (Lovestone et al., 1994; Tanaka et al., 1998) and in vivo models (Spittaels et al., 2000). τ Hyperphosphorylation by PP2A inhibition induces a disruption of microtubules causing cell death in SH-SY5Y cells treated with OA (Tanaka et al., 1998). This microtubule destabilization compromises axonal transport and induces neurodegeneration of neurons with tangles, a widely accepted characteristic in brains of AD patients (Alonso et al.,

1994, 1997). We have observed that OA almost doubled τ phosphorylation on its Ser-396/404 and that the α7 and β2* nicotinic agonists, together with the GSK-3β inhibitor, reduced Ser-396/404-τ-hyperphosphorylation. Furthermore, cytoskeleton alterations caused by OA were partially reverted with nAChR agonist treatment (Fig. 10).

Apart from reducing τ phosphorylation, nicotinic stimulation also confers neuroprotection and anti-inflammatory actions by activation of some of the intracellular pathways here described (Egea et al., 2007; Kawamata and Shimohama, 2011; Marrero and Bencherif, 2009; Parada et al., 2010), which could also be contributing to the protective effects observed by both α7 and β2 nAChR activation.

In conclusion, both α7 and β2* nAChR activation can mediate protection against OA. α7 nAChR-mediated protection involves a Ca^{2+}-independent JAK2/PI3K/Akt/GSK-3β signaling pathway. In contrast, the Ca^{2+}-dependent protective mechanism mediated by β2* nAChR relates to MEK/ERK 1/2, PI3K/Akt activation, and GSK-3β inactivation. However, both signaling pathways converge on GSK-3β downregulation and reduction of phosphorylation of τ in cells exposed to OA, which represents a good model of neuronal death related to the characteristic τ hyperphosphorylation found in AD patients.

FUNDING
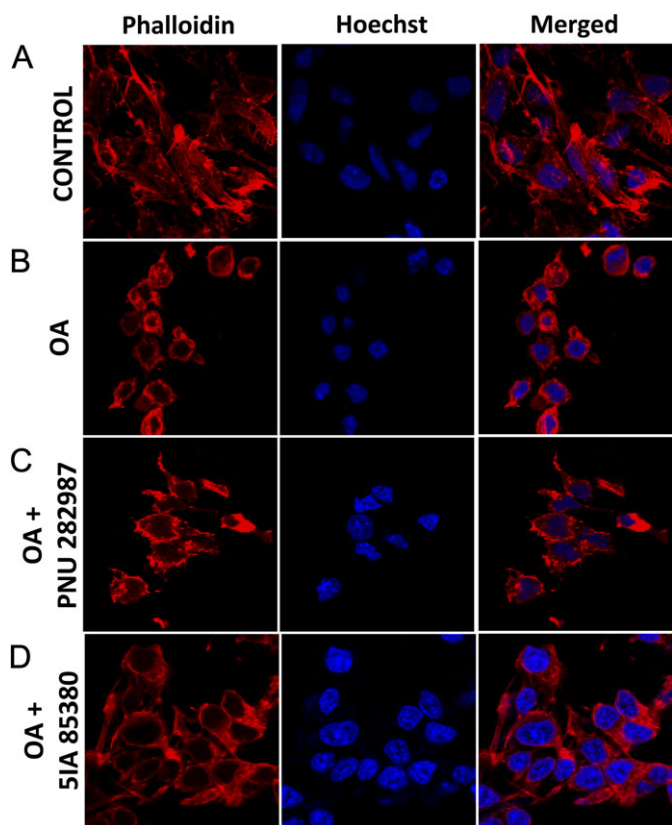
FIG. 10. PNU 282987 and 5IA 85380 partially prevent cytoarchitecture disruption elicited by OA. Photomicrographs of double staining of SH-SY5Y marked with rhodamine-phalloidin (filamentous actin marker) and Hoechst (nuclear marker). Cells were incubated with PNU 282987 (10µM) (C) or 5IA 85380 (100µM) (D) during 24 h, and then 30nM OA was added for 5 h. A control group without any treatment (A) and another just treated with 30nM OA for 5 h were run in parallel (B). As shown in the photomicrographs, PNU 282987 and 5IA 85380 prevented partially the cytoarchitecture alterations caused by OA.

ACKNOWLEDGMENTS

REFERENCES

Alonso, A. C., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K. (1994). Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 5562–5566.

Alonso, A. D., Grundke-Iqbal, I., Barra, H. S., and Iqbal, K. (1997). Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 298–303.

Alvarez, G., Muñoz-Montaño, J. R., Satrústegui, J., Avila, J., Bogónez, E., and Díaz-Nido, J. (2002). Regulation of tau phosphorylation and protection against beta-amyloid-induced neurodegeneration by lithium. Possible implications for Alzheimer's disease. *Bipolar Disord.* **4,** 153–165.

Arias, E., Gallego-Sandin, S., Villarroya, M., Garcia, A. G., and 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,** 1346–1353.

Avila, J. (2000). Tau aggregation into fibrillar polymers: taupathies. *FEBS Lett.* **476,** 89–92.

Bitner, R. S., Nikkel, A. L., Markosyan, S., Otte, S., Puttfarcken, P., and Gopalakrishnan, M. (2009). Selective alpha7 nicotinic acetylcholine receptor activation regulates glycogen synthase kinase3beta and decreases tau phosphorylation in vivo. *Brain Res.* **1265,** 65–74.

Bodnar, A. L., Cortes-Burgos, L. A., Cook, K. K., Dinh, D. M., Groppi, V. E., Hajos, M., Higdon, N. R., Hoffmann, W. E., Hurst, R. S., Myers, J. K., *et al.* (2005). Discovery and structure-activity relationship of quinuclidine benzamides as agonists of alpha7 nicotinic acetylcholine receptors. *J. Med. Chem.* **48,** 905–908.

Buckingham, S. D., Jones, A. K., Brown, L. A., and Sattelle, D. B. (2009). Nicotinic acetylcholine receptor signalling: roles in Alzheimer's disease and amyloid neuroprotection. *Pharmacol. Rev.* **61,** 39–61.

Busciglio, J., Lorenzo, A., Yeh, J., and Yankner, B. A. (1995). Beta-amyloid fibrils induce tau phosphorylation and loss of microtubule binding. *Neuron* **14,** 879–888.

Cabado, A. G., Leira, F., Vieytes, M. R., Vieites, J. M., and Botana, L. M. (2004). Cytoskeletal disruption is the key factor that triggers apoptosis in okadaic acid-treated neuroblastoma cells. *Arch. Toxicol.* **78,** 74–85.

Canas, N., Valero, T., Villarroya, M., Montell, E., Verges, J., Garcia, A. G., and Lopez, M. G. (2007). Chondroitin sulfate protects SH-SY5Y cells from oxidative stress by inducing heme oxygenase-1 via phosphatidylinositol 3-kinase/Akt. *J. Pharmacol. Exp. Ther.* **323,** 946–953.

Chernyavsky, A. I., Arredondo, J., Qian, J., Galitovskiy, V., and Grando, S. A. (2009). Coupling of ionic events to protein kinase signaling cascades upon activation of alpha7 nicotinic receptor: cooperative regulation of alpha2-integrin expression and Rho kinase activity. *J. Biol. Chem.* **284,** 22140–22148.

Cho, J. H., and Johnson, G. V. (2004). Glycogen synthase kinase 3 beta induces caspase-cleaved tau aggregation in situ. *J. Biol. Chem.* **279,** 54716–54723.

Cole, A., Frame, S., and Cohen, P. (2004). Further evidence that the tyrosine phosphorylation of glycogen synthase kinase-3 (GSK3) in mammalian cells is an autophosphorylation event. *Biochem. J.* **377,** 249–255.

Cowan, C. M., Chee, F., Shepherd, D., and Mudher, A. (2010). Disruption of neuronal function by soluble hyperphosphorylated tau in a Drosophila model of tauopathy. *Biochem. Soc. Trans.* **38,** 564–570.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378,** 785–789.

Cummings, J. L. (2004). Treatment of Alzheimer's disease: current and future therapeutic approaches. *Rev. Neurol. Dis.* **1,** 60–69.

Dajas-Bailador, F., and Wonnacott, S. (2004). Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol. Sci.* **25,** 317–324.

Dajas-Bailador, F. A., Mogg, A. J., and Wonnacott, S. (2002a). Intracellular Ca2+ signals evoked by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca2+ channels and Ca2+ stores. *J. Neurochem.* **81,** 606–614.

Dajas-Bailador, F. A., Soliakov, L., and Wonnacott, S. (2002b). Nicotine activates the extracellular signal-regulated kinase 1/2 via the alpha7 nicotinic acetylcholine receptor and protein kinase A, in SH-SY5Y cells and hippocampal neurones. *J. Neurochem.* **80,** 520–530.

De Vos, K. J., Grierson, A. J., Ackerley, S., and Miller, C. C. (2008). Role of axonal transport in neurodegenerative diseases. *Annu. Rev. Neurosci.* **31,** 151–173.

del Barrio, L., Egea, J., Leon, R., Romero, A., Ruiz, A., Montero, M., Alvarez, J., and Lopez, M. G. (2011). Calcium signalling mediated through

alpha7 and non-alpha7 nAChR stimulation is differentially regulated in bovine chromaffin cells to induce catecholamine release. *Br. J. Pharmacol.* **162,** 94–110.

Dickinson, J. A., Hanrott, K. E., Mok, M. H., Kew, J. N., and Wonnacott, S. (2007). Differential coupling of alpha7 and non-alpha7 nicotinic acetylcholine receptors to calcium-induced calcium release and voltage-operated calcium channels in PC12 cells. *J. Neurochem.* **100,** 1089–1096.

Donnelly-Roberts, D. L., Xue, I. C., Arneric, S. P., and Sullivan, J. P. (1996). In vitro neuroprotective properties of the novel cholinergic channel activator (ChCA), ABT-418. *Brain Res.* **719,** 36–44.

Egea, J., Rosa, A. O., Cuadrado, A., Garcia, A. G., and Lopez, M. G. (2007). Nicotinic receptor activation by epibatidine induces heme oxygenase-1 and protects chromaffin cells against oxidative stress. *J. Neurochem.* **102,** 1842–1852.

Engel, T., Hernandez, F., Avila, J., and Lucas, J. J. (2006). Full reversal of Alzheimer's disease-like phenotype in a mouse model with conditional overexpression of glycogen synthase kinase-3. *J. Neurosci.* **26,** 5083–5090.

Ferchmin, P. A., Perez, D., Eterovic, V. A., and de Vellis, J. (2003). Nicotinic receptors differentially regulate N-methyl-D-aspartate damage in acute hippocampal slices. *J. Pharmacol. Exp. Ther.* **305,** 1071–1078.

Gentry, C. L., and Lukas, R. J. (2002). Regulation of nicotinic acetylcholine receptor numbers and function by chronic nicotine exposure. *Curr. Drug Targets CNS Neurol. Disord.* **1,** 359–385.

Gong, C. X., Grundke-Iqbal, I., and Iqbal, K. (1994). Dephosphorylation of Alzheimer's disease abnormally phosphorylated tau by protein phosphatase-2A. *Neuroscience* **61,** 765–772.

Gong, C. X., Shaikh, S., Wang, J. Z., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K. (1995). Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. *J. Neurochem.* **65,** 732–738.

Gong, C. X., Singh, T. J., Grundke-Iqbal, I., and Iqbal, K. (1993). Phosphoprotein phosphatase activities in Alzheimer disease brain. *J. Neurochem.* **61,** 921–927.

Grimes, C. A., and Jope, R. S. (2001). CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. *J. Neurochem.* **78,** 1219–1232.

Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., and Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 4913–4917.

Grundke-Iqbal, I., Vorbrodt, A. W., Iqbal, K., Tung, Y. C., Wang, G. P., and Wisniewski, H. M. (1988). Microtubule-associated polypeptides tau are altered in Alzheimer paired helical filaments. *Brain Res.* **464,** 43–52.

Haass, C., and Selkoe, D. J. (2007). Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat. Rev. Mol. Cell Biol.* **8,** 101–112.

Haydar, S. N., and Dunlop, J. (2010). Neuronal nicotinic acetylcholine receptors—targets for the development of drugs to treat cognitive impairment associated with schizophrenia and Alzheimer's disease. *Curr. Top. Med. Chem* **10,** 144–152.

He, P., and Shen, Y. (2009). Interruption of beta-catenin signaling reduces neurogenesis in Alzheimer's disease. *J. Neurosci.* **29,** 6545–6557.

Hughes, K., Nikolakaki, E., Plyte, S. E., Totty, N. F., and Woodgett, J. R. (1993). Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *EMBO J.* **12,** 803–808.

Hurst, R. S., Hajos, M., Raggenbass, M., Wall, T. M., Higdon, N. R., Lawson, J. A., Rutherford-Root, K. L., Berkenpas, M. B., Hoffmann, W. E., Piotrowski, D. W., *et al.* (2005). A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. *J. Neurosci.* **25,** 4396–4405.

Irizarry, M. C., Soriano, F., McNamara, M., Page, K. J., Schenk, D., Games, D., and Hyman, B. T. (1997). Abeta deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse. *J. Neurosci.* **17,** 7053–7059.

Kawamata, J., and Shimohama, S. (2011). Stimulating nicotinic receptors trigger multiple pathways attenuating cytotoxicity in models of Alzheimer's and Parkinson's diseases. *J. Alzheimers Dis.* **24,** 95–109.

Kihara, T., Shimohama, S., Sawada, H., Honda, K., Nakamizo, T., Shibasaki, H., Kume, T., and Akaike, A. (2001). Alpha 7 nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block A beta-amyloid-induced neurotoxicity. *J. Biol. Chem.* **276,** 13541–13546.

Kihara, T., Shimohama, S., Urushitani, M., Sawada, H., Kimura, J., Kume, T., Maeda, T., and Akaike, A. (1998). Stimulation of alpha4beta2 nicotinic acetylcholine receptors inhibits beta-amyloid toxicity. *Brain Res.* **792,** 331–334.

Lau, K. F., Miller, C. C., Anderton, B. H., and Shaw, P. C. (1999). Molecular cloning and characterization of the human glycogen synthase kinase-3beta promoter. *Genomics* **60,** 121–128.

Lee, V. M., and Trojanowski, J. Q. (1992). The disordered neuronal cytoskeleton in Alzheimer's disease. *Curr. Opin. Neurobiol.* **2,** 653–656.

Liu, T., Perry, G., Chan, H. W., Verdile, G., Martins, R. N., Smith, M. A., and Atwood, C. S. (2004). Amyloid-beta-induced toxicity of primary neurons is dependent upon differentiation-associated increases in tau and cyclin-dependent kinase 5 expression. *J. Neurochem.* **88,** 554–563.

Lovestone, S., Reynolds, C. H., Latimer, D., Davis, D. R., Anderton, B. H., Gallo, J. M., Hanger, D., Mulot, S., Marquardt, B., Stabel, S., *et al.* (1994). Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. *Curr. Biol.* **4,** 1077–1086.

Lucas, J. J., Hernandez, F., Gomez-Ramos, P., Moran, M. A., Hen, R., and Avila, J. (2001). Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J.* **20,** 27–39.

Luo, S., Kulak, J. M., Cartier, G. E., Jacobsen, R. B., Yoshikami, D., Olivera, B. M., and McIntosh, J. M. (1998). Alpha-conotoxin AuIB selectively blocks alpha3 beta4 nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release. *J. Neurosci.* **18,** 8571–8579.

Marrero, M. B., and Bencherif, M. (2009). Convergence of alpha 7 nicotinic acetylcholine receptor-activated pathways for anti-apoptosis and anti-inflammation: central role for JAK2 activation of STAT3 and NF-kappaB. *Brain Res.* **1256,** 1–7.

Mendes, C. T., Mury, F. B., de Sa Moreira, E., Alberto, F. L., Forlenza, O. V., Dias-Neto, E., and Gattaz, W. F. (2009). Lithium reduces Gsk3b mRNA levels: implications for Alzheimer disease. *Eur. Arch. Psychiatry Clin. Neurosci.* **259,** 16–22.

Mukhin, A. G., Gundisch, D., Horti, A. G., Koren, A. O., Tamagnan, G., Kimes, A. S., Chambers, J., Vaupel, D. B., King, S. L., Picciotto, M. R., *et al.* (2000). 5-Iodo-A-85380, an alpha4beta2 subtype-selective ligand for nicotinic acetylcholine receptors. *Mol. Pharmacol.* **57,** 642–649.

Nakayama, H., Numakawa, T., Ikeuchi, T., and Hatanaka, H. (2001). Nicotine-induced phosphorylation of extracellular signal-regulated protein kinase and CREB in PC12h cells. *J. Neurochem.* **79,** 489–498.

Parada, E., Egea, J., Romero, A., del Barrio, L., Garcia, A. G., and Lopez, M. G. (2010). Poststress treatment with PNU282987 can rescue SH-SY5Y cells undergoing apoptosis via alpha7 nicotinic receptors linked to a Jak2/Akt/HO-1 signaling pathway. *Free Radic. Biol. Med.* **49,** 1815–1821.

Peng, X., Katz, M., Gerzanich, V., Anand, R., and Lindstrom, J. (1994). Human alpha 7 acetylcholine receptor: cloning of the alpha 7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha 7 homomers expressed in Xenopus oocytes. *Mol. Pharmacol.* **45,** 546–554.

Perez, M., Hernandez, F., Gomez-Ramos, A., Smith, M., Perry, G., and Avila, J. (2002). Formation of aberrant phosphotau fibrillar polymers in neural cultured cells. *Eur. J. Biochem.* **269,** 1484–1489.

Picciotto, M. R., and Zoli, M. (2008). Neuroprotection via nAChRs: the role of nAChRs in neurodegenerative disorders such as Alzheimer's and Parkinson's disease. *Front. Biosci.* **13,** 492–504.

Pizzi, M., Valerio, A., Arrighi, V., Galli, P., Belloni, M., Ribola, M., Alberici, A., Spano, P., and Memo, M. (1995). Inhibition of glutamate-induced neurotoxicity by a tau antisense oligonucleotide in primary culture of rat cerebellar granule cells. *Eur. J. Neurosci.* **7,** 1603–1613.

Price, J. L., Davis, P. B., Morris, J. C., and White, D. L. (1991). The distribution of tangles, plaques and related immunohistochemical markers in healthy aging and Alzheimer's disease. *Neurobiol. Aging* **12,** 295–312.

Quesada, A., Lee, B. Y., and Micevych, P. E. (2008). PI3 kinase/Akt activation mediates estrogen and IGF-1 nigral DA neuronal neuroprotection against a unilateral rat model of Parkinson's disease. *Dev. Neurobiol.* **68,** 632–644.

Shaw, S., Bencherif, M., and Marrero, M. B. (2002). Janus kinase 2, an early target of alpha 7 nicotinic acetylcholine receptor-mediated neuroprotection against Abeta-(1-42) amyloid. *J. Biol. Chem.* **277,** 44920–44924.

Soletti, R. C., del Barrio, L., Daffre, S., Miranda, A., Borges, H. L., Moura-Neto, V., Lopez, M. G., and Gabilan, N. H. (2010). Peptide gomesin triggers cell death through L-type channel calcium influx, MAPK/ERK, PKC and PI3K signaling and generation of reactive oxygen species. *Chem. Biol. Interact.* **186,** 135–143.

Spittaels, K., Van den Haute, C., Van Dorpe, J., Geerts, H., Mercken, M., Bruynseels, K., Lasrado, R., Vandezande, K., Laenen, I., Boon, T., *et al.* (2000). Glycogen synthase kinase-3beta phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice. *J. Biol. Chem.* **275,** 41340–41349.

Srivastava, A. K., and Pandey, S. K. (1998). Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. *Mol. Cell. Biochem.* **182,** 135–141.

Stambolic, V., and Woodgett, J. R. (1994). Mitogen inactivation of glycogen synthase kinase-3 beta in intact cells via serine 9 phosphorylation. *Biochem. J.* **303**(Pt 3), 701–704.

Tanaka, T., Zhong, J., Iqbal, K., Trenkner, E., and Grundke-Iqbal, I. (1998). The regulation of phosphorylation of tau in SY5Y neuroblastoma cells: the role of protein phosphatases. *FEBS Lett.* **426,** 248–254.

Tian, D., Zhu, M., Li, J., Ma, Y., and Wu, R. (2009). Cigarette smoke extract induces activation of beta-catenin/TCF signaling through inhibiting GSK3beta in human alveolar epithelial cell line. *Toxicol. Lett.* **187,** 58–62.

Tian, Q., Lin, Z. Q., Wang, X. C., Chen, J., Wang, Q., Gong, C. X., and Wang, J. Z. (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.

Tizabi, Y., Manaye, K. F., Smoot, D. T., and Taylor, R. E. (2004). Nicotine inhibits ethanol-induced toxicity in cultured cerebral cortical cells. *Neurotox. Res.* **6,** 311–316.

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

Ueda, M., Iida, Y., Kitamura, Y., Kawashima, H., Ogawa, M., Magata, Y., and Saji, H. (2008). 5-Iodo-A-85380, a specific ligand for alpha 4 beta 2 nicotinic acetylcholine receptors, prevents glutamate neurotoxicity in rat cortical cultured neurons. *Brain Res.* **1199,** 46–52.

Vale, C., and Botana, L. M. (2008). Marine toxins and the cytoskeleton: okadaic acid and dinophysistoxins. *FEBS J.* **275,** 6060–6066.

Wang, J. Z., Gong, C. X., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K. (1995). Dephosphorylation of Alzheimer paired helical filaments by protein phosphatase-2A and -2B. *J. Biol. Chem.* **270,** 4854–4860.

Wang, Q. M., Fiol, C. J., DePaoli-Roach, A. A., and Roach, P. J. (1994). Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J. Biol. Chem.* **269,** 14566–14574.

Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., and Delon, M. R. (1982). Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science* **215,** 1237–1239.

Yoon, S. Y., Choi, J. E., Choi, J. M., and Kim, D. H. (2008). Dynein cleavage and microtubule accumulation in okadaic acid-treated neurons. *Neurosci. Lett.* **437,** 111–115.