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Neurotoxicity induced by okadaic acid in the human neuroblastoma SH-SY5Y line can be differentially prevented by α7 and β2* nicotinic stimulation

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Running title: nAChR reduce okadaic acid neurotoxicity

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ABSTRACT

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 5Iodo-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 Ca^{2+} and involved the intracellular signaling pathway JAK2/PI3K/Akt. When Ca^{2+} 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 Ca^{2+}-dependent and implicated the signaling pathways PI3K/Akt and ERK1/2. Both α7 and β2* nAChR activation converged on down-regulation of GSK3β 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.

INTRODUCTION

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 since beta-amyloid can trigger tau phosphorylation and the subsequent degeneration of the affected neurons (Busciglio et al. 1995; Alvarez et al., 2002; Liu et al., 2004). It has also been shown that beta-amyloid fibrils can induce neurotoxicity and tau hyperphosphorylation without plaque formation; it therefore seems that beta-amyloid pathology could precede hyperphophorylation of tau 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; Grundke-Iqbal *et al.* 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; Alonso *et al.* 1997; De Vos *et al.* 2008) and also for cell death in *"in vitro"* models of τ hyperphosphorylation (Cowan *et al.*; 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 PP-1, PP-2A and PP-2B (Gong *et al.* 1994; Wang *et al.* 1995) and their activity has been shown to be diminished in brain tissue from Alzheimer´s disease patients (Gong *et al.* 1995; Gong *et al.* 1993).

Okadaic acid, 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 hyperphosphorylation 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 Alzheimer's disease (Arias *et al.* 2005; Cowan *et al.*; Perez *et al.* 2002; Tanaka *et al.* 1998), in order to investigate neuroprotective strategies.

There is a body of evidence for nAChRs exerting neuroprotective effects against a variety of insults (Dajas-Bailador and Wonnacott 2004; Picciotto and Zoli 2008). With respect to Alzheimer's disease, 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). 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 independent 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 ERK1/2. 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, PD 98059 were purchased from Tocris Cookson Inc. (Bristol, UK). Okadaic acid, 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 nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release (Lou 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, 1 mM 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 sub-cultured in 48-well plates at a seeding density of 1 x 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 intracellular lactate dehydrogenase (LDH) activity was measured spectrophotometrically 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 $NaHCO_3$, 5.5 glucose; pH 7.4) and incubated with the membrane-permeable Ca^{2+}-sensitive dye fluo-3 AM (10 μM) 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, 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 40 mM $MnCl_2$ (F_{min}). Maximum fluorescence responses were calculated as a percentage of F_{max} - F_{min}.

Immunoblotting

SH-SY5Y cells were washed once with cold phosphate-buffered saline 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, 1mmol/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 (SDS–PAGE) and transferred to PVDF membranes (Amersham). Membranes were incubated with the following antibodies: anti-extracellular regulated kinase 1/2 (ERK 1/2) and anti-phospho-ERK1/2 at 1:1000 (Santa Cruz Biotechnology Inc., Heidelberg, Germany); anti-JAK-2 , anti-phospho-Tyr-211-JAK-2, 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 Signalling, Izasa S.A., Barcelona, Spain); anti-β-actin at 1:100000 (Sigma, Madrid, Spain). Membranes were also incubated with Anti-phospho-Ser-396/404-Tau and Anti-Tau at 1:1000 which were previously used by Lucas et al. (2001). Appropriate peroxidase-conjugated secondary antibodies at 1:10000 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 4 groups: two were treated with EMEM, one with 10 μM PNU282987 and another one with 100 μM 5IA 85380 for 24 h. Then, the control group was maintained in EMEM and the rest of the groups were treated with okadaic acid for 5 h. After exposure of

the cells to the different treatments, they were rinsed three times with PBS (NaC1 9g/L, NaH_2PO_4 10 mM, K_2HPO_4 10 mM). 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 phalloidin (Sigma-Aldrich, Madrid, Spain) 1:1000 in PBS. Coverslips were then thoroughly washed with PBS. In the second rinsing, Hoechst (Invitrogen, Madrid, Spain) 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 30 nM 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.*) or 5IA 85380, respectively, or with the α7 allosteric modulator PNU 120596. With pre-incubation 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 significant but partial protection against OA-induced toxicity at 10 and 30 μM (fig. 1 A). 5IA 85380 also produced significant protection at 100 μM (fig. 1 B). The α7 positive allosteric modulator PNU 120596 alone showed no protection at any of the concentration assayed (0.3 to 10 μM) (fig. 1 C)

Intracellular Ca^{2+} 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 10 μM. However, when the agonist was added to cells pre-

incubated 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 by activation of nAChR since the signal was significantly attenuated by the non-selective nAChR antagonist mecamylamine. In particular, these Ca^{2+} signals could be attributed to stimulation of the α7 subtype nAChR since the selective α7 nAChR antagonists αBGT and MLA completely inhibited the increases in fluorescence (fig. 2 A).

5IA 85380, at the protective concentration of 100 μM, produced a significant intracellular Ca^{2+} signal by itself (fig. 2B). Selective β2* antagonists such as DHβE and α-conotoxin MII or mecamylamine were able to significantly block Ca^{2+} signals induced by 5IA 85380. However, α3β4 (α-conotoxin AuIB) or α7 (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 β2-containing nAChRs while PNU 282987 is activating α7 nAChRs.

Ca^{2+}-dependence in the protective effects of 5IA 85380 and PNU 282987

To determine the influence of Ca^{2+} entry mediated by α7 and β2* nAChRs in the neuroprotective effects afforded by PNU 282987 and 5IA 85380, we performed experiments in the absence or presence of extracellular Ca^{2+}. Since 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 pre-incubation 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 hours before exposing the cells to 16 h to OA (fig. 3 A). In the case of 5IA 85380, protection was significant following 1 h pre-incubation (fig. 3 B). For

comparability we selected a 4 h preincubation period for both agonists since this length of exposure produced a level of protection similar to that seen following 24 h pre-incubation. 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. 3 D) while that induced by PNU 282987 was not (fig. 3 C). Moreover, when cells were pre- and co-incubated with the α7 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. 3 C). These results suggest that Ca^{2+} entry via β2* nAChR activation is required to afford a neuroprotective effect. However, in the case of the α7 nAChR, PNU 282987 exerted a Ca^{2+}-independent neuroprotective effect. Furthermore, as shown by the experiments with the α7 agonist in the presence of the α7 nAChR allosteric modulator that promotes Ca^{2+} entry though the α7 nAChR subtype (Fig. 2A), protection was lost in a Ca^{2+} dependent way (Fig. 3 C).

JAK-2 is involved in protection mediated by α7 but not by β2* nAChRs

JAK-2 is a protein that can be physically associated with α7 nAChRs and activated when nicotine binds to this receptor subtype (Shaw *et al.* 2002). To determine if α7 and/or β2* nAChR subtype stimulation could activate this kinase under our experimental conditions, SH-SY5Y cells were incubated with PNU 282987 10 μM, PNU 282987 10 μM plus PNU 120596 10 μM or 5IA 85380 100 μM for different times (5, 15, 30 min and 1 or 24 h) and JAK-2 phosphorylation was then analyzed by western blotting. Cells treated with PNU 282987 10 μM showed maximally phosphorylated JAK-2 after 15 min incubation; thereafter, phospho-JAK-2 decreased to basal levels (fig. 4 A). In contrast, 5IA 85380 failed to promote

the activation of JAK-2 at any of the times tested (fig. 4 B). Also, no alteration in phospho-JAK-2 levels was observed when the α7 agonist was incubated in the presence of the α7 allosteric modulator (fig. 4 C).

JAK-2 has been previously shown to be involved in neuroprotection 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 JAK-2 phosphorylation, we evaluated if the protective effect of PNU 282987 against OA would be lost in the presence of a JAK-2 inhibitor, AG-490. To corroborate that JAK-2 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 4 D, the protective effect of PNU 282987 was completely prevented by pre- (15 minutes before) and co-incubation with AG-490, but this compound did not affect protection afforded by 5IA 85380. Therefore, these results indicate that JAK-2 is linked to α7 nAChR-mediated survival. Moreover, the presence of the α7 allosteric modulator prevented PNU 282987-induced JAK phosphorylation (fig. 4 C), 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 JAK-2 (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 minutes for both agonists (fig. 5 A and B). To determine if protection afforded by α7 and β2* nAChR was dependent on the PI3K/Akt pathway, cells were pre-(15 minutes before) and co-incubated with a PI3K

inhibitor, LY 294002 (3 μM). Protection elicited by PNU 282987 and 5IA 85380 against OA-toxicity was prevented by the inhibitor LY 294002 (fig. 5 C). Taken together, these results indicate that protection obtained with both nicotinic agonists is dependent on activation of PI3K/Akt.

ERK 1/2 is involved in the neuroprotective effect mediated by 5IA 85380 but not by PNU 282987.

The 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 6 A, PNU 282987 10 μM 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. 6 B). To corroborate the involvement of MAPK activation in the neuroprotective effect mediated by 5IA 85380 and not by PNU 282987, we employed a MEK inhibitor, PD 98059. As shown in figure 6 C, 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 kinase(s) 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 minutes incubation, respectively (fig. 7 A and B). Subsequently, this phosphorylation decreased over time and after 24 hours 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 7 C shows how AR-A014418 alone produced protection against OA and such protection was not statistically different from that obtained with the nicotinic agonists alone. Since 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, pre-incubated for 24 h before adding OA, induced neuroprotection we evaluated the activation and the expression 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 hours alone or in the presence of the neuroprotective compounds. As shown in figure 8 A, the expression of total GSK-3β increased after OA treatment and nicotinic agonists prevented this increased expression to almost basal levels (fig. 8 A); 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. 8 B).

α7 and β2* 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 Ser396/404 (Cho and Johnson 2004). The question of how GSK-3β activity affected the phosphorylation on τ Ser396/404 residue was raised. After OA treatment for 16 h in the presence or absence of PNU 282987, 5IA85380 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 Ser396/404 residue.

Effect of α7 and β2* nicotinic agonists on the cytoskeleton alterations caused by okadaic acid

AO, besides causing hyperphosphorylation of tau, induces changes in the cytoskeleton that are identified with strong retraction and rounding in cell morphology (Vale and Botana, 2008; Yoon et al., 2008; Cabado et al., 2004). 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 pre-incubated for 24 h with PNU282987 (10 μM) (Fig. 10C) or 5IA 85380 (100 μM) (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 (Fig. 1 A and B).

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 signalling pathways converge on the regulation of GSK3β and reduction of τ phosphorylation.

To analyse 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 10 μM did not induce a measureable 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.*) 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 while β2* nAChR protection is Ca^{2+}-dependent.

Regarding the intracellular signalling 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, JAK-2 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 induce JAK-2 phosphorylation. Activation of the JAK-2/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 JAK-2 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 JAK-2 under similar conditions, as determined by the western blot experiments (Fig. 4C).

Continuing with the intracellular signalling 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 signalling pathway is shared by both nAChR subtypes. In the case of α7 nAChR, it has been described that one of the targets of JAK-2 is precisely

PI3K/Akt (Shaw *et al.* 2002); these kinases can be activated in a Ca^{2+}-dependent (Soletti *et al.*) 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, ERK1/2 activation was only induced by 5IA 85380. Participation of MAPK in β2* nAChR-mediated protection was supported with the experiments with the inhibitor PD98059 (Fig. 6C).

Since both agonists activated Akt, we tried to identify Akt targets which 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 24h 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 since 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 two-fold. Increases in the expression of GSK-3β have been reported in glial progenitor cells of Alzheimer´s disease patients treated with Aβ (He and Shen 2009) as well as in post-mortem brain of Alzheimer patients (Lau *et al.* 1999). In fact, there are several transgenic models that over-express GSK-3β and reproduce many pathophysiological features of Alzheimer disease (Engel *et al.* 2006). Taken together, OA seems to increase the expression of GSK-3β as observed in Alzheimer´s disease 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 mRNA 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 destabilisation compromises axonal transport and induces neurodegeneration of neurons with tangles, a widely accepted characteristic in brains of Alzheimer´s disease patients (Alonso *et al.* 1994; Alonso *et al.* 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 okadaic acid were partially reverted with nAChR agonist treatment (Fig. 10).

Apart from reducing tau phosphorylation, nicotinic stimulation also confers neuroprotection and anti-inflammatory actions by activation of some of the intracellular pathways here described (Marrero and Bencherif, 2009; Kawamata and Shimohama, 2011; Parada et al., 2010; Egea et al., 2007), 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-JAK-2/PI3K/Akt/GSK-3β signalling pathway. In contrast, the Ca^{2+}-dependent protective mechanism mediated by β2* nAChR relates to MEK/ERK1/2, PI3K/Akt activation and GSK-3β inactivation. However,

both signalling pathways converge on GSK-3β down regulation and reduction of phosphorylation of τ in cells exposed to okadaic acid which represents a good model of neuronal death related to the characteristic tau hyperphosphorylation found in Alzheimer´s disease patients.

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LEGENDS TO FIGURES

Figure 1. Protective effects mediated by the nicotinic agonists PNU 282987 and 5IA 85380 and the α7 allosteric modulator PNU 120596 against okadaic acid-induced toxicity. Cells were pre-treated for 24 hours with increasing concentrations of the α7 nicotinic agonist PNU282987, the β2* nicotinic agonist 5IA 85380 or the α7 positive allosteric modulator PNU 120596. Thereafter, SH-SY5Y were incubated for 16 h with okadaic acid (OA) 30 nM and at the end of the experiment cell death was evaluated by measuring the release of LDH release 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 ± S.E.M. of triplicates of at least 4 different batches of cells. * $p< 0.05$,** $p< 0.01$ with respect to OA alone.

Figure 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 10 μM PNU 282987 alone or in cells pre-incubated during 15 min with the α7 allosteric modulator PNU 120596 (10 μM); PNU 120596 was also co-incubated with various nicotinic antagonist such as mecamylamine ("Meca" 30 μM), α-bungarotoxin ("BGT" 100 nM) or methyllycaconitine ("MLA" 100 nM). (B) Cytosolic Ca^{2+} increases induced by 5IA 85380 at 100 μM alone or in the presence of the nicotinic antagonist Meca 30 μM, BGT 100 nM, α-conotoxin-AuIB ("AuIB" 10 μM), dihydro-β-eritroidine ("DHβE" 10 μM) or α-conotoxin-MII ("MII" 200 nM). The data represent the average of maximum fluorescence increases during 10 seconds after the implementation of PNU 282987 10 μM **(A)** or 5IA 85380 100 μM **(B)**. Experiments were conducted in triplicate on at least 6 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)**.

Figure 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 (10 μM) **(A)** or 5IA 85380 (100 μM) **(B)** during 5 min, 1 h, 4 h or 24 h, then the agonist was removed and fresh EMEM was added until to complete 24 h; after this period, EMEM was replaced by OA (30 nM) 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 pre-incubated for 4 h in the presence or absence of extracellular Ca^{2+} using the same protocol described above. PNU 120596 ("PNU 12" 10 μM) was pre-incubated for 15 min and co-incubated during the application of PNU 282987 **(C)**. Data represents the mean of LDH values normalized with respect to OA alone ± S.E.M. of triplicates of at least 6 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.

Figure 4. Differential phosphorylation and implication of JAK-2 in neuroprotection mediated by PNU 282987 and 5IA 85380. PNU282987 **(A)**, 5IA 85380 **(B)** and PNU 282987 plus the allosteric modulator PNU 120596 were incubated during different time periods (5 min, 15 min, 30 min, 1 h and 24 h); at the end, P-JAK-2 (immunoblots on the top) and JAK-2 total (immunoblots at the bottom) were quantified by western blot. (**D)** Represents the effect of the JAK-2 inhibitor, AG-490, on the neuroprotective action of PNU 282987 10 μM ("PNU 28") and 5IA 85380 100 μM ("5IA"). Data are the mean ± S.E.M. of at least 4 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).**

Figure 5. Phosphorylation of Akt and implication of PI3K in neuroprotection mediated by PNU 282987 and 5IA 85380. Akt phosphorylation by PNU 282987 10 μM **(A)** and 5IA 85380 100 μM **(B)** at different incubation periods. **(C)** Shows the effect of the PI3K inhibitor 3 μM LY 294002 on the neuroprotection mediated by PNU 282987 10 μM ("PNU 28") and 5IA 85380 100 μM ("5IA") against OA-induced toxicity. Each value is a mean of at least 4 different cell cultures ± S.E.M. $*p < 0.05$ 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, $^{\$\$}p < 0.01$ with respect to 5IA 85380 **(C)**.

Figure 6. Phosphorylation of ERK 1/2 and implication of MEK in neuroprotection mediated by PNU 282987 and 5IA 85380. ERK 1/2 phosphorylation by PNU 282987 10 μM **(A)** and 5IA 85380 100 μM **(B)** at different incubation periods. **(C)** Shows the effect of the MEK inhibitor PD 98059 1μM on neuroprotection mediated by PNU 282987 10 μM ("PNU 28") and 5IA 85380 100 μM ("5IA") against OA-induced toxicity. Each value is a mean of at least 4 different cell cultures ± S.E.M. $*p < 0.05$ with respect to the basal in **(B)**; $***p < 0.001$ with respect to OA alone, $^{\#\#}p < 0.01$ with respect to 5IA 85380 **(C)**.

Figure 7. GSK-3β phosphorylation and implication in neuroprotection mediated by PNU 282987 and 5IA 85380. Time course of Ser-9 were analysed by western blot in protein extracts obtained from cells treated with PNU 282987 10 μM (**A**) and 5IA 85380 100 μM (**B**) during different time periods. **(C)** Shows the effect of the GSK-3β inhibitor AR-A014418 1μM on the neuroprotection mediated by PNU 282987 10 μM ("PNU 28") and 5IA 85380 100 μM ("5IA") against OA-induced toxicity. Each value is a mean of at least 4 different cultures ± S.E.M. $*p < 0.05$ 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)**.

Figure 8. GSK-3β expression/phosphorylation under the treatment of OA. Protein samples were collected from cells that were pre-incubated for 24 h with PNU 282987 10 μM, 5IA 85380 100 μM or AR-A014418 1 μM and, subsequently this pre-treatment was replaced by medium with OA 30 nM for 16 hours. 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 4 different cultures ± S.E.M. $*p < 0.05$, $**p < 0.01$ with respect to basal levels and, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ with respect to OA alone.

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

Figure 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, red) and Hoechst (nuclear marker, blue). Cells were incubated with PNU 282987 (10 μM) (C) or 5IA 85380 (100 μM) (D) during 24 h, then OA 30 nM was added for 5h. A control group without any treatment (A) and another just treated with OA 30 nM 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.

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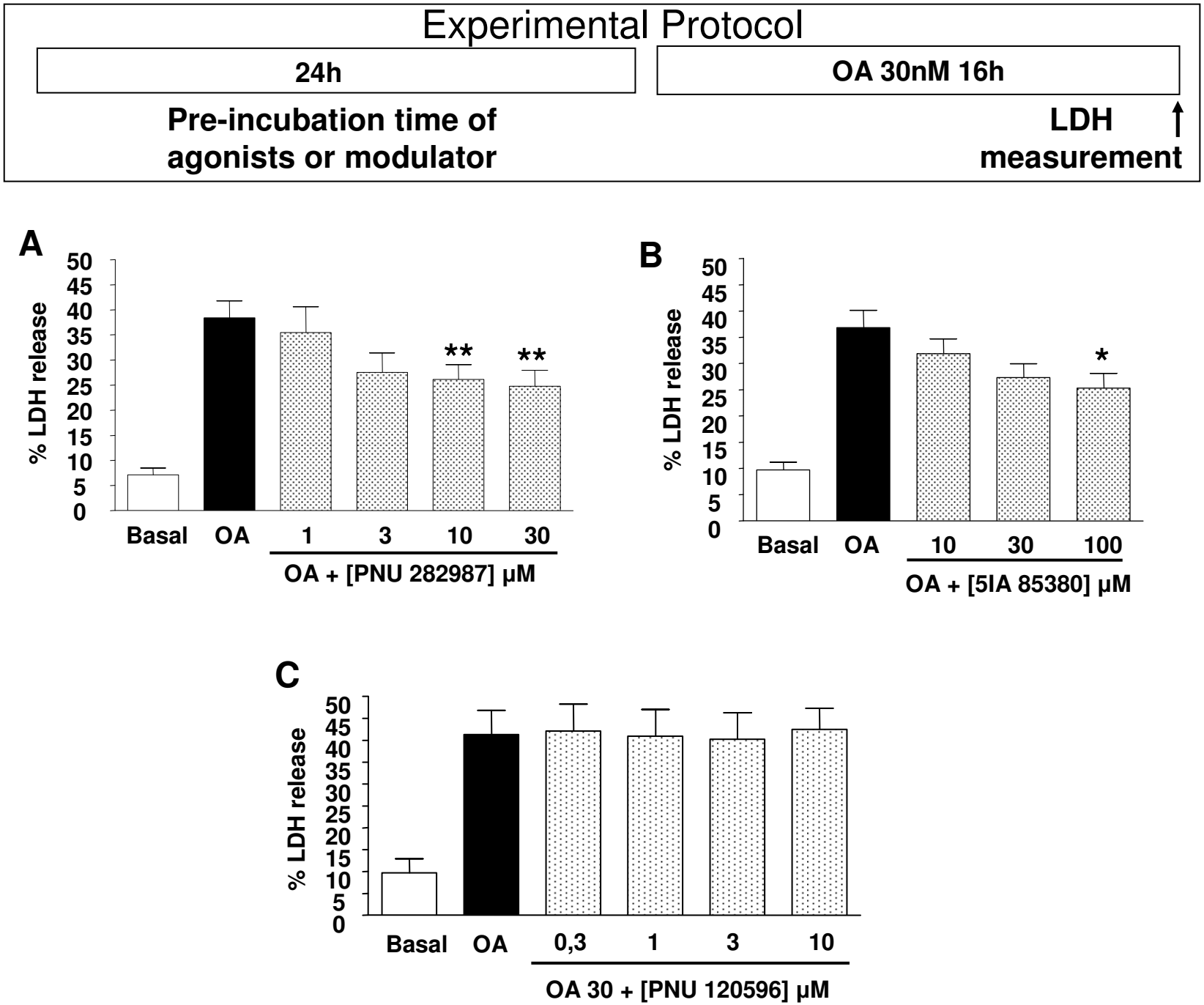

Experimental Protocol
24h
OA 30nM 16h
Pre-incubation time of agonists or modulator
LDH measurement
A
% LDH release
50
45
40
35
30
25
20
15
10
5
0
Basal
OA
1
3
10
30
**
**
OA + [PNU 282987] µM
B
% LDH release
Basal
OA
10
30
100
*
OA + [5IA 85380] µM
C
% LDH release
Basal
OA
0,3
1
3
10
OA 30 + [PNU 120596] µM

Fig. 1

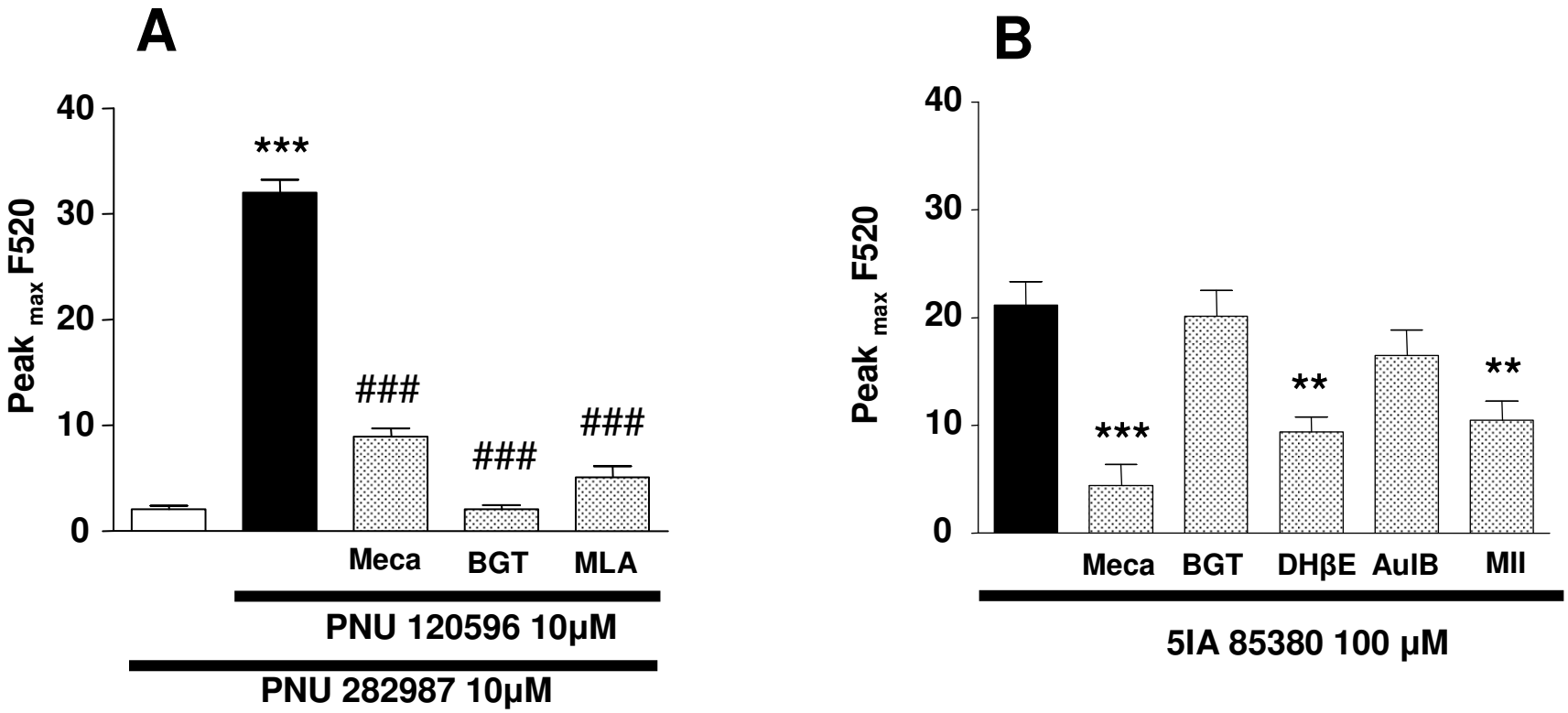
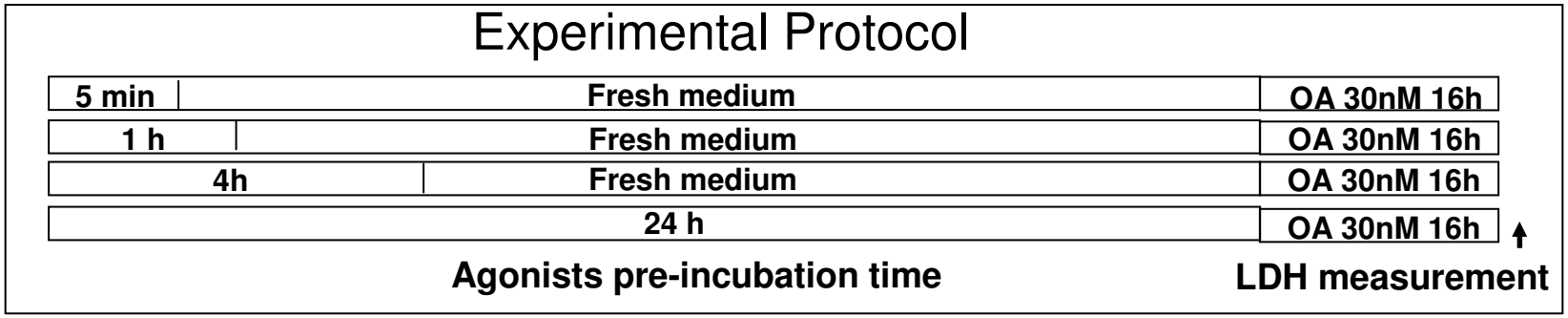
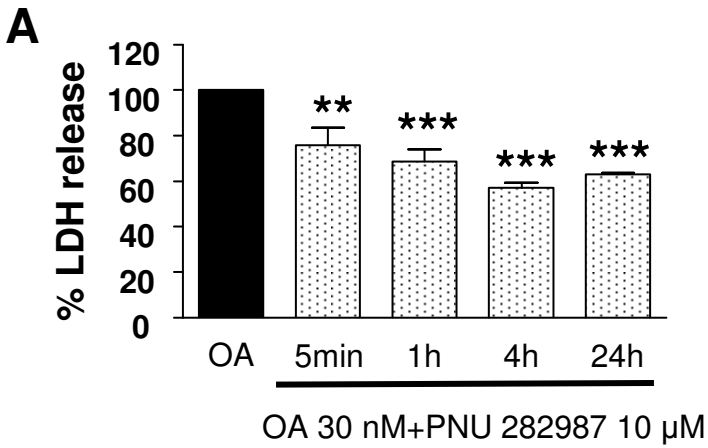
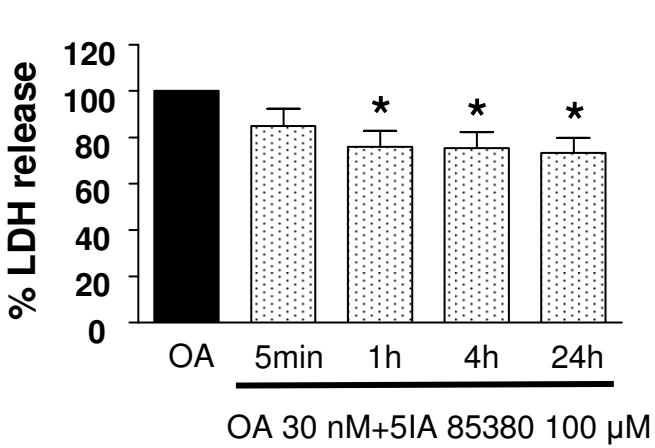
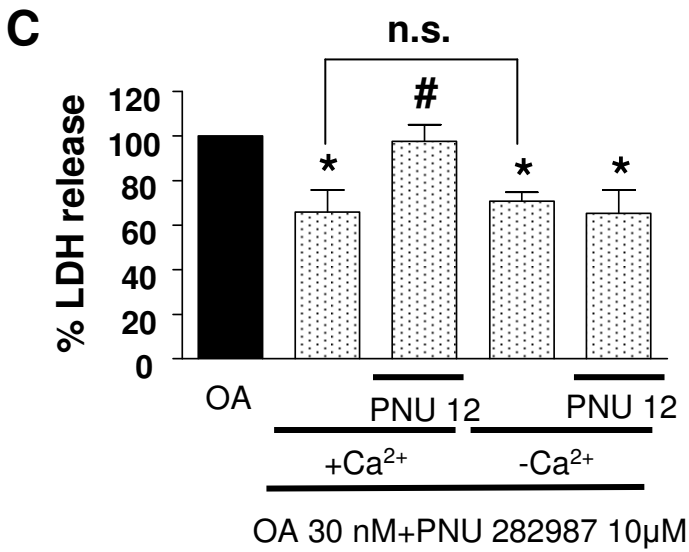

A
40
30
20
10
0
Peak max F520
***
###
###
###
Meca
BGT
MLA
PNU 120596 10μM
PNU 282987 10μM
B
40
30
20
10
0
Peak max F520
***
**
**
Meca
BGT
DHβE
AuIB
MII
5IA 85380 100 μM

Fig. 2


Experimental Protocol
5 min
Fresh medium
OA 30nM 16h
1 h
Fresh medium
OA 30nM 16h
4h
Fresh medium
OA 30nM 16h
24 h
OA 30nM 16h
Agonists pre-incubation time
LDH measurement


A
% LDH release
120
100
80
60
40
20
0
**
***
***
***
OA
5min
1h
4h
24h
OA 30 nM+PNU 282987 10 µM


B
% LDH release
120
100
80
60
40
20
0
*
*
*
OA
5min
1h
4h
24h
OA 30 nM+5IA 85380 100 µM


C
n.s.
#
% LDH release
120
100
80
60
40
20
0
*
*
*
OA
PNU 12
PNU 12
+Ca2+
-Ca2+
OA 30 nM+PNU 282987 10µM

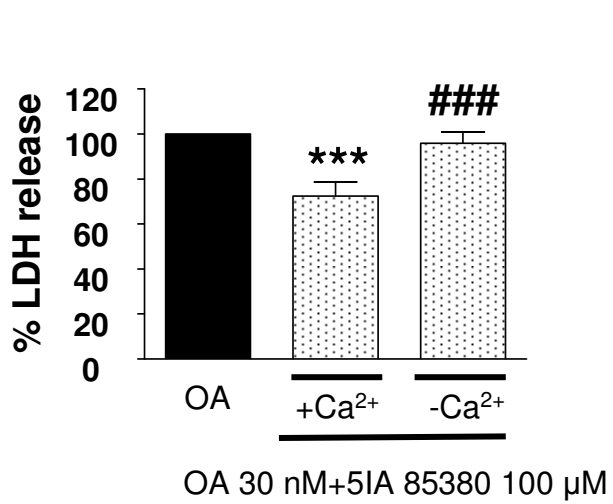
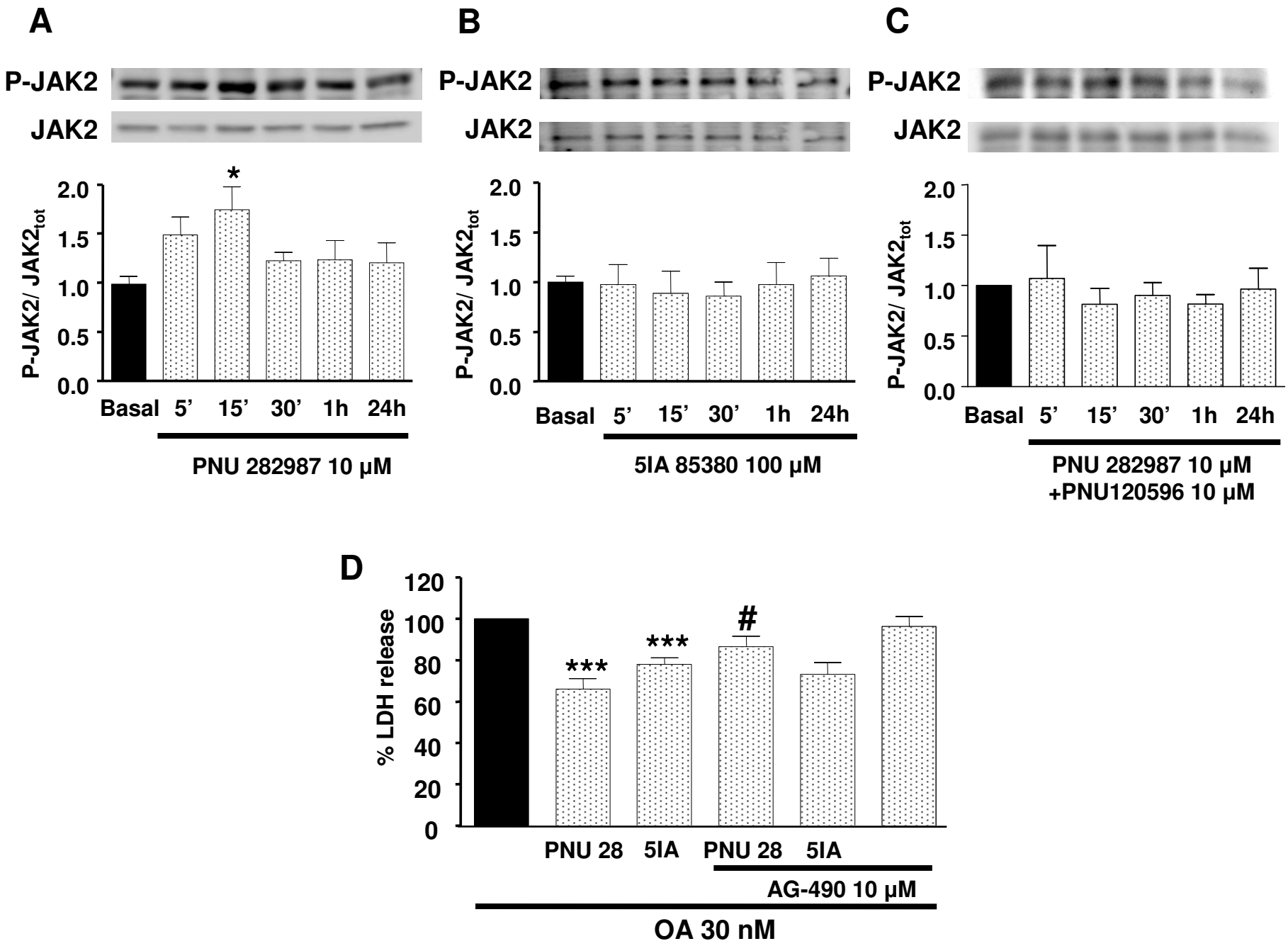

D
###
% LDH release
120
100
80
60
40
20
0
***
OA
+Ca2+
-Ca2+
OA 30 nM+5IA 85380 100 µM

Fig. 3


A
P-JAK2
JAK2
P-JAK2/ JAK2tot
2.0
1.5
1.0
0.5
0.0
*
Basal
5'
15'
30'
1h
24h
PNU 282987 10 µM
B
P-JAK2
JAK2
P-JAK2/ JAK2tot
2.0
1.5
1.0
0.5
0.0
Basal
5'
15'
30'
1h
24h
5IA 85380 100 µM
C
P-JAK2
JAK2
P-JAK2/ JAK2tot
2.0
1.5
1.0
0.5
0.0
Basal
5'
15'
30'
1h
24h
PNU 282987 10 µM
+PNU120596 10 µM
D
% LDH release
120
100
80
60
40
20
0
***
***
#
PNU 28
5IA
PNU 28
5IA
AG-490 10 µM
OA 30 nM

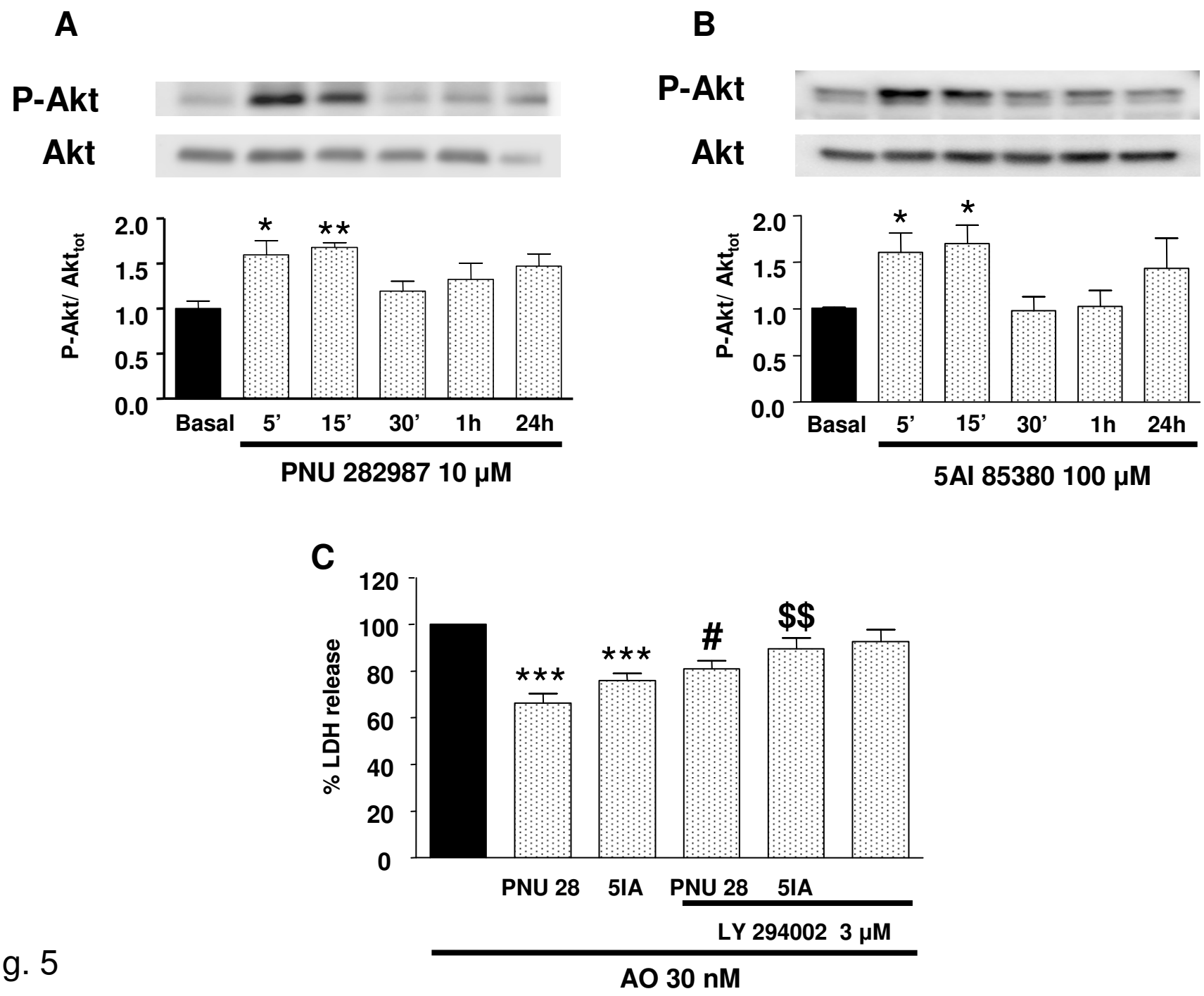

A
P-Akt
Akt
P-Akt/ Akt$_{tot}$
2.0
1.5
1.0
0.5
0.0
*
**
Basal
5'
15'
30'
1h
24h
PNU 282987 10 µM
B
P-Akt
Akt
P-Akt/ Akt$_{tot}$
2.0
1.5
1.0
0.5
0.0
*
*
Basal
5'
15'
30'
1h
24h
5AI 85380 100 µM
C
% LDH release
120
100
80
60
40
20
0
***
***
#
$$
PNU 28
5IA
PNU 28
5IA
LY 294002 3 µM
AO 30 nM

Fig. 5

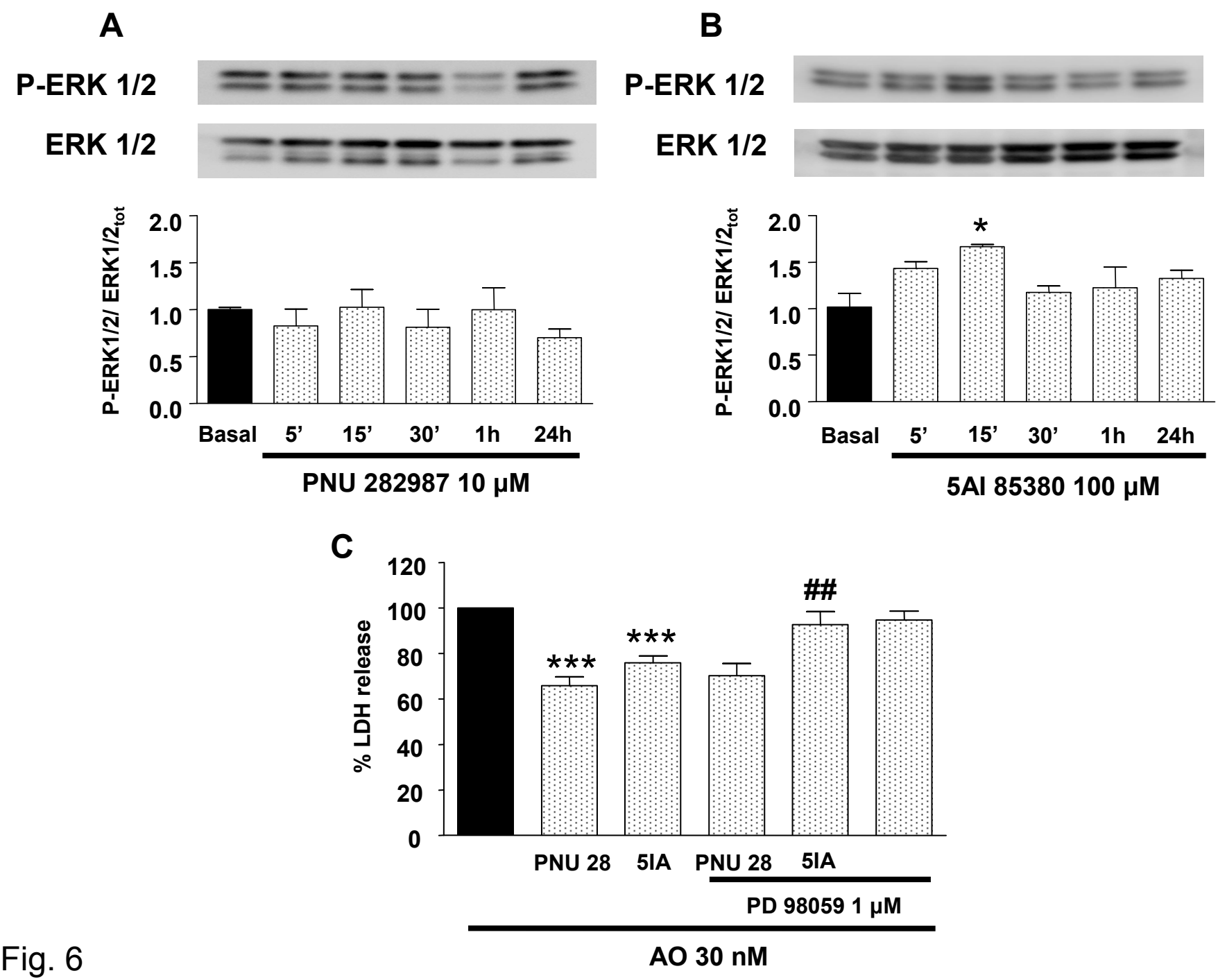
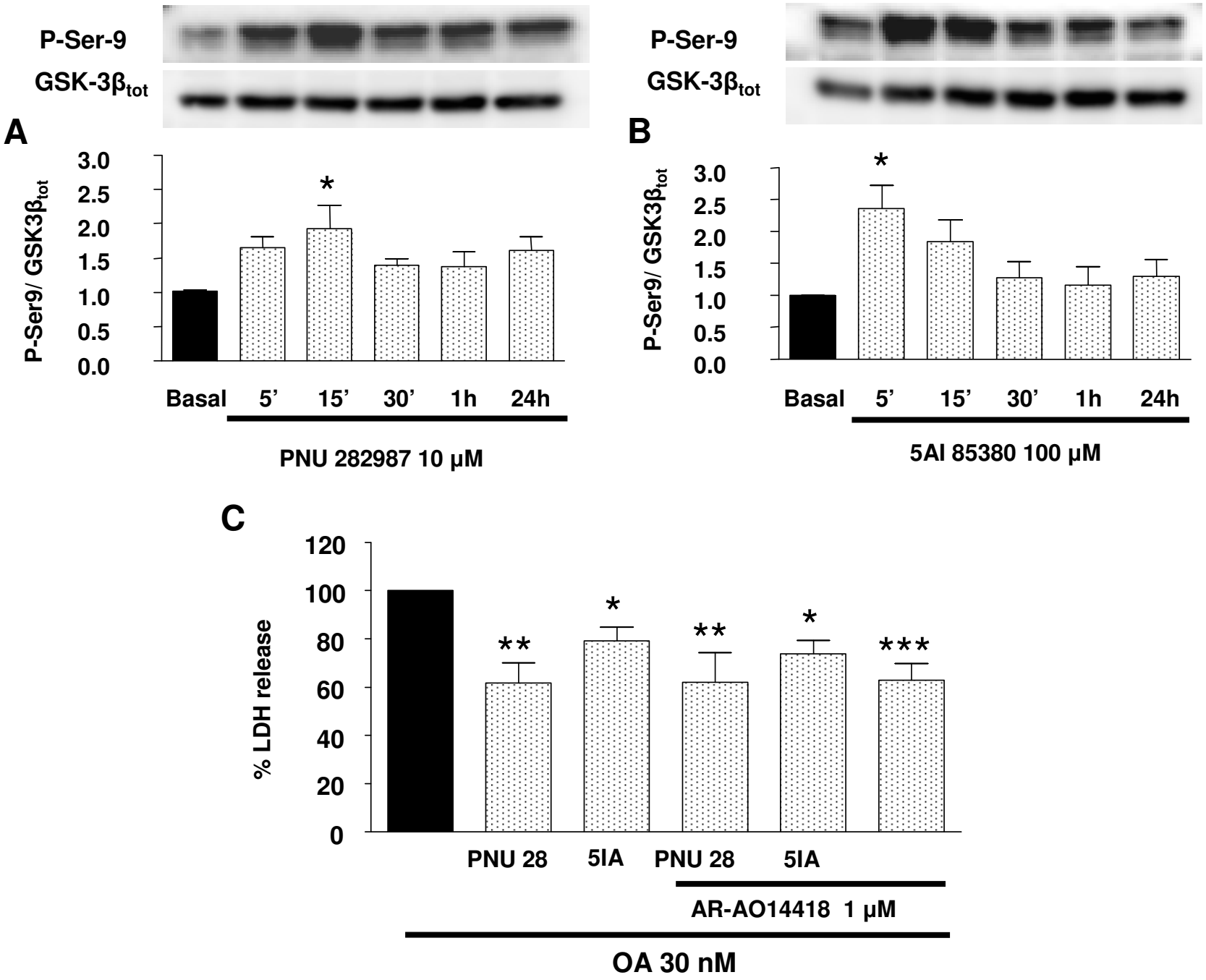
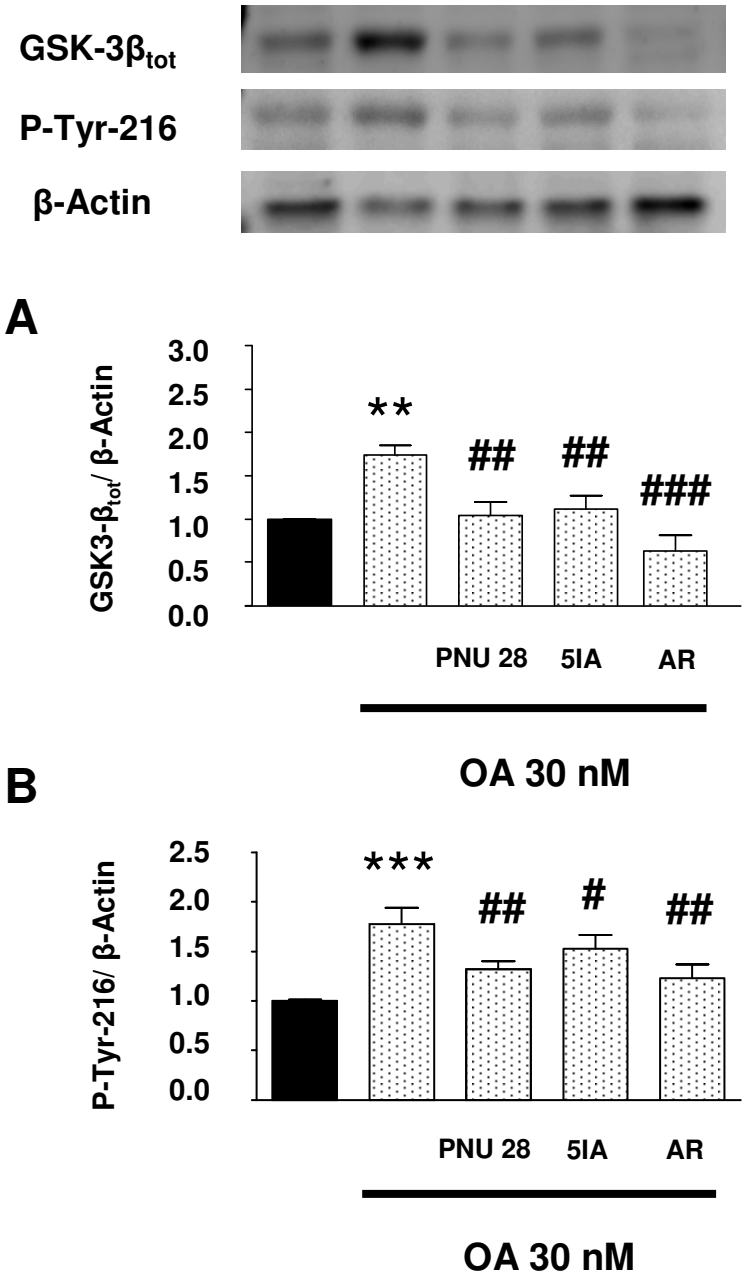
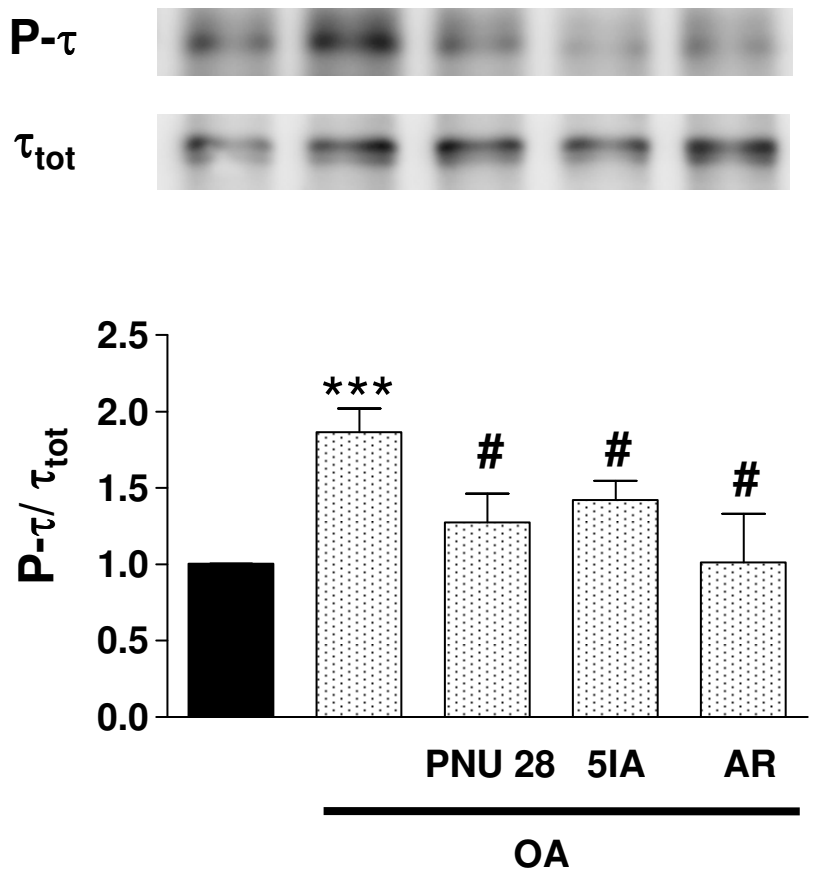

A
P-ERK 1/2
ERK 1/2
P-ERK1/2/ ERK1/2tot
2.0
1.5
1.0
0.5
0.0
Basal
5'
15'
30'
1h
24h
PNU 282987 10 µM
B
P-ERK 1/2
ERK 1/2
P-ERK1/2/ ERK1/2tot
*
5AI 85380 100 µM
C
% LDH release
120
100
80
60
40
20
0
***
***
##
PNU 28
5IA
PNU 28
5IA
PD 98059 1 µM
AO 30 nM

Fig. 6


P-Ser-9
GSK-3β tot
A
P-Ser9/ GSK3β tot
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Basal
5'
15'
30'
1h
24h
PNU 282987 10 μM
P-Ser-9
GSK-3β tot
B
P-Ser9/ GSK3β tot
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Basal
5'
15'
30'
1h
24h
5AI 85380 100 μM
C
% LDH release
120
100
80
60
40
20
0
**
*
**
*
***
PNU 28
5IA
PNU 28
5IA
AR-AO14418 1 μM
OA 30 nM


GSK-3β$_{tot}$
P-Tyr-216
β-Actin
A
GSK3-β$_{tot}$/ β-Actin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
##
##
###
PNU 28
5IA
AR
OA 30 nM
B
P-Tyr-216/ β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
***
##
#
##
PNU 28
5IA
AR
OA 30 nM


P-τ
τtot
P-τ/ τtot
2.5
2.0
1.5
1.0
0.5
0.0
***
#
#
#
PNU 28
5IA
AR
OA

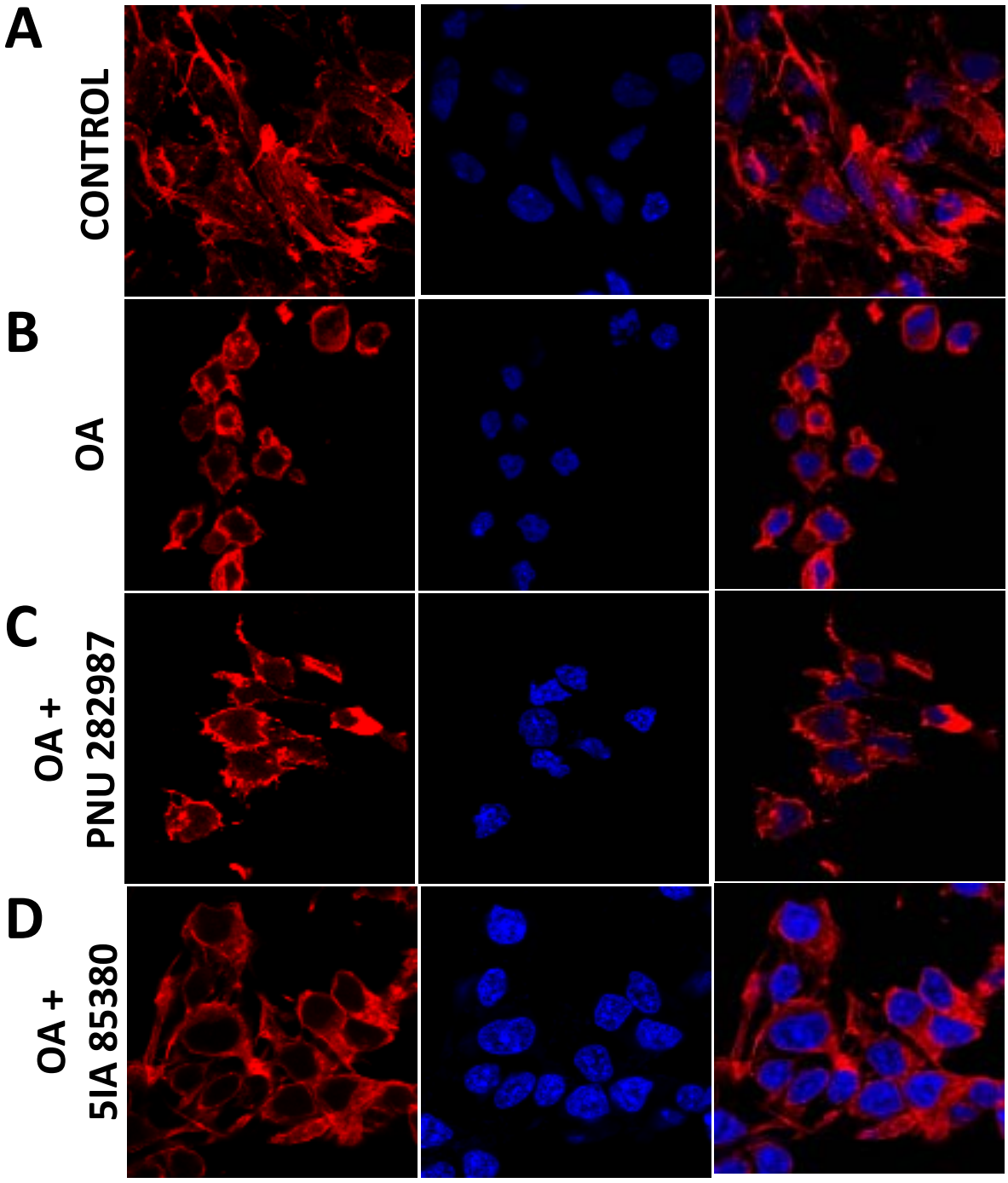

Phalloidin
Hoechst
Merged
A
CONTROL
B
OA
C
OA +
PNU 282987
D
OA +
5IA 85380

Fig. 10