

RESEARCH PAPER

Positive allosteric modulation of $\alpha 7$ neuronal nicotinic acetylcholine receptors: lack of cytotoxicity in PC12 cells and rat primary cortical neurons

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Background and purpose: $\alpha 7$ -Nicotinic acetylcholine receptors ($\alpha 7$ nAChRs) play an important role in cognitive function. Positive allosteric modulators (PAMs) amplify effects of $\alpha 7$ nAChR agonist and could provide an approach for treatment of cognitive deficits in neuropsychiatric diseases. PAMs can either predominantly affect the apparent peak current response (type I) or increase both the apparent peak current response and duration of channel opening, due to prolonged desensitization (type II). The delay of receptor desensitization by type II PAMs raises the possibility of Ca^{2+} -induced toxicity through prolonged activation of $\alpha 7$ nAChRs. The present study addresses whether type I and II PAMs exhibit different cytotoxicity profiles.

Experimental approach: The present studies evaluated cytotoxic effects of type I PAM [N-(4-chlorophenyl)- α -(4-chlorophenyl)-aminomethylene]-3-methyl-5-isoxazoleacetamide (CCMI) and type II PAM 1-[5-chloro-2,4-dimethoxy-phenyl]-3-[5-methyl-isoxazol-3-yl]-urea (PNU-120596), or 4-[5-(4-chloro-phenyl)-2-methyl-3-propionyl-pyrrol-1-yl]-benzenesulphonamide (A-867744). The studies used cultures of PC12 cells and primary cultures of rat cortical neuronal cells.

Key results: Our results showed that neither type I nor type II PAMs had any detrimental effect on cell integrity or cell viability. In particular, type II PAMs did not affect neuron number and neurite outgrowth under conditions when $\alpha 7$ nAChR activity was measured by Ca^{2+} influx and extracellular signal-regulated kinases 1 and 2 phosphorylation, following exposure to $\alpha 7$ nAChR agonists.

Conclusions and implications: This study demonstrated that both type I and type II $\alpha 7$ nAChR selective PAMs, although exhibiting differential electrophysiological profiles, did not exert cytotoxic effects in cells endogenously expressing $\alpha 7$ nAChRs. *British Journal of Pharmacology* (2009) 158, 1857–1864; doi:10.1111/j.1476-5381.2009.00474.x; published online 27 November 2009

Keywords: $\alpha 7$ nAChR; positive allosteric modulator; ERK1/2; cytotoxicity

Abbreviations: $\alpha 7$ nAChRs, $\alpha 7$ nicotinic acetylcholine receptors; ERK1/2, extracellular signal-regulated kinases 1 and 2; HCS, high content screen; PAM, positive allosteric modulators

Introduction

$\alpha 7$ -Nicotinic acetylcholine receptors (nAChRs; nomenclature follows Alexander *et al.*, 2008) are acetylcholine-gated cation channels that exhibit biophysical and pharmacological profiles distinct from other nAChR subtypes and are located in brain regions critical to learning and memory (see Dajas-Bailador and Wonnacott, 2004; Hogg and Bertrand, 2004). Preclinical validation of $\alpha 7$ nAChRs as a drug target for potential treatment of cognitive deficits such as in Alzheimer's disease and schizophrenia largely stems from pharmacological studies in animal models. For example, $\alpha 7$ nAChR

selective agonists have been shown to improve cognitive performance across a variety of preclinical models relevant to Alzheimer's disease and schizophrenia (Van Kampen *et al.*, 2004; Bodnar *et al.*, 2005; Wishka *et al.* 2006; Bitner *et al.* 2007; Boess *et al.* 2007; Pichat *et al.* 2007) whereas antagonists or antisense have been shown to impair cognitive function (Felix and Levin, 1997; Bettany and Levin, 2001; Curzon *et al.*, 2006). More recently, $\alpha 7$ nAChR selective positive allosteric modulators (PAMs) have also been described and shown to be effective in animal models, further validating the role of $\alpha 7$ nAChRs.

PAMs are chemically heterogeneous and capable of differentially modifying the biophysical properties of $\alpha 7$ nAChRs without interacting at the orthosteric binding site (for a review, see Bertrand and Gopalakrishnan, 2007). PAMs, in principle, can enhance $\alpha 7$ nAChR functions without altering the temporal integrity of neurotransmission. Based on

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biophysical properties, at least two different profiles of $\alpha 7$ PAMs can be distinguished: type I – exemplified by 5-hydroxyindole, genistein, NS-1738 and CCMI (Compound 6 in Ng *et al.*, 2007) – predominantly increases peak current amplitude response, and type II – represented by PNU-120596 (Hurst *et al.*, 2005), TQS (Grønlien *et al.*, 2007) and A-867744 (Malysz *et al.*, 2009) – affecting both the peak current response and the current decay profile. Both type I and II PAMs have shown efficacy in cognition and sensory gating deficit models *in vivo* (Hurst *et al.*, 2005; Timmermann *et al.*, 2007; Ng *et al.* 2007; Faghih *et al.* 2009).

Although types I and II PAMs with different electrophysiological profiles have demonstrated *in vivo* behavioural efficacy, the physiological and pharmacological effects following the allosteric modulation needs to be further defined. Especially, changes in desensitization profiles of $\alpha 7$ nAChR and the associated changes in Ca^{2+} dynamics can be triggered by type II PAMs through delaying desensitization of the receptor, but not by type I PAMs (see Bertrand and Gopalakrishnan, 2007). It has been suggested that the altered kinetics and increased sustained levels of Ca^{2+} may lead to activation of various cytotoxic events. For example, in the characterization of a type I PAM CCMI, it was shown that PNU-120596 was cytotoxic, whereas CCMI did not significantly alter nAChR kinetics and had no effect on cell viability (Ng *et al.*, 2007). With the emergence of many type I and II PAMs, the question remains whether Ca^{2+} entry resulted from delayed $\alpha 7$ nAChR desensitization by type II PAMs leads to Ca^{2+} -induced cytotoxicity. This study aimed to address whether type I and type II PAMs can reveal differential cytotoxicity profiles. Our data suggest that both type I and type II PAMs do not cause toxic effect when enhancing the activation of $\alpha 7$ nAChR in PC12 and rat cortical neuronal cells.

Methods

Cell culture and treatment

All animal care and experimental procedures complied with Institutional guidelines.

Rat cortical neuronal cells were isolated and cultured as previously described (Hu *et al.*, 2007). Briefly, postnatal day 0 (P0) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were anesthetized with inhaled CO_2 and killed by decapitation. The meninges were removed and the cortices were excised and subsequently incubated at 37°C for 30 min in Hanks' Balanced Salt Solutions (HBSS) containing $35 \text{ U}\cdot\text{mL}^{-1}$ papain before the addition of $0.2 \text{ mg}\cdot\text{mL}^{-1}$ DNase I buffer in HBSS. Tissues were then mechanically triturated by repeated passages through a 5-mL pipette. Cell debris was removed by passing cells through a $70 \mu\text{m}$ nylon filter. The cell suspension was centrifuged at $200 \times g$ for 5 min at room temperature and the resulting pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. Cortical neuronal cell cultures were plated at a density of $5 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$ in poly-D-lysine coated 96-well plates and maintained at 37°C with 5% CO_2 .

PC12 cells (American Type Culture Collection, Manassas, VA) were cultured as previously described (Hu *et al.*, 2008).

Briefly, PC12 cells were plated on poly-D-lysine coated 96-well plates, cultured in Ham's F12K medium supplemented with 15% horse serum, 2.5% fetal bovine serum (FBS), 2 mM L-glutamine, $100 \text{ U}\cdot\text{mL}^{-1}$ penicillin and $100 \mu\text{g}\cdot\text{mL}^{-1}$ streptomycin at 37°C with 5% CO_2 .

Calcium mobilization assay (FLIPR)

$\alpha 7$ nAChR-mediated elevation of intracellular Ca^{2+} levels was measured using the FLIPR calcium 4 assay kit. PC12 cells and rat P0 cortical neuronal cells were grown in the poly-D-lysine coated 96-well plates for 3 and 6 days respectively. Prior to the assay, the culture media were removed and cells were loaded with $100 \mu\text{L}$ of calcium-4 no-wash dye prepared in N-methyl D-glucosamine (NMDG) buffer (10 mM HEPES, pH 7.4, 140 mM NMDG, 5 mM KCl, 1 mM MgCl_2 , 10 mM CaCl_2) for 1 h at room temperature. $\alpha 7$ nAChR PAMs and PNU-282987 were also prepared in NMDG buffer. The effects of $\alpha 7$ nAChR PAMs were determined by first adding PAMs for 5 min followed by addition of $10 \mu\text{M}$ PNU-282987 for another 5 min. Changes in fluorescence intensity were recorded over time at 25°C in FLIPR ($\lambda_{\text{EX}} = 488 \text{ nm}$, $\lambda_{\text{EM}} = 540 \text{ nm}$). Concentration–response data were generated.

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) in-cell

Western blot assays

Undifferentiated PC12 cells were cultured for 3 days. Prior to experiments, the culture media were replaced with serum-free media, to starve cells overnight. On the day of the experiment, cell media were removed and cells were treated with $\alpha 7$ nAChR PAMs prepared in Dulbecco's phosphate buffer saline (D-PBS) for 10 min at 37°C followed by addition of $10 \mu\text{M}$ $\alpha 7$ nAChR agonist PNU-282987 for 5 min. Cells were then fixed with 4% paraformaldehyde for 60 min, followed by four washes using phosphate-buffered saline (PBS) (pH 7.4), permeabilized with 0.1% Triton X-100/PBS and blocked with the Odyssey blocking buffer for 3 h at room temperature. The cells were then incubated overnight at 4°C with mouse anti-phospho-ERK (pERK) monoclonal antibody (1:500) and rabbit anti-total ERK (tERK) antibody (1:1000). On the following day, cells were washed three times with 0.1% Tween-20/PBS and incubated with the Alexa Fluor 680 anti-rabbit antibody (1:1000) for detection of total ERK (tERK) or with IRDye 800CW anti-mouse IgG antibody (1:1000) for 1 h at room temperature for detection of pERK. Cells were washed three times, and target signals were simultaneously visualized using Odyssey Infrared Imaging Scanner (Li-Cor Biosciences, Lincoln, NE, USA) with the 680-nm fluorophore emitting an image of red color (tERK) and the 800-nm fluorophore emitting an image of green color (pERK). The integrated fluorescence intensities were calculated and analysed using the Odyssey Infrared Imaging System Application Software version 1.2.15 (Li-Cor Biosciences). Data analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). tERK and pERK signals were quantitated and pERK/tERK ratio were normalized to the control response of $10 \mu\text{M}$ PNU-120596 in presence of $10 \mu\text{M}$ PNU-282987.

Cytotoxicity assays

Cytotoxicity was measured by cell integrity and cell viability. PC12 cells and P0 cortical neuronal cells were cultured in

complete growth medium for 3 and 6 days, respectively, prior to treatment with varying concentrations of PAMs for 24 h. Cell integrity was assessed using Toxilight assay (Cambrex, Rockland, ME, USA) that measures activity of the enzyme adenylate kinase (AK) released into the medium from damaged cells. Toxilight bioassay was conducted as previously described (Miret *et al.*, 2006). Briefly, 20 μ L of cell culture media were transferred to a white walled 96-well plate, and 100 μ L of AK working solution was added to each well. The plates were incubated for 5 min at room temperature. Luminescence was measured in the Wallac Victor 21 420 multilabel counter (PerkinElmer, Waltham, MA, USA). Cell viability was assessed using the Cell Titer 96 Aqueous One Solution kit (Promega, Madison, WI, USA) that measures activity of mitochondrial dehydrogenase in metabolically active cells. Cell Titer 96 Aqueous One Solution assay was conducted as previously described (Woods *et al.*, 2008). The colorimetric methyl tetrazolium salt reagent was added to each well of the plate, and incubated for 1–4 h. The absorbance values were read at 490 nm.

High content screen (HCS) microscopy analysis

Rat P0 primary cortical neuronal cells were cultured for 6 days and treated with varying concentrations of either PNU-120 596 or A-867744 alone and in presence of 10 μ M PNU-282987 for 3 days. Cells were then fixed and stained as previously described (Hu *et al.*, 2007). Briefly, neurons were stained by mouse anti-tubulin monoclonal antibody (1:100) and then recognized by FITC-labelled anti-mouse antibody (1:1000). Nuclei (360/40 nm excitation and 465/30 nm emission filters) and neuron (475/35 nm excitation and 535/40 nm emission filters) images were collected using the ImageXpress Micro automatic fluorescent microscopy system. Typically, four wells were used for each treatment condition on a 96-well plate, and images were collected from four fields within each well. The neurite outgrowth module in the Imaging software was used to automatically count neuron numbers and the extent of neurite outgrowth. Experiments were conducted independently three times, in quadruplicate for each treatment group.

Data analysis

Data are expressed as mean \pm SEM. Student's *t*-tests (paired and two-tailed) were performed to assess significant differences. Wherever applicable, *P* values less than 0.05 were considered statistically significant.

Materials

DMEM, Ham's F12K medium, PBS, D-PBS, FBS, horse serum, L-glutamine and penicillin/streptomycin were purchased from Gibco Life Technologies Inc. (Grand Island, NY, USA). Papain (Worthington, Lakewood, NJ, USA), Deoxyribonuclease I (DNase I), monoclonal mouse anti-phospho-ERK 1/2 and polyclonal rabbit anti-ERK1/2 antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). IRDye 800CW conjugated affinity-purified anti-mouse IgG antibody (Rockland Immunochemicals, Gilbertsville, PA, USA), Alexa Fluor 680

goat anti-rabbit IgG antibody (H+L) (Molecular Probes, Eugene, OR, USA), Hoechst 33 342 (Molecular Probes), Anti-tubulin monoclonal antibody (Upstate, Lake Placid, NY, USA), Rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Chemicon International Inc, Temecula, CA, USA), Fluorescein (FITC)-conjugated affinity-purified donkey anti-mouse IgG antibody and rhodamine (TRITC)-conjugated affinity-purified donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were purchased from indicated sources. Poly-D-lysine coated 96-well plates were purchased from Becton Dickinson Labware (Bedford, MA, USA). Nylon filters (70 mm) were purchased from BD Biosciences Discovery Labware (Bedford, MA, USA). Test compounds including A-867744, PNU-120596, PNU-282987 and CCMI were synthesized in house. Odyssey Infrared Imaging Scanner and blocking buffer were from Li-Cor Biosciences. The ImageXpress Micro automated fluorescent microscopy system, fluometric imaging plate reader (FLIPR) and FLIPR calcium4 assay kit were from Molecular Devices Corporation (Sunnyvale, CA, USA).

Results

Effect of PAMs on intracellular Ca^{2+} changes in PC12 and cortical neurons

PC12 and rat cortical neuronal cells have been shown to express endogenous functional $\alpha 7$ nAChRs either by high affinity binding of $\alpha 7$ nAChR ligands or by functional responsiveness to $\alpha 7$ nAChR ligands (Dickinson *et al.*, 2007; Anderson *et al.*, 2008; El Kouhen *et al.*, 2009), or by $\alpha 7$ nAChR-mediated calcium signalling (Ren *et al.*, 2005; Dickinson *et al.* 2007). Initially, we determined the differential modulation of $\alpha 7$ nAChR agonist activity by type I and II PAMs by both FLIPR-based Ca^{2+} and ERK1/2 phosphorylation measurements. Both PNU-120596 and A-867 744 evoked robust increases in intracellular Ca^{2+} in the presence of $\alpha 7$ nAChR agonist PNU-282987 in both PC12 [Figure 1A, EC_{50} = 0.2 μ M (0.13–0.27 μ M, 95% CI, *n* = 3) and EC_{50} = 0.3 μ M (0.20–0.39 μ M, 95% CI, *n* = 3) respectively] and rat cortical neuronal cells [Figure 1B, EC_{50} = 0.3 μ M (0.14–0.60 μ M, 95% CI, *n* = 3) and EC_{50} = 0.1 μ M (0.07–0.16 μ M, 95% CI, *n* = 3) respectively]. No robust intracellular Ca^{2+} changes were observed with PAM or agonist alone as expected (data not shown). When applied with agonist, CCMI did not induce substantial intracellular Ca^{2+} changes in either cell line (Figure 1A,B). These observations indicated that types I and II PAMs differentially modulated $\alpha 7$ nAChR agonist activity in the PC12 and rat cortical neuronal cells, which was consistent with the differential effects of type I and type II PAMs on agonist-evoked Ca^{2+} dynamics.

Effect of PAMs on phosphorylation of ERK1/2 in PC12 cells

Next, we assessed the effects of PAMs on ERK1/2 phosphorylation in PC12 cells. A robust increase in ERK1/2 phosphorylation was induced by $\alpha 7$ nAChR agonists in the presence of type II PAMs (El Kouhen *et al.*, 2009). As illustrated in Figure 2, ERK phosphorylation was enhanced in a concentration-dependent manner by both PNU-120596 and

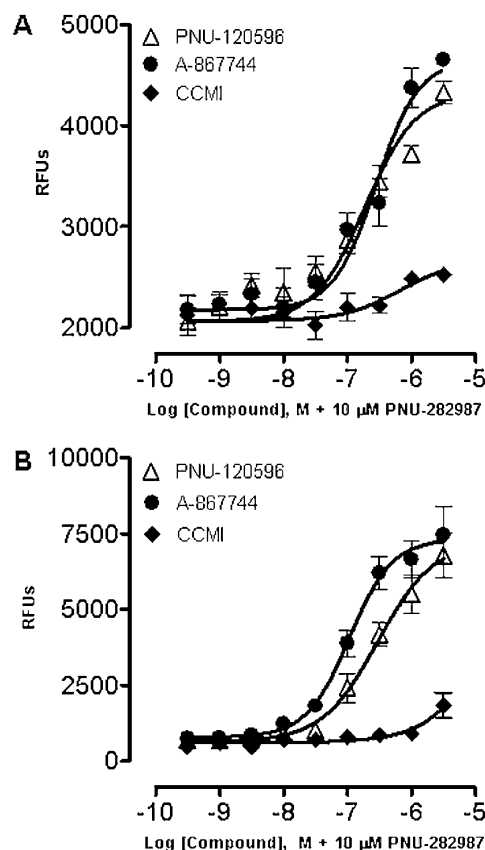


Figure 1 Effect of $\alpha 7$ nAChR type I and type II PAMs on intracellular Ca^{2+} levels in PC12 cells and rat cortical neuronal cells in culture. PC12 cells (A) or rat cortical neuronal cells (B) were treated with a range of concentrations of PNU-120596, A-867744 or CCMI for 10 min before the addition of 10 μM PNU-282987 for 5 min. Ca^{2+} influx was measured by FLIPR calcium 4 assay and expressed as relative fluorescence units (RFUs). Each point represents the mean \pm SEM of three independent experiments.

A-867744 in the presence of the agonist PNU-282987 [Figure 2A,B, $\text{EC}_{50} = 1.7 \mu\text{M}$ (1.1–2.5 μM , 95% CI, $n = 3$) and $\text{EC}_{50} = 0.4 \mu\text{M}$ (0.35–0.52 μM , 95% CI, $n = 3$ respectively)]. However, CCMI did not increase pERK levels in the presence of PNU-282987. The agonist or PAM alone did not increase pERK, as described previously (El Kouhen *et al.*, 2009). These observations, consistent with the differential effects on agonist-evoked Ca^{2+} dynamics, further indicate distinctions between type I and type II PAMs in modulating $\alpha 7$ nAChR functions.

Effects of PAMs on cell integrity and viability in PC12 cells and cortical neurons

Potential cytotoxic effects of PAMs were assessed in PC12 and rat cortical neurons by two measurements: cell integrity and viability (see Methods). Thapsigargin was used as a positive toxicity control for both assays.

PC12 cells treated with varying concentrations of PAMs in the absence or presence of the agonist PNU-282987 for 24 h did not show significant loss of cell integrity (Figure 3A) or significant decreases in cell viability (Figure 3B). Under

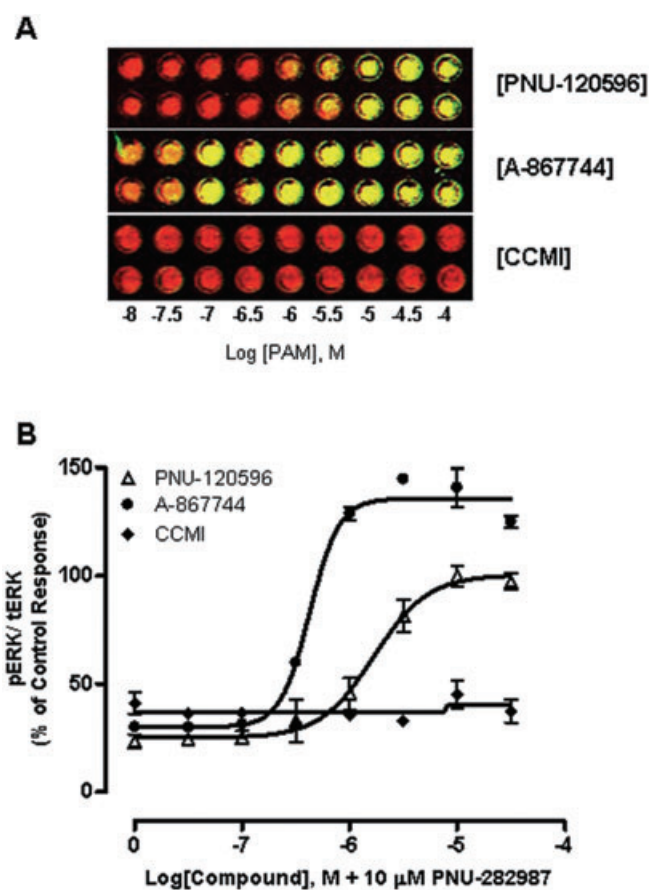


Figure 2 Effect of $\alpha 7$ nAChR type I and type II PAMs on ERK1/2 phosphorylation in PC12 cells. (A) Representative images of ERK1/2 phosphorylation measured by InCell Western in a 96-well format. PC12 cells were treated with varying concentrations of PNU-120596, A-867744 or CCMI for 10 min at 37°C prior to the addition of 10 μM PNU-282987 for 5 min. Cells were then imaged for ERK1/2 phosphorylation signals. The superimposed image indicates co-staining of pERK (green) and tERK (red). (B) ERK1/2 phosphorylation. tERK and pERK signals were quantified and pERK/tERK ratio were normalized to the control response to 10 μM PNU-120596 + 10 μM PNU-282987. Each point represents the mean \pm SEM of three independent experiments.

these conditions, concentration-dependent cytotoxicity was observed with thapsigargin in both assays (Figure 3A,B).

Cell integrity and cell viability were also measured in rat primary cortical neuronal cells following treatment with varying concentrations of PAMs (PNU-120596 or CCMI) in the absence or presence of the agonist PNU-282987. Again, PAMs alone or in combination with PNU-282987 revealed no cytotoxicity in both assays, while thapsigargin showed concentration-dependent cytotoxic effects under these conditions (Figure 4A,B).

To further assess for potentially subtle effects of the PAMs, we utilized high-content microscopy techniques to determine changes in neuron number and neurite outgrowth in rat primary cortical neuronal cell cultures. Cells were treated with varying concentrations of PNU-120596 or A-867744 in the presence or absence of the $\alpha 7$ nAChR agonist PNU-282987 for 3 days. Figure 5 demonstrates that PNU-120596 or A-867744 (up to 30 μM) alone or in combination with PNU-282987 did

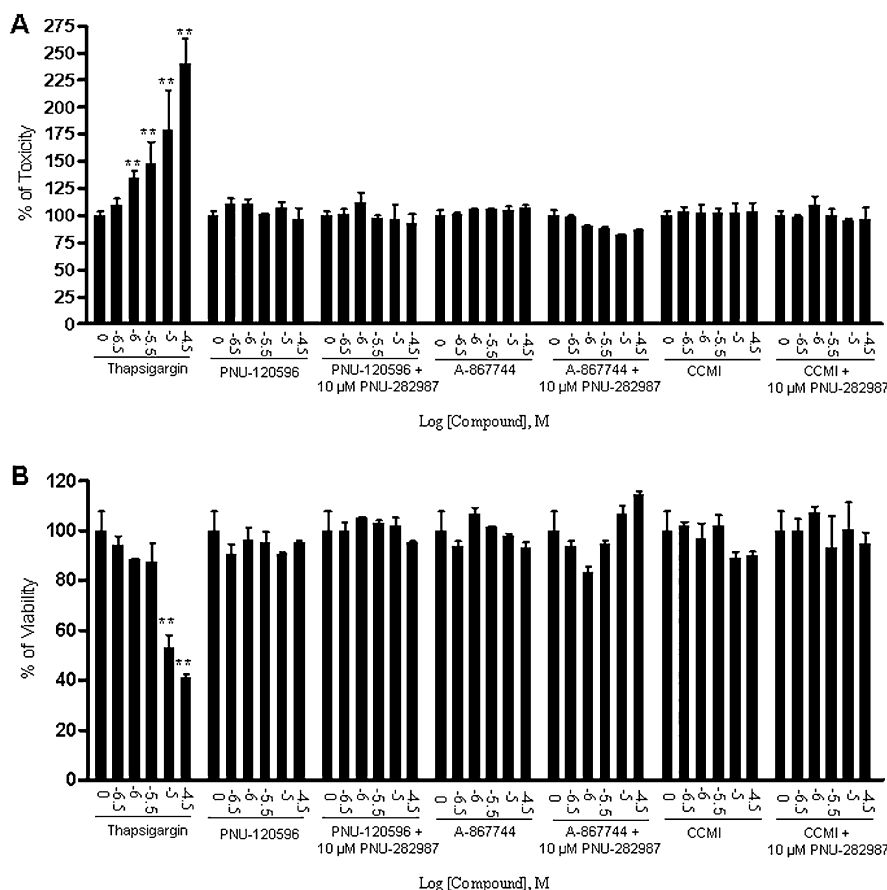


Figure 3 $\alpha 7$ nAChR type I and type II PAMs did not show cytotoxic effect on cell integrity or cell viability of PC12 cells. PC12 cells were cultured for 3 days and then treated with a range of concentrations of PNU-120596, A-867744 or CCMI alone or in the presence of 10 μ M PNU-282987 for 24 h followed by measurements of cell integrity determined by the release of adenylate kinase and cell viability determined by mitochondrial dehydrogenase enzyme activity. (A) Cell integrity: PAMs alone or in combination with agonist did not increase the release of adenylate kinase, whereas thapsigargin reduced cell integrity in a concentration-dependent manner. (B) Cell viability: PAMs alone or in combination with the agonist did not affect mitochondrial dehydrogenase enzyme activity, whereas thapsigargin significantly reduced cell viability in a concentration-dependent manner. The values are shown as the percentage of untreated cells. Each point represents the mean \pm SEM of 5 independent experiments. $**P < 0.01$ versus the untreated group.

not alter morphology of the cells (Figure 5A), neuron number (Figure 5B) or neurite outgrowth (Figure 5C).

Discussion

In the present study, we investigated the effect of type I and type II PAMs in PC12 and rat cortical neuronal cells expressing endogenous $\alpha 7$ nAChR. Our results showed that type II PAMs PNU-120596 and A-867744 did not exhibit detrimental effects on cell integrity, cell viability, neuron number or neurite outgrowth under conditions when receptor activity was measured by downstream cellular responses such as $\alpha 7$ nAChR-sensitive Ca^{2+} influx and ERK1/2 phosphorylation.

In the recent years, a chemically heterogeneous class of molecules has been identified as selective PAMs of $\alpha 7$ nAChRs (Bertrand and Gopalakrishnan, 2007). Initial reports have suggested that important considerations be given regarding development of PAMs, particularly those belonging to type II, as therapeutic agents, as the effects of type II PAMs on desensitization could lead to cytotoxic effects. For example, Ng

et al. (2007) showed that PNU-120 596 was cytotoxic in SHSY-5Y cells overexpressing $\alpha 7$ nAChRs, whereas CCMI was not. In addition, in L250T mutant mice, the mutation in the $\alpha 7$ nAChR that reduced desensitization promoted apoptosis (Orr-Urtreger *et al.*, 2000). With the emergence of many more type I and II PAMs, the question remains whether Ca^{2+} entry due to preventing or reversing $\alpha 7$ nAChR desensitization by type II PAMs leads to Ca^{2+} -induced cytotoxicity.

PNU-120596 and A-867744 are examples of prototypical type II PAMs that increases both agonist-evoked peak currents and prolongs the evoked response in the continued presence of the agonist (Hurst *et al.*, 2005; Grønlien *et al.*, 2007) whereas compounds like CCMI and NS-1738 are examples of type I PAMs with no effect on desensitization kinetics. In the present study, we showed that both of the type II PAMs dramatically enhanced Ca^{2+} responses and ERK phosphorylation in a concentration-dependent manner whereas CCMI, a type I PAM, did not. ERK1/2 phosphorylation in response to $\alpha 7$ nAChR agonists has been recently demonstrated both *in vitro* (Steiner *et al.*, 2007; Dickinson *et al.*, 2008; El Kouhen *et al.*, 2009) and *in vivo* (Bitner *et al.*, 2007). For example, in

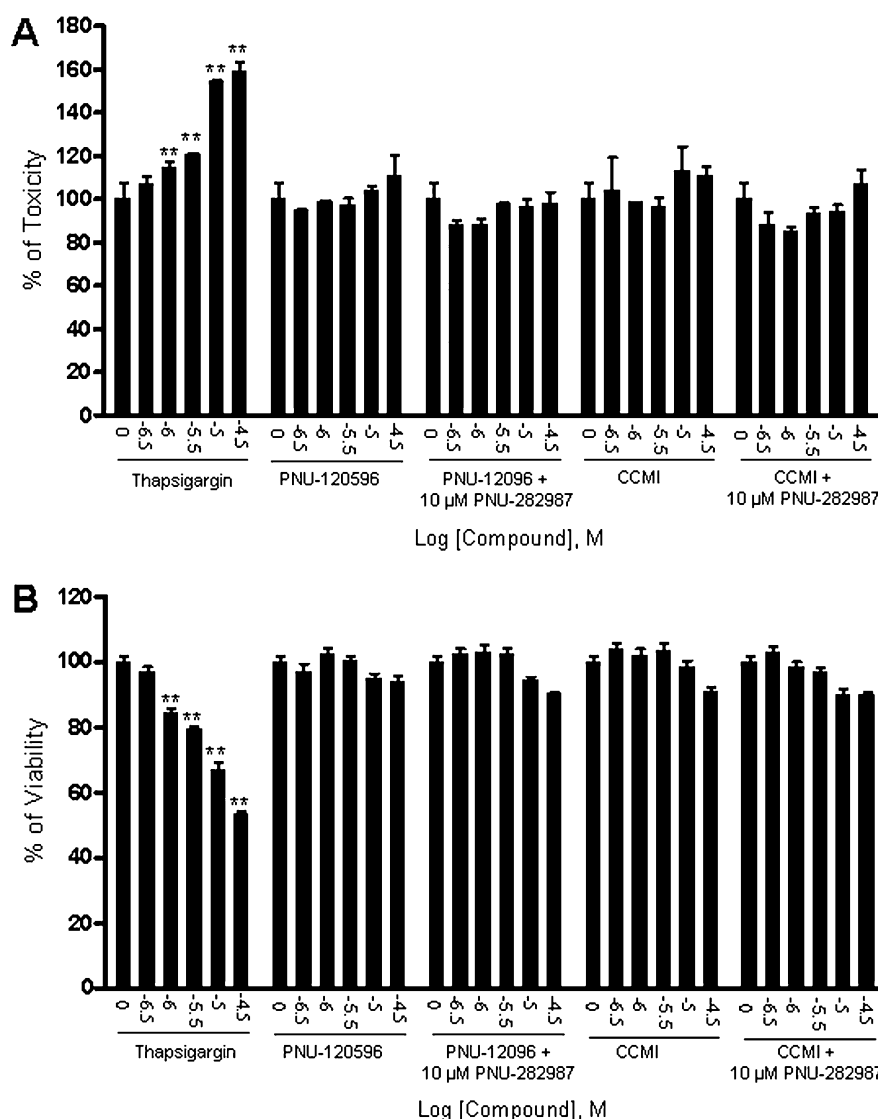


Figure 4 $\alpha 7$ nAChR type I and type II PAMs did not show cytotoxic effect on integrity or viability of rat cortical neuronal cells. Rat primary cortical neuronal cells were cultured for 6 days and then treated with a range of concentrations of PNU-120596 or CCMI alone or in the presence of 10 μ M PNU-282987 for 24 h. Cell integrity was determined by the release of adenylate kinase and cell viability was determined by mitochondrial dehydrogenase enzyme activity. (A) Cell integrity: PAMs alone or in combination with the agonist did not increase the release of adenylate kinase, whereas thapsigargin reduced cell integrity in a concentration-dependent manner. (B) Cell viability: PAMs alone or in combination with the agonist did not affect mitochondrial dehydrogenase enzyme activity, whereas thapsigargin significantly reduced cell viability in a concentration-dependent manner. The values are shown as the percentage of the untreated group. Each point represents the mean \pm SEM of four independent experiments. ** $P < 0.01$ versus the untreated group.

PC12 cells, robust increase in ERK1/2 phosphorylation can be revealed by $\alpha 7$ nAChR agonists in the presence of type II PAMs (El Kouhen *et al.*, 2009). The observation that type I PAMs do not increase Ca^{2+} response or ERK phosphorylation changes *in vitro* is attributable to the lack of effect of such compounds on receptor desensitization, in addition to amplification of peak current responses, detected using electrophysiological approaches (Ng *et al.*, 2007; Timmermann *et al.*, 2007).

No cytotoxic effects were detected when type I or type II PAMs were applied alone or combined with a selective $\alpha 7$ agonist, PNU-282987, as assessed by changes in adenylate kinase release or mitochondrial dehydrogenase activity in both PC12 cells and in cortical neurons, under conditions

where thapsigargin evoked concentration-dependent cytotoxic effects. We also employed HCS, a sensitive method for detecting neuronal degeneration and enabling quantitative analysis of a larger amount of morphology data than conventional microscopy (Hu *et al.*, 2008). Treatment of cortical neuronal cells with type II PAMs in the absence or presence of $\alpha 7$ nAChR agonist PNU-282987 for 3 days did not alter cell morphology, neuron number or neurite outgrowth (Figure 5). These observations indicating lack of cytotoxicity with PAMs of either profile is in contrast to the report showing that PNU-120596 was cytotoxic whereas CCMI had no effect on cell viability in SH-SY5Y cells transfected with $\alpha 7$ nAChRs (Ng *et al.*, 2007). The basis of this discrepancy is unclear at present, but might be attributed to the potential differences

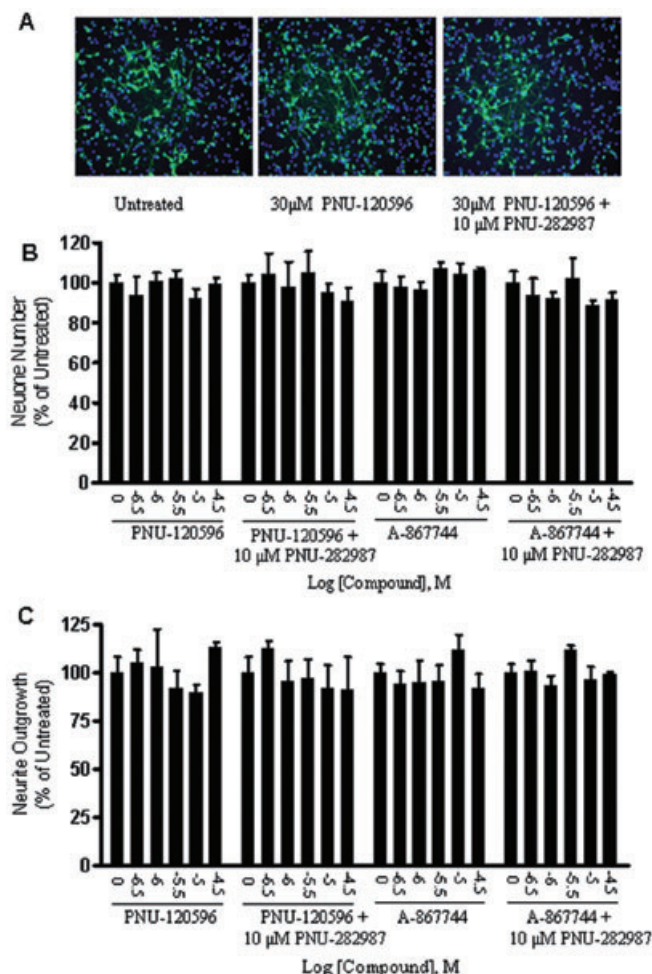


Figure 5 $\alpha 7$ nAChR type II PAMs did not show cytotoxic effect on morphology, cell number or neurite outgrowth of rat primary cortical neurons. Rat primary cortical neuronal cells were cultured for 6 days and then treated with a range of concentrations of PNU-120596 or A-867744 in the presence or absence of the $\alpha 7$ nAChR agonist PNU-282987 for 3 days. Neurons were stained with anti-tubulin antibody (green) and nuclei with Hoechst 33342 (blue). (A) Representative superimposed images of neurons and nuclei treated with buffer control, 30 μ M PNU-120596 or 30 μ M PNU-120596 in combination with 10 μ M PNU-282987 respectively. The PAMs alone or in combination with the agonist did not affect neuron morphology (A), neuron number (B), or neurite outgrowth (C). The values are shown as the percentage of the untreated group. Each point represents the mean \pm SEM of three independent experiments.

between rodent and human cell lines, overexpression of $\alpha 7$ nAChRs *per se* or limitations in the assays. For example, the cytotoxic effect of thapsigargin on SH-SY5Y cells [10 μ M yielding 20–30% viability; Ng *et al.* (2007)] appears to be greater than that on PC12 cells (10 μ M yielding 50–70% viability) examined in this study. In addition to cell viability, our present study thoroughly analysed cell integrity and morphology in both PC12 and primary rat cortical neuronal cells. Thus, these approaches may be more relevant to physiological conditions although it should be noted that *in vivo* studies following chronic treatment with PAMs will be necessary to define their longer-term effects on safety and tolerability. In conclusion, our study demonstrated that $\alpha 7$ nAChR PAMs do

not exhibit cytotoxic properties in cells expressing $\alpha 7$ nAChRs. PAMs derived from both profiles could represent viable opportunities as therapeutic approaches for treatment of cognitive dysfunction and other indications.

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