

MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Memantine protects rat cortical cultured neurons against β -amyloid-induced toxicity by attenuating tau phosphorylation

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Abstract

It has been suggested that accumulation of beta-amyloid (A β) peptide triggers neurodegeneration, at least in part, via glutamate-mediated excitotoxicity in Alzheimer's disease (AD) brain. This is supported by observations that toxicity induced by A β peptide in cultured neurons and in adult rat brain is known to be mediated by activation of glutamatergic *N*-methyl-D-aspartate (NMDA) receptors. Additionally, recent clinical studies have shown that memantine, a noncompetitive NMDA receptor antagonist, can significantly improve cognitive functions in some AD patients. However, very little is currently known about the potential role of memantine against A β -induced toxicity. In the present study, we have shown that A β_{1-42} -induced toxicity in rat primary cortical cultured neurons is accompanied by increased extracellular and decreased intracellular glutamate levels. We subsequently demonstrated that A β toxicity is induced by increased phosphorylation of tau protein and activation of tau kinases, i.e. glycogen synthase kinase-3 β and extracellular signal-related kinase 1/2. Additionally, A β treatment induced cleavage of caspase-3 and decreased phosphorylation of cyclic AMP response element binding protein, which are critical in determining survival of neurons. Memantine treatment significantly protected cultured neurons against A β -induced toxicity by attenuating tau-phosphorylation and its associated signaling mechanisms. However, this drug did not alter either conformation or internalization of A β_{1-42} and it was unable to attenuate A β -induced potentiation of extracellular glutamate levels. These results, taken together, provide new insights into the possible neuroprotective action of memantine in AD pathology.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the presence of two hallmark lesions: extracellular β -amyloid (A β)-containing neuritic plaques and intracellular phospho-tau-positive neurofibrillary tangles (Hardy, 1997; Brion *et al.*, 2001; Selkoe & Schenk, 2003). Loss of neurons and synapses in selected brain regions represents another invariant feature that correlates with the memory and cognitive decline observed in AD patients (Lopez & DeKosky, 2003). Of all the brain regions, the glutamatergic pyramidal neurons of the cerebral cortex and hippocampus are affected early in the disease process in a pattern that corresponds to the distribution of plaques and tangles (Butterfield & Pocernich, 2003; Francis, 2003). Given the established role of glutamate and its receptors in synaptic plasticity such as long-term potentiation (LTP), a cellular basis of learning and memory, it has been suggested that dysfunction of glutamatergic neurons, together with the loss of the basal forebrain cholinergic neurons that provide innervation to the hippocampus and

cortex, contribute to the progressive memory impairment associated with AD patients (Bartus *et al.*, 1982; Cacabelos *et al.*, 1999; Kar *et al.*, 2004; Waxman & Lynch, 2005). However, very little information is currently available on the cause of dysfunction and degeneration of these neurons or their possible relationships to A β -containing neuritic plaques or tau-positive neurofibrillary tangles.

A large body of evidence indicates that A β peptides, the primary constituents of neuritic plaques, may initiate the process of neurodegeneration in AD brains (Clippingdale *et al.*, 2001; Casas *et al.*, 2004; Oakley *et al.*, 2006; Smith *et al.*, 2006). The mechanisms associated with A β toxicity are not clearly defined, but appear to involve alterations in intracellular calcium, production of free radicals, phosphorylation of tau protein and/or activation of a caspase cascade culminating in programmed cell death (Roth, 2001; Mattson & Chan, 2003; Blurton-Jones & Laferla, 2006). More recently, a number of studies have indicated that A β toxicity is mediated, at least in part, by glutamate-mediated excitotoxicity. This is supported by data showing that: (i) A β peptides can potentiate glutamate release from cultured microglia and can inhibit its uptake in cultured glial as well as neuronal cells (Harris *et al.*, 1996; Fernandez-Tome *et al.*, 2004); (ii) A β peptides can enhance glutamate-mediated toxicity in cultured

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neurons, whereas transient inactivation of the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor by antagonists can protect neurons from A β toxicity (Mattson *et al.*, 1992; Tremblay *et al.*, 2000; Floden *et al.*, 2005); (iii) A β -induced neurodegeneration in the adult rat brain is mediated, in part, by activation of the NMDA receptor (Harkany *et al.*, 2000; Miguel-Hidalgo *et al.*, 2002); and (iv) transgenic mice exhibiting high levels of A β peptide show increased vulnerability to excitotoxicity (Guo *et al.*, 1999; Moechars *et al.*, 1999). These results are substantiated by the approval of memantine, a noncompetitive, low- to moderate-affinity NMDA receptor antagonist for the treatment of AD patients. This antagonist, which prevents pathological activation of the NMDA receptor without affecting its physiological functions, has been shown to significantly improve cognitive behavioral functions in moderate to severe AD patients (Sonkusare *et al.*, 2005; Schmitt *et al.*, 2007). However, very little is currently known about the potential role of memantine against A β -induced toxicity. In the present study, using rat primary cortical cultures, we have shown that memantine can protect cultured neurons against A β -induced toxicity by attenuating phosphorylation of tau protein and its associated signaling mechanisms, thus providing an underlying basis for some of its beneficial effect in AD patients.

Materials and methods

Materials

Timed-pregnant Sprague–Dawley rats purchased from Charles River (St Constant, Quebec, Canada) were maintained according to the Animal Care and Use Committee of the University of Alberta and the Canadian Council for Animal Care Committee guidelines. A β _{1–42} and its reverse sequence A β _{42–1} were purchased from American Peptides (Sunnyvale, CA, USA), fluorescein-conjugated A β _{1–42} (i.e., fluorescein-A β _{1–42}) was from Anaspec (San Jose, CA, USA), the Cell Titer 96 Cell Proliferation Assay kit was from Promega (Madison, WI, USA) and the Live/Death assay kit (L-3224) was from Molecular Probes (Eugene, OR, USA). Memantine was obtained as a generous gift from Lundbeck (Copenhagen, Denmark), and AP-5 was purchased from Cedarlane (Burlington, Ontario, Canada). Polyacrylamide electrophoresis gels (4–20%) were obtained from Invitrogen (Burlington, Ontario, Canada) and the enhanced chemiluminescence (ECL)⁺ kit was from Amersham (Mississauga, Ontario, Canada). Antiphospho-Ser⁹glycogen synthase kinase-3 β (GSK-3 β ; catalogue no. 9323), antiphospho-Ser⁴⁷³Akt (catalogue no. 9271), antiphospho-Thr²⁰²/Tyr²⁰⁴ extracellular-signal related kinase 1/2 (ERK1/2; catalogue no. 9101), antiphospho-Ser¹³³cyclic AMP response element binding (CREB) protein (catalogue no. 9191), anticlaved caspase-3 (catalogue no. 9961) and anti-Akt antisera (catalogue no. 9272) were from Cell Signaling (Mississauga, Ontario, Canada), whereas anti-GSK-3 β (catalogue no. 610201) was from BD Transduction Labs (Mississauga, Ontario, Canada) and antiphospho-Tyr²¹⁶GSK-3 β (catalogue no. 05-413) from Upstate Biotechnology (Lake Placid, NY, USA). Antiphospho-Tau AT180 (Thr²³¹, catalogue no. MN-1040) and AT270 (Thr¹⁸¹, catalogue no. MN-1050) were from Polymedco Inc (Cortlandt Manor, NY, USA), anti-A β (6E10; recognizes 1–17 fragment of human A β) was from Signet (Dedham, MA, USA), and anti-Rab7 (catalogue no. sc-10767) and antimicrotubule associated protein-2 (MAP2; catalogue no. sc-20172) as well as all secondary antibodies were from Santa Cruz Biotechnology (San Diego, CA, USA). Cell culture reagents B27, penicillin and streptomycin were purchased from Invitrogen (Burlington, Ontario, Canada) and Hoechst 33258 and LiCl were from Sigma–Aldrich (Mississauga, Ontario, Canada). Reagents and solvents required for high performance liquid

chromatography (HPLC) such as methanol, tetrahydrofuran (THF), 2-mercaptoethanol and acetonitrile were purchased from Waters Ltd (Milford, MA, USA) or Fisher Scientific (Pittsburgh, PA, USA), whereas *o*-phthalaldehyde (OPA) was obtained from Pierce Chemicals (Rockford, IL, USA). Formar carbon-coated grids, 0.5% Formvar solution and uranyl acetate were from EM Science (Hatfield, PA, USA). All other reagents were from Sigma–Aldrich or Fisher Scientific.

Primary cortical neuronal culture

Primary rat cortical cultures were prepared from 16- or 17-day-old embryos of timed-pregnant Sprague–Dawley rats as described previously (Zheng *et al.*, 2002; Wei *et al.*, 2008), with minor modification. In brief, the pregnant rats were anaesthetized with halothane (2–5%, 10 min) and then decapitated. The entorhinal cortex (excluding frontal cortex and occipital cortex) from the pup brain was then dissected in Hanks's balanced salt solution (HBSS) supplemented with 15 mM HEPES, 10 U/mL penicillin and 10 mg/mL streptomycin, and digested with 0.25% each of trypsin and EDTA. The cell suspension was filtered through a cell strainer (40 μ m Nylon) and then plated on either 96-well plates (2×10^3 cells per well for survival/death assay), 24-well plates (2×10^4 cells per well for biochemical analysis and HPLC assay to measure glutamate release) or 12-mm glass coverslips (2×10^4 cells per coverslip for immunocytochemical staining). The cultures were grown at 37°C in a 5% CO₂ humidified atmosphere in Neurobasal medium supplemented with B27, 50 μ M glutamine, 15 mM HEPES, 10 U/mL penicillin and 10 mg/mL streptomycin. The medium was replaced 1 day later without glutamine and all experiments were performed on day 6 or 7 after plating.

Treatments

The oligomeric form of A β _{1–42}, used in all experiments related to toxicity in primary cultured neurons, was prepared as described in detail earlier (Song *et al.*, 2006). In brief, A β _{1–42} was initially dissolved at a 1-mM concentration in hexafluoroisopropanol (HFIP), aliquoted in sterile microcentrifuge tubes, dried under a stream of N₂ and then stored at –80°C. Just before use, the peptide was resuspended in dimethylsulfoxide to 5 mM and then added to cultured neurons to the required final concentrations (0.01–20 μ M) for different periods of time (i.e. 1, 3, 6, 18, 24 or 48 h). In some experiments, cultured neurons were exposed to 10 μ M A β _{1–42} for 24 h either with various concentrations of memantine (0.1–20 μ M) or AP-5 (10–100 μ M) or following different periods (2 or 24 h) of pretreatment with the antagonist. Some cultured neurons were exposed to A β _{1–42} together with optimal concentrations of memantine (20 μ M) and AP-5 (10 μ M). In a parallel set of experiments, cultured neurons were exposed to 10 μ M A β _{1–42} for 24 h with or without 1 mM LiCl. Control and A β -treated cultures were then processed for cell viability or toxicity, immunoblotting, immunostaining, glutamate release and/or internalization of the labeled peptide. Fluorescein-A β _{1–42}, which was used to evaluate peptide internalization, was also prepared in HFIP. To determine the conformation state of the peptide, 10 μ M A β _{1–42} was dissolved in HFIP or in distilled water, incubated in culture medium at 37°C for 24 h and then assessed by Western blot or electron microscopy (EM).

Neuronal viability or toxicity assays

Viability of neurons was assessed using the Cell Titer 96 cell proliferation colorimetric assay that converts 3-(4,5-dimethylthiazolyl)-

2,5-diphenyl-tetrazolium bromide (MTT) from a yellow to a blue formazan crystal by dehydrogenase enzymes in metabolically active cells (Zheng *et al.*, 2002). Control and A β -treated culture plates were replaced with new medium containing 0.25% MTT and then incubated for 2 h in a CO₂ incubator at 37°C. The reaction was terminated and measured spectrophotometrically at 570 nm. The experiment was repeated three to five times in triplicate. Neuronal viability was also assessed using the Live/Death assay kit containing calcein AM and ethidium homodimer (EthD-1) as the fluorescent probes. Calcein AM is a cell-permeant dye that fluoresces in live cells with a functional intracellular esterase whereas EthD-1 is a membrane-impermeable DNA-binding dye that is excluded from live cells with an intact plasma membrane. In this paradigm, control and A β -treated cultures were incubated with medium containing 2 μ M calcein AM and 4 μ M EthD-1 for 30 min in a CO₂ incubator at 37°C, fixed in 4% paraformaldehyde (PFA) and then visualized under a Zeiss Axioskop-2 epifluorescence microscope. In a parallel series of experiments, neuronal apoptosis was assessed by using the nuclear marker Hoechst 33258 as described earlier (Song & de Chaves, 2003). In brief, control and A β -treated cultures were fixed with 4% PFA for 20 min, washed in Tris-buffered saline (TBS) and then stained with Hoechst 33258 (50 ng/mL) for 10 min. The chromatin staining pattern was analyzed for individual cells under a Zeiss Axioskop-2 epifluorescence microscope. The experiment was repeated three times in triplicate. The percentage of apoptotic cells was calculated by counting condensed and/or fragmented nuclei versus evenly stained nuclei of normal cells. The data, which are presented as mean \pm SEM, were analyzed using one-way ANOVA followed by Newman–Keuls *post hoc* analysis with significance set at $P < 0.05$ and $P < 0.001$.

Western blotting

For Western blotting, control and A β -treated cells from different experimental paradigms were rinsed with cold TBS and then harvested in radioimmunoprecipitation assay buffer (TBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 10% glycerol with inhibitors 50 mM NaF, 1 mM NaVO₃, 10 μ g/mL aprotinin and 10 μ g/mL leupeptin). Samples were then denatured in modified Laemlli sample buffer (40 mM Tris–HCl, pH 6.8, 1% SDS, 4% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue) and boiled for 2–5 min, and equal amounts of proteins (20 μ g) were separated by 4–20% polyacrylamide gel electrophoresis as described earlier (Hawkes *et al.*, 2005). The proteins were subsequently transferred to PVDF or nitrocellulose membranes, blocked with TTBS (TBS with 0.1% Tween-20) containing 5% non-fat milk and incubated overnight at 4°C with antiphospho-Tau AT180 (1 : 1000), antiphospho-Tau AT270 (1 : 1000), antiphospho-Ser⁴⁷³Akt (1 : 500), antiphospho-Thr²⁰²/Tyr²⁰⁴ERK1/2 (1 : 1000), antiphospho-Ser⁹GSK-3 β (1 : 1000), antiphospho-Tyr²¹⁶GSK-3 β (1 : 1000), anticaspace-3 (1 : 1000) and antiphospho-Ser¹³³CREB (1 : 1000) antibodies. The specificity and characterization of the antibodies used in this study have been described earlier (Greenberg & Davies, 1990; Zheng *et al.*, 2002; Garnier *et al.*, 2003; Hawkes *et al.*, 2005; Wei *et al.*, 2008). Membranes were washed in TTBS, incubated for 1 h at 22°C with the appropriate secondary antibody and visualized using an ECL⁺ detection kit. Blots were stripped and reprobed with either anti-Akt (1 : 1000), anti-ERK (1 : 1000), anti-GSK-3 β (1 : 1000), anti-tau (1 : 3000) or anti- β -tubulin (1 : 1000) antibodies. All blots were quantified using an MCID image analysis system (Hawkes *et al.*, 2005). The data, which are presented as mean \pm SEM, were analyzed using one-way ANOVA followed by Newman–Keuls *post hoc* analysis

using GRAPHPAD PRISM software. The level of significance was set at $P < 0.05$ and $P < 0.001$.

Immunocytochemistry

Control and A β -treated neurons plated on coverslips were processed for phospho-tau immunoreactivity as described earlier (Zheng *et al.*, 2002). In brief, neurons were fixed with 4% PFA, blocked with 2% bovine serum albumin in TTBS and incubated overnight at 4°C with phospho-tau antibodies i.e. AT180 (1 : 500; Thr²³¹) and AT270 (1 : 500; Thr¹⁸¹), with or without anti-MAP2 (1 : 500) antibody. The cells were washed in TTBS and exposed to appropriate secondary antibodies for 2 h at room temperature. The coverslips were then mounted in Vectashield with or without Hoechst 33258 labeling and examined under a Zeiss Axioskop-2 epifluorescence microscope. The percentage of cells labeled with phospho-tau and Hoechst 33258 was calculated in two separate sets of control cultures as well as in cultures that were treated with 10 μ M A β _{1–42} with or without 20 μ M memantine.

HPLC analysis

Primary rat cortical cultured neurons were treated with 10 μ M A β _{1–42} for different periods of time (20 min, 1 h and 24 h) and then culture media and cell lysates were collected to measure glutamate levels. In brief, collected media and cell lysates were centrifuged at 13400 *g* for 5 min, supernatants were diluted (1 : 2) with methanol and then samples were analyzed for glutamate levels by reverse-phase HPLC (Waters Ltd., Milford, MA, USA) using OPA as the derivatizing agent and 2-mercaptoethanol as a reducing agent (Parent *et al.*, 2001). For our assay, we used a Waters Alliance 2690XE instrument equipped with an autosampler (Waters Ltd), thermally controlled sample and column compartments at 4 and 30°C respectively, and a Waters 474 fluorescence detector. Separation was carried out on a Symmetry C18 column (3.5 mm, 4.6 \times 150 mm) coupled with a guard column containing the same stationary phase (5 mm, 3.9 \times 150 mm). Fluorescence emitted by the thioalkyl derivatives was detected using a Waters TM Scanning Fluorescence detector (excitation wavelength 260 nm and emission wavelength 455 nm) following elution of the derivatized amino acids from the column. For mobile phases, solvent A (pH 6.2) consisted of 900 mL 0.08 M NaH₂PO₄, 240 mL MeOH and 10 mL THF, whereas solvent B (pH 6.2) consisted of 1340 mL 0.04 M NaH₂PO₄, 1110 mL MeOH and 60 mL THF. The mobile phase gradient was set at 60% A and 40% B. Mobile phase B was increased to 100% over 20 min. In parallel with culture samples, a series of varying concentrations (10 nM–10 μ M) of glutamate standards were run through the HPLC. EMPOWER[®] software was used to collect and analyze the data. Glutamate levels in cell lysates were normalized to protein amounts and were expressed as ng/ μ g protein.

Determination of A β conformation

The conformation state of 10 μ M A β _{1–42} dissolved in HFIP or in water was determined by Western blotting and EM analysis after 24 h exposure in the culture medium with or without 20 μ M memantine. For Western blotting, the medium containing A β _{1–42} was diluted in sample buffer and then run on a 4–20% polyacrylamide gel electrophoresis system. The proteins were then transferred to nitrocellulose membranes, incubated overnight at 4°C with 6E10 A β antibody (1 : 1000), washed with TTBS, exposed to the secondary antibody and detected using an ECL⁺ kit. For EM experiments, culture

media containing 10 μ L of A β_{1-42} (10 μ M) with or without 20 μ M memantine were placed on Formvar-carbon coated grids for 1 min. Ten microlitres of 0.5% glutaraldehyde was added to each grid and incubation was continued for an additional 1 min. The grid was washed with distilled water and wick-dried. Finally, grids were stained with 2% uranyl acetate for 2 min, wick-dried, air-dried and then viewed on a JEOL CX 100 electron microscope and photographed with a Mega View III digital camera as described earlier (Wei *et al.*, 2008).

Endocytosis of A β_{1-42}

Earlier studies have shown that A β peptide following internalization is targeted to lysosomes (Ditaranto *et al.*, 2001). To determine possible internalization of A β peptide, cultured neurons were first exposed to 10 μ M fluorescein-A β_{1-42} for 24 h, fixed in 4% PFA and then incubated with the early endosomal marker anti-Rab7 (1 : 250) antibody overnight at 4°C. The cells were then exposed to fluorescein isothiocyanate-labeled goat antirabbit IgG (1 : 500) for 2 h and mounted in Vectashield with or without Hoechst 33258 labeling, and pictures were taken with a Zeiss LSM 510 two-photon confocal microscope equipped with an Plan-Neofluar 40/1.3 oil objective using appropriate filters (LP 560, BP 500–550 IR and BP 390–465 IR) and excitation wavelengths (488, 543 and 780 nm). For colocalization images, background signal was eliminated using the threshold tool for the best-fit threshold value. Images were stored for subsequent analysis using a Zeiss LSM image analysis system (Carl Zeiss, Welwyn Garden City, UK). To establish the potential influence of memantine on A β endocytosis, cortical cultured neurons were exposed to 10 μ M fluorescein-A β_{1-42} for different periods of time (5 min–24 h) in the presence or absence of 20 μ M memantine, washed with TBS and then fixed in 4% PFA. The cells were then stained with Hoechst 33258, mounted in Vectashield and examined with a Zeiss Axioskop-2 epifluorescence microscope as well as a Zeiss LSM 510 confocal microscope, or evaluated with a fluorescence plate reader.

Results

A β_{1-42} -induced toxicity in rat cortical cultured neurons

Rat primary cortical cultured neurons are vulnerable to A β_{1-42} -mediated toxicity, as evident from a reduction in MTT values and concurrent decrease in viable neurons in the Hoechst 33258 nuclear staining and live/death assays (Fig. 1A–F). A concentration-dependent (0.01–20 μ M) effect of A β_{1-42} over a 24-h treatment revealed a significant decrease in MTT values from 1 μ M onwards, reaching a plateau at an \sim 10 μ M concentration of the peptide. Exposure of 10 μ M A β_{1-42} to cultured neurons decreased MTT values in a time-dependent (1–48 h) manner, with a significant reduction in cell viability observed at 3 h and reaching maximal effect (\sim 50% decrease) at 2 days post-treatment (Fig. 1A and B). The toxic potency of A β_{1-42} on cortical cultured neurons was substantiated by a similar time (1–48 h)- and dose (0.01–20 μ M)-dependent increase in the number of Hoechst 33258-positive apoptotic neurons (Fig. 1C–E). Our live/death assay also revealed that exposure of cultured neurons to 10 μ M A β_{1-42} over 24 h can induce a marked increase in the number of dead cells (Fig. 1F). The reverse-sequence peptide A β_{42-1} (10 μ M; see Fig. 1A and C), in contrast to the regular peptide sequence, did not significantly affect the survival of neurons in either the MTT assay or the Hoechst 33258 nuclear staining or live/death assay, thus confirming the specificity of the observed effects.

A β_{1-42} and tau phosphorylation

To determine whether A β -induced toxicity in rat cortical cultured neurons was associated with increased phosphorylation of tau protein, we evaluated tau phosphorylation by immunoblotting and immunocytochemistry. An increased phosphorylation of tau by 10 μ M A β_{1-42} (the concentration selected from aforementioned dose- and time-dependence study of the peptide on neuronal viability) was observed using both AT-180 and AT-270 antibodies as early as 3 h and remained elevated until 24 h of treatment (Fig. 2A and B). At the cellular level, phospho-tau immunoreactivity in control cultures was detected mostly in neurites, whereas in A β -treated cultures staining was apparent in neurites as well as soma of the neurons; this is confirmed by our dual-labeling experiment with MAP2 (Fig. 2C and D). Furthermore, many phospho-tau-positive neurons displayed beaded neurites, consistent with neuronal degeneration (Fig. 2C). Our double labeling experiments followed by quantification of neurons labeled with phospho-tau and Hoechst 33258 showed that the number of neurons which demonstrated accumulation of phospho-tau in cell soma prior to the appearance of nuclear fragmentation is significantly higher in A β -treated cultures than in control cultures (Fig. 2D and S1).

A β_{1-42} and intracellular signaling cascades

Earlier studies have indicated that A β -induced tau phosphorylation is mediated by the activation of various kinases, including mitogen-activated protein kinase (MAPK) and GSK-3 β (Brion *et al.*, 2001; Ferrer *et al.*, 2005; Wang *et al.*, 2007). To determine whether these kinases are involved in A β -induced tau phosphorylation, rat cortical cultured neurons were exposed to 10 μ M A β_{1-42} for various times (1, 3, 6 and 24 h) and then activation of MAPK and GSK-3 β was assessed by immunoblotting using phospho-Thr²⁰²/Tyr²⁰⁴ERK1/2, phospho-Ser⁹GSK-3 β and phospho-Tyr²¹⁶GSK-3 β antisera. A significant induction of the MAPK, i.e. phospho-ERK1/2, was evident as early as 1 h and remained elevated over the 24-h treatment period (Fig. 3A and B). While the levels of phospho-Tyr²¹⁶GSK-3 β also exhibited a protracted increase over the 24-h treatment paradigm, a marked reduction in phospho-Ser⁹GSK-3 β levels was not apparent until 24 h following exposure to A β_{1-42} (Fig. 3C and D). Activation of GSK-3 β via decreased Ser⁹ and/or increased Tyr²¹⁶ phosphorylation could be mediated by multiple mechanisms including inhibition of phosphatidylinositol 3 (PI-3)-dependent Akt kinase activity (Jope & Johnson, 2004). Our results showed that levels of phospho-Ser⁴⁷³Akt were decreased in a time-dependent manner in A β_{1-42} -treated cortical cultured neurons. Concomitant with the kinases, we also evaluated the activation of caspase-3 and CREB, which are critical in determining death or survival of neurons (Fan *et al.*, 2005; Waxman & Lynch, 2005). Our results clearly indicated that 10 μ M A β_{1-42} increased the proteolytically active form of caspase-3 (i.e. 17 kDa) but decreased phospho-Ser¹³³CREB levels in cortical cultured neurons (Fig. 3E and F).

A β_{1-42} and glutamate release

Some earlier studies have suggested that A β peptides, by inhibiting uptake, can increase extracellular glutamate levels which can render neurons vulnerable to degeneration (Fernandez-Tome *et al.*, 2004). To determine whether A β_{1-42} can increase extracellular glutamate levels in rat cortical cultured neurons, we first measured glutamate levels in culture media using HPLC following exposure to 10 μ M A β_{1-42} . Our results show that A β_{1-42} was able to significantly increase glutamate levels in culture medium over a 24-h treatment paradigm (Fig. 3G). It

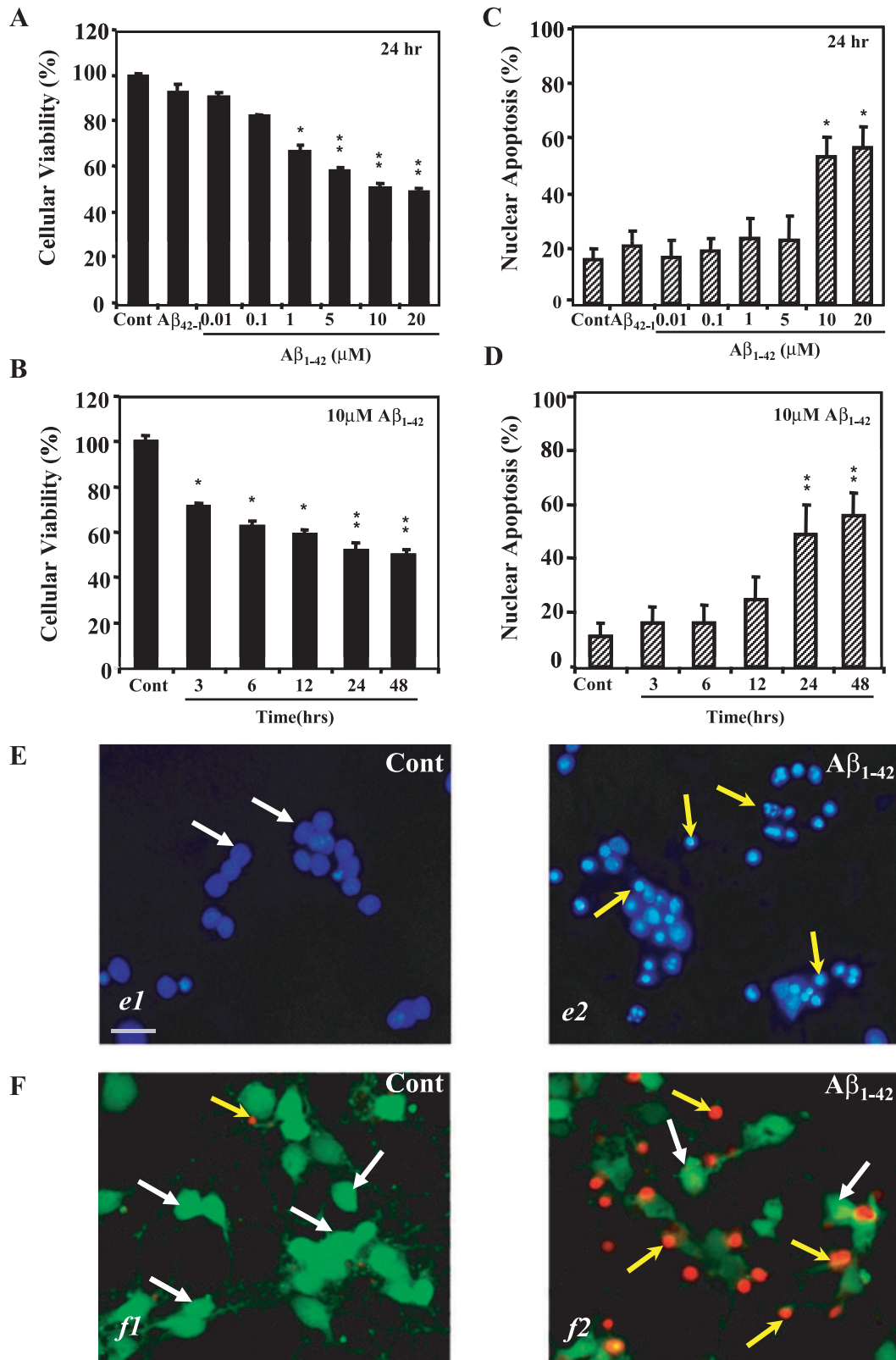


FIG. 1. A β_{1-42} -induced toxicity in rat primary cortical cultured neurons. Histograms showing the dose and time-course effects of A β_{1-42} on viability of cortical cultured neurons as revealed by (A and B) MTT reduction and (C and D) Hoechst nuclear fragmentation. Images from (E) Hoechst staining and (F) the live/death assay are also shown. (e1) White arrows indicate healthy nuclei and (e2) yellow arrows indicate apoptotic nuclei in Hoechst-labeled cultured neurons. (F: f1 and f2) Calcein AM staining (green; white arrows) represents intracellular esterase activity in living neurons, while EthD-1 (red; yellow arrows) shows dead neurons with disintegrated plasma membrane. Control cultured neurons were treated with 10 μ M A β_{42-1} . Data are presented as % of control (means \pm SEM) and were obtained from three to five separate experiments, each performed in triplicate. Details of statistical tests are given in Materials and methods. Cont, control; * P < 0.05 and ** P < 0.001 compared to control value. Scale bar, 20 μ m.

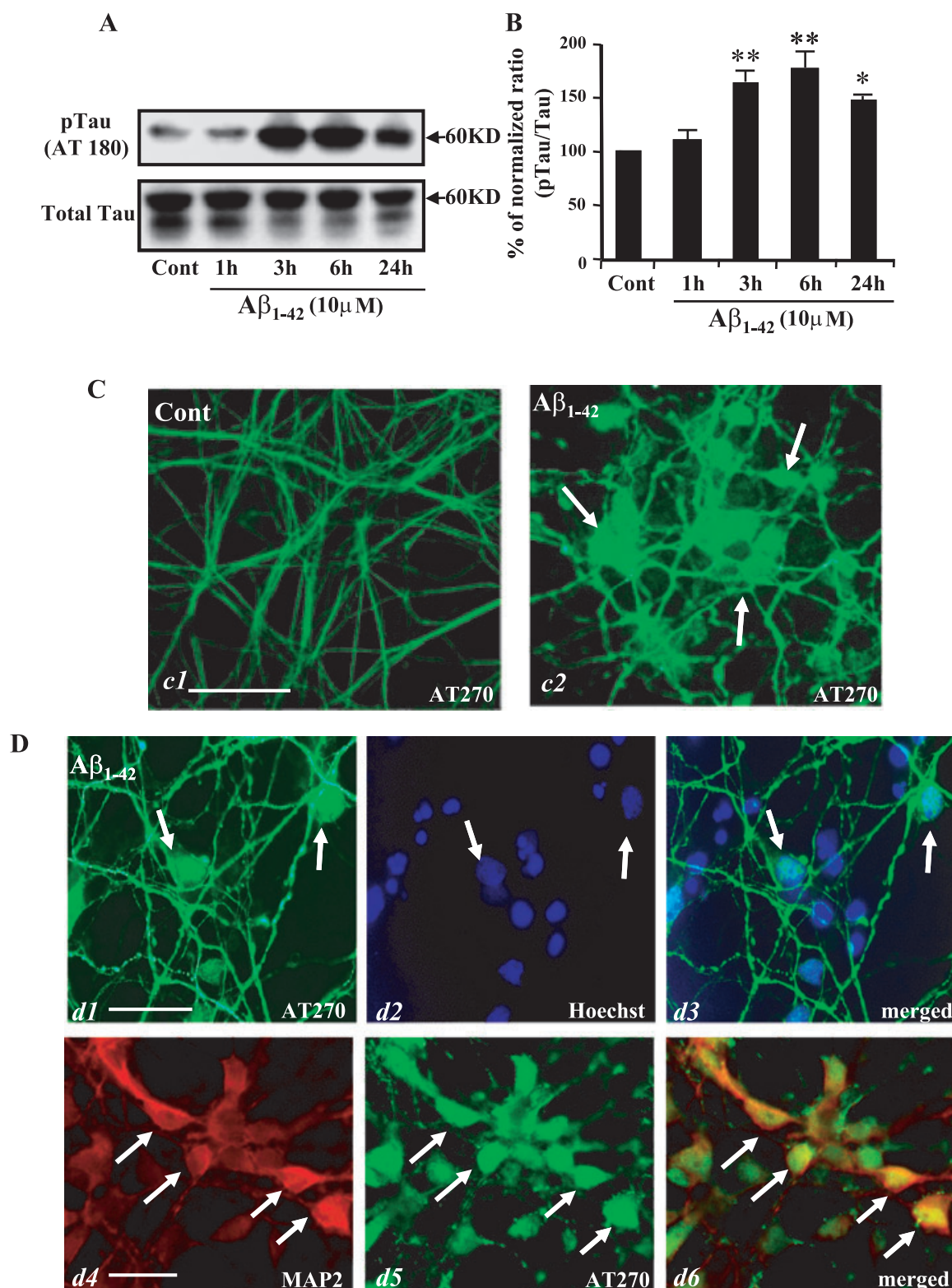


FIG. 2. $A\beta_{1-42}$ and tau phosphorylation. (A) Western blot and (B) corresponding histogram showing increased levels of phospho-tau from 3 to 24 h following 10 μ M $A\beta_{1-42}$ treatment. (C, c2) At the cellular level, phospho-tau immunoreactivity was apparent in soma (arrows) as well as neurites of the neurons in $A\beta$ -treated cultures, whereas (c1) staining in control cultures was observed mostly in neurites. (D, d1–3) Double-labeling experiments with phospho-tau and Hoechst 33258 revealed that cellular accumulation of phospho-tau occurred prior to the appearance of nuclear fragmentation in $A\beta$ -treated cultured neurons. (D, d4–6) Double-labeling experiments with MAP2 and phospho-tau showed that cellular accumulation of phospho-tau occurred in neuronal cell bodies. Quantification of the Western blot data, which are presented as percentages of control in the histogram, include results from three to five separate experiments. Cont, control; * $P < 0.05$ and ** $P < 0.001$ compared to control value. Scale bar, 20 μ m.

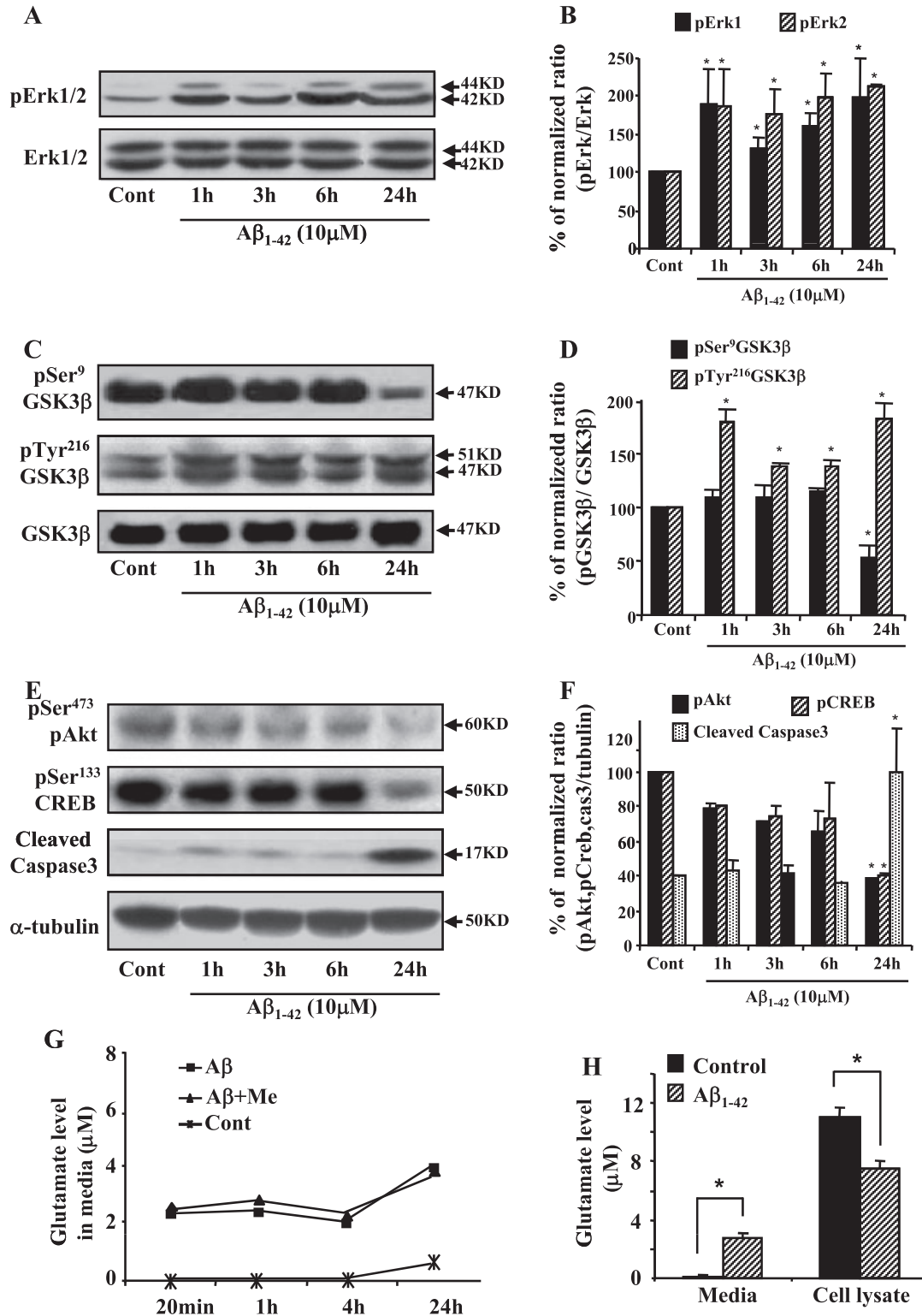


FIG. 3. A β_{1-42} -induced intracellular signaling and glutamate levels. (A, C and E) Western blots and (B, D and F) corresponding histograms showing time-dependent alterations in the levels of (A and B) phospho-ERK1/2, (C and D) phospho-Ser⁹GSK-3 β and phospho-Tyr²¹⁶GSK-3 β , and (E and F) phospho-Akt, phospho-CREB and cleaved caspase-3 in 10 μ M A β_{1-42} -treated cultured neurons. (A and B) A significant induction of phospho-ERK1/2 protein was evident as early as 1 h and remained elevated over the 24-h treatment period. (C and D) The levels of phospho-Tyr²¹⁶GSK-3 β showed a prolonged increase over the 24-h treatment period, whereas a marked reduction in phospho-Ser⁹GSK-3 β levels was not apparent until 24 h following A β_{1-42} treatment. (E and F) Levels of phospho-Akt and phospho-CREB were decreased, but cleaved caspase-3 product was found to increase in A β -treated cultures in a time-dependent manner. Quantification of the Western blot data, which are presented as percentages of control in the histograms, include results from three to five separate experiments. (G and H) A β_{1-42} (10 μ M) was found to increase glutamate levels in the medium over a 24-h treatment paradigm and (G) memantine treatment did not alter glutamate levels. (H) An increase in extracellular glutamate levels following treatment with A β_{1-42} (10 μ M) was accompanied by a parallel decrease in the intracellular level. Cont, control; * P < 0.05.

is also of significance to note that an increase in extracellular glutamate levels was accompanied by a parallel decrease in the intracellular levels at 24 h following exposure to 10 μM $\text{A}\beta_{1-42}$ (Fig. 3H).

Memantine and $\text{A}\beta_{1-42}$ -induced toxicity

To determine the potential neuroprotective role of memantine, cortical cultured neurons were treated with various concentrations (1, 5, 10 and 20 μM) of this antagonist either at the same time as or 2 and 24 h prior to exposure of the neurons to 10 μM $\text{A}\beta_{1-42}$, and then cell viability was assessed using MTT, the live/death assay and Hoechst 33258 staining. The concentration of memantine used in the study was based on earlier data obtained from cultured neurons (see Tremblay *et al.*, 2000). Our results, in agreement with a previous study (Tremblay *et al.*, 2000), showed that 10 and 20 μM memantine can significantly protect cultured neurons against $\text{A}\beta$ -induced toxicity. As the effect was more pronounced with 20 than with 10 μM memantine, we selected a 20- μM concentration for all our subsequent experiments (Fig. 4A–C). Additionally, our results indicate that either pre- or co-treatment with 20 μM memantine could significantly improve survival of neurons and there was no difference between the two experimental paradigms (Fig. 4A). The competitive NMDA receptor antagonist AP-5 at 10 μM concentration was also found to protect cultured neurons when co-applied with $\text{A}\beta_{1-42}$ (Fig. 4A). Interestingly, treatments with optimal concentrations of memantine and AP-5 together were found to protect cultured neurons against $\text{A}\beta_{1-42}$ -mediated toxicity to a similar extent as observed following treatment with either memantine or AP-5 alone (Fig. 4A). Subsequently, to define whether the protective effect of memantine is mediated by attenuating the aforementioned signaling mechanisms, we first measured phospho-tau levels in cultured neurons treated with $\text{A}\beta$ peptide in the presence or absence of memantine. It is evident from our results that memantine treatment not only decreased the levels of phospho-tau as observed using AT180 and AT270 antibodies but also significantly attenuated its somatodendritic accumulation in $\text{A}\beta$ -treated cultured neurons (Fig. 4D–F and S1). This was accompanied by a significant reversal of phospho-ERK1/2, phospho-Ser⁹GSK-3 β and phospho-Tyr¹⁶GSK-3 β levels and cleaved caspase-3 product in $\text{A}\beta$ -treated cultured neurons. The levels of phospho-Ser⁴⁷³Akt were increased, but not significantly, in memantine-treated $\text{A}\beta$ -cultured neurons (Fig. 5A–F). In addition to memantine, 1 mM LiCl, a potent inhibitor of GSK-3 β activity, was found to protect cortical cultured neurons against $\text{A}\beta$ -mediated toxicity (see Fig. S2) as reported earlier (Alvarez *et al.*, 2002).

Effects of memantine on $\text{A}\beta$ conformation, glutamate release and endocytosis

Several lines of experimental approach have shown that the neurotoxic potential of $\text{A}\beta$ peptides depends on their ability to form oligomers and/or fibrils (Clippingdale *et al.*, 2001; Smith *et al.*, 2006). To

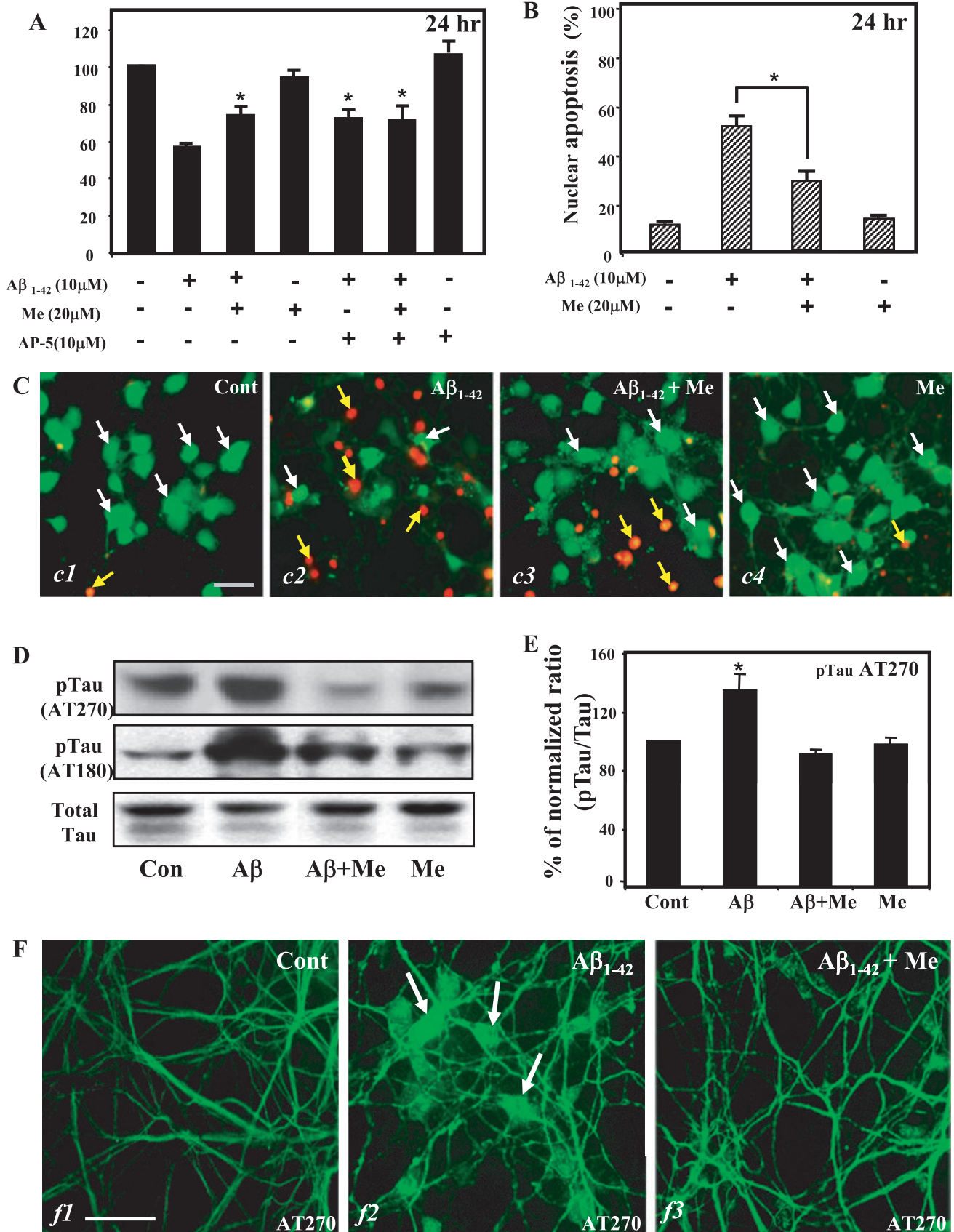
determine whether the concentration of memantine that protects neurons against $\text{A}\beta$ -mediated toxicity can influence peptide conformation, 10 μM $\text{A}\beta_{1-42}$ (i.e. the concentration used for the toxicity assay) dissolved in HFIP or water was incubated for 24 h in culture medium with or without 20 μM memantine and then processed for Western blotting. Monomeric and various oligomeric $\text{A}\beta_{1-42}$ forms were apparent in the medium and 20 μM memantine did not affect conformation of the peptide (Fig. 6A). However, it remains to be established whether concentrations of memantine other than 20 μM can influence conformation of $\text{A}\beta$ peptide. Previously we (Jhamandas *et al.*, 2005) and others (Lashuel *et al.*, 2002; De Felice *et al.*, 2004) have used the EM method to determine the potential effects of a drug on the conformation of $\text{A}\beta$ peptides. In the present study our EM results showed that oligomeric and fibrillar forms of $\text{A}\beta_{1-42}$ in the culture media were not influenced by treatment with 20 μM memantine (Fig. 6B). In parallel, we also evaluated whether memantine can protect neurons by regulating extracellular glutamate levels. Interestingly, $\text{A}\beta$ -induced potentiation of glutamate levels in cortical cultured neurons was not found to be altered by treatment with the antagonist (Fig. 3G).

Some earlier studies have shown that, following internalization, $\text{A}\beta_{1-42}$ is targeted to lysosomes via late endosomes and then it induces toxicity by triggering an intracellular signaling cascade (Yang *et al.*, 1998; Ditaranto *et al.*, 2001). To determine the internalization of $\text{A}\beta$ peptide in our experimental paradigm, cultured neurons were first exposed to 10 μM fluorescein- $\text{A}\beta_{1-42}$ and then labeled with late-endosomal marker Rab-7 and Hoechst 33258. Our results clearly revealed that a subset of fluorescein- $\text{A}\beta_{1-42}$ is localized with Rab-7 labeling in cortical cultured neurons (Fig. 6C). Subsequently, to determine whether $\text{A}\beta$ internalization can be influenced by memantine at the concentration that protects neurons against toxicity, we performed a time-course experiment with fluorescein-conjugated $\text{A}\beta_{1-42}$ (10 μM) in cultured neurons in the presence or absence of 20 μM memantine. It is apparent from our results obtained from confocal microscopy as well as the fluorescence plate reader that intracellular accumulation of fluorescein- $\text{A}\beta_{1-42}$, which occurred in a time-dependent manner in cultured neurons (Fig. 6C), was not affected by memantine treatment (Fig. 6D and E).

Discussion

The present study revealed, for the first time, that the NMDA receptor antagonist memantine, which has been approved for the treatment of AD patients, can protect rat primary cortical cultured neurons against $\text{A}\beta$ -mediated toxicity by attenuating phosphorylation of tau protein and associated signaling mechanisms. Additionally, we have shown that memantine at a concentration that protects neurons against toxicity does not alter conformation or internalization of $\text{A}\beta$ peptide and it is unable to attenuate $\text{A}\beta$ -induced potentiation of extracellular glutamate levels. These results, taken together, provide new insights into the neuroprotective action of memantine and its possible beneficial effects in AD pathology.

FIG. 4. Effects of memantine on $\text{A}\beta_{1-42}$ -induced toxicity. Rat cortical cultured neurons were treated with 10 μM $\text{A}\beta_{1-42}$ in the presence or absence of 20 μM memantine and then (A) cellular viability and (B) nuclear fragmentation were assessed by MTT reduction and Hoechst staining, respectively. Additionally, effects of 10 μM AP-5 alone and AP-5 (10 μM) + memantine (20 μM) against $\text{A}\beta$ -induced toxicity were evaluated using the MTT assay (A). Data presented as percentages of control (means \pm SEM) were obtained from three to five separate experiments, each performed in triplicate. (C, c1–4) Images from the live/death assay of, respectively, control cultured neurons, cultured neurons treated with $\text{A}\beta_{1-42}$, $\text{A}\beta_{1-42}$ + memantine and memantine alone. White arrows indicate living neurons and yellow arrows indicate dead neurons in the live/death assay. Memantine and AP-5 significantly protected cultured neurons against $\text{A}\beta_{1-42}$ -induced toxicity but the effects of memantine and AP-5 were not found to be additive. (D–F) $\text{A}\beta_{1-42}$ -induced alterations in (D and E) the levels and (F) the distribution profile of immunoreactive phospho-tau were found to be attenuated by memantine treatment. Quantification of the Western blot data, which are presented as percentages of control in the histogram, include results from three to five separate experiments. Cont, control; Me, memantine; * $P < 0.05$ compared to control value. Scale bar, 20 μm .



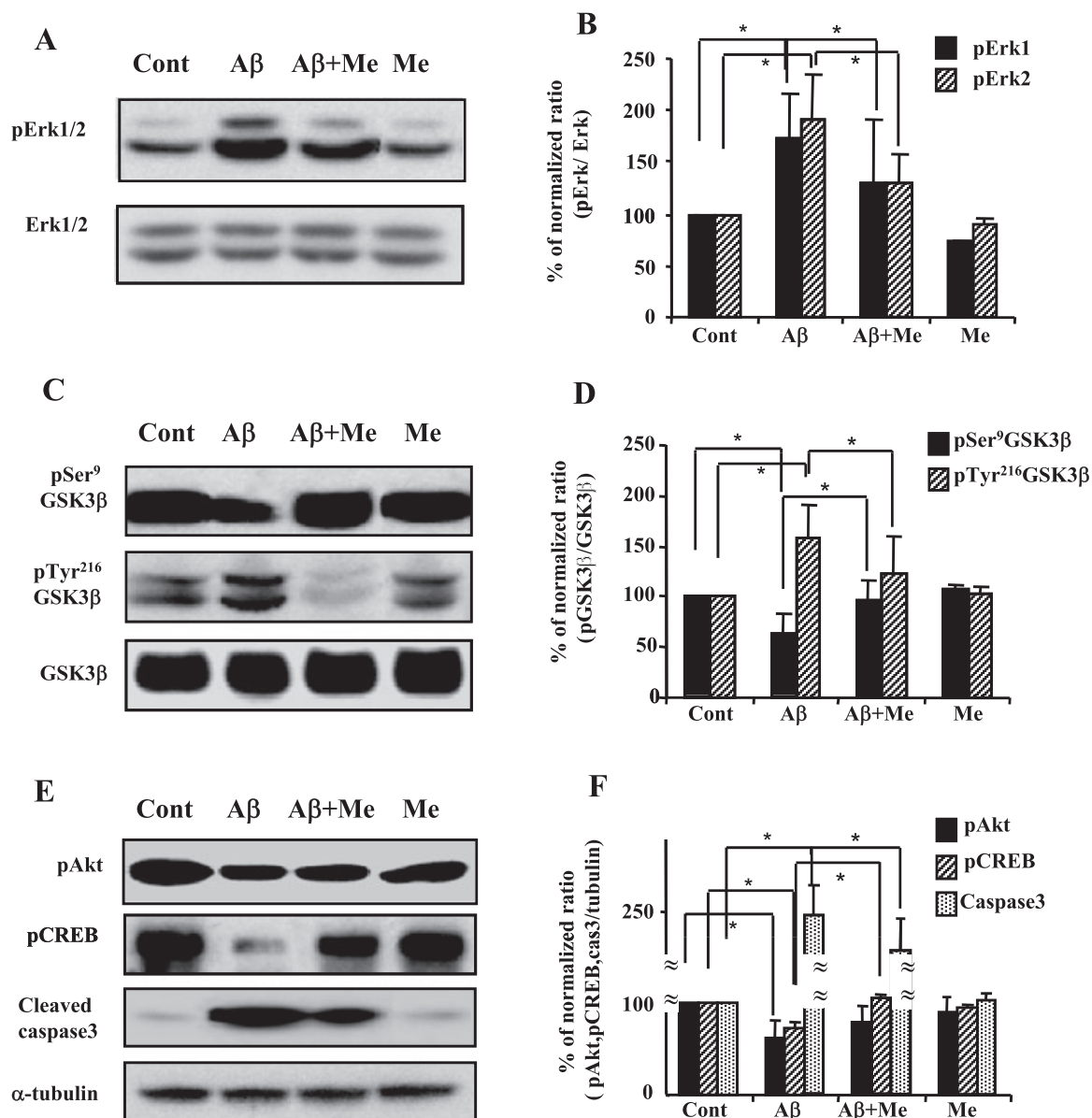


FIG. 5. Memantine and A β_{1-42} -induced intracellular signaling. (A, C and E) Western blots and (B, D and F) corresponding histograms showing memantine-induced reversal in the levels of (A and B) phospho-ERK1/2, (C and D) phospho-Ser⁹GSK-3 β and phospho-Tyr²¹⁶GSK-3 β , and (E and F) phospho-Akt, phospho-CREB and cleaved caspase-3 products in 10 μ M A β -treated rat cortical cultured neurons. Quantification of the Western blot data, which are presented as percentages of control in the histograms, include results from three to five separate experiments. * $P < 0.05$; Cont, control; Me, memantine.

A number of studies have shown that A β toxicity in cultured neurons is mediated, at least in part, via increased phosphorylation of tau protein induced by activation of multiple kinases including ERK1/2 and GSK-3 β (Alvarez *et al.*, 2002; Ferrer *et al.*, 2005; Stoothoff & Johnson, 2005). A critical role of the phosphorylated tau in A β -induced toxicity has been established by the evidence that: (i) cells undergoing A β toxicity exhibit increased levels of tau phosphorylation (Brion *et al.*, 2001; Alvarez *et al.*, 2002); (ii) inhibition of tau phosphorylation by blocking tau kinases can prevent cell death (Ferrer *et al.*, 2005; Stoothoff & Johnson, 2005) and (iii) neurons cultured from tau protein-knockout mice are resistant to A β toxicity (Rapoport *et al.*, 2002). More recently, it has been shown that a reduction in endogenous tau protein levels in the mutant amyloid precursor protein transgenic mouse can lead to amelioration of cognitive behavioral deficits without altering the high A β levels in

brain (Roberson *et al.*, 2007). Using immunocytochemistry and immunoblotting, we observed that A β_{1-42} treatment can not only increase tau phosphorylation but also evoke an AD-like accumulation of phosphorylated tau in the cell soma of the cultured cortical neurons. Tau phosphorylation is accompanied by a protracted increase in the levels of phospho-ERK1/2. Additionally, A β treatment increases the levels of phospho-Tyr²¹⁶GSK-3 β but decreases the levels of phospho-Ser⁹GSK-3 β and phospho-Akt, thus suggesting that an increased activation of GSK-3 β via downregulation of the PI-3/Akt kinase-dependent pathway may be involved in the phosphorylation of tau protein in cortical cultured neurons. This is supported in part by the evidence that the GSK-3 inhibitor LiCl can partially protect cultured neurons against A β -induced toxicity. Given the evidence that phosphorylation of tau protein occurs prior to the loss of neurons and inhibition of tau phosphorylation can prevent A β -induced neurode-

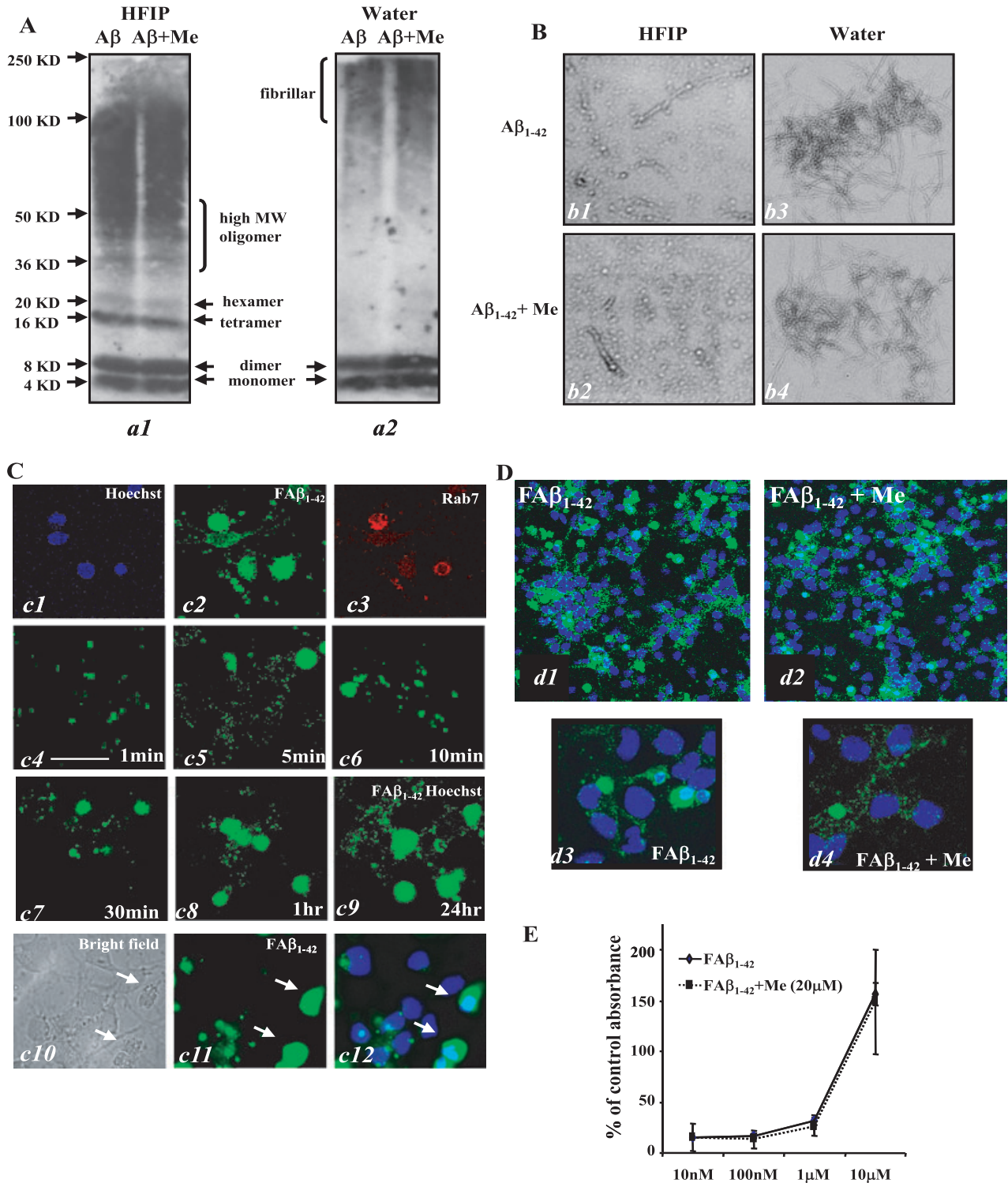


FIG. 6. Effects of memantine on A β ₁₋₄₂ conformation and internalization. (A and B) A β ₁₋₄₂ (10 μ M) prepared in HFIP or water was incubated with or without 20 μ M memantine in culture medium for 24 h and then its conformational state was evaluated by (A) Western blotting and (B) electron microscopy. (A) Monomeric and various oligomeric forms of A β ₁₋₄₂ that are evident in Western blots of control culture medium were not altered in the presence of memantine. This experiment was repeated three times and the Western blot in A represents a typical result obtained from an experiment. (B) Similarly, electron microscopy studies revealed that (*b1* and *b2*) HFIP-prepared oligomeric and (*b3* and *b4*) water-prepared fibrillar forms of A β ₁₋₄₂ were not altered by memantine treatment. (C–E) Confocal microscopic images showing the localization of fluorescein-conjugated A β ₁₋₄₂ (10 μ M; green) with (*c1–3*) late-endosomal marker Rab7 (red) and (*c4–9*) time-dependent increase in the internalization of fluorescein-conjugated A β ₁₋₄₂ (green) into the rat cortical cultured neurons. (*c10–12*) Images of phase-contrast microscopy and Hoechst staining depicting internalization of labeled A β ₁₋₄₂ into the cultured neurons are also shown. Exposure of cultured neurons to 10 nM–10 μ M fluorescein-conjugated A β ₁₋₄₂ in the presence or absence of 20 μ M memantine for 24 h did not alter internalization of the labeled A β peptide, (D) as evident at the cellular level and (E) following quantification using a fluorescence plate reader. Panels *d3* and *d4* are parts of *d1* and *d2* at higher magnification, respectively. All the experiments were performed three or four times. Quantification data, which are presented in (E) as percentages of control, include results from three separate experiments. Cont, control; Me, memantine. Scale bar, 20 μ m.

generation (Brion *et al.*, 2001; Alvarez *et al.*, 2002; Zheng *et al.*, 2002; Ferrer *et al.*, 2005), it is likely that increased levels of phosphorylated tau can contribute to the death of cortical cultured neurons via triggering loss of microtubule binding. Our results further showed that A β_{1-42} can induce cleavage of caspase-3 and decrease phospho-CREB levels. Cleaved caspase-3 product can play a critical role in the degeneration of neurons by breaking down its downstream death substrates and stimulation of endonucleases that cleave genomic DNA into fragments (Enari *et al.*, 1998; Fan *et al.*, 2005). Conversely, decreased phosphorylation of CREB, which possibly occurs via various pathways such as cAMP-, PKA-, ERK1/2- and/or PI-3/Akt kinase-dependent pathways (Walton & Dragunow, 2000; Tong *et al.*, 2004; Waxman & Lynch, 2005), can interfere with the transcription of genes regulating survival of neurons. In the present study we showed that decreased phosphorylation of CREB was not concomitant with ERK activation, suggesting that ERK1/2 might not be the major upstream target of CREB activation in these neurons. This is supported in part by a recent study which revealed that intraneuronal A β accumulation in transgenic rats exhibiting no extracellular amyloid plaques, in contrast to other animal models of AD (Minkeviciene *et al.*, 2004; Van Dam & De Deyn, 2006), can lead to enhanced phosphorylation of ERK2 and decreased phosphorylation of CREB kinase p90RSK (Echeverria *et al.*, 2004). Thus, it is likely that exposure of the rat cortical cultured neurons to A β_{1-42} can not only induce signaling pathways involved in the degeneration of neurons but can also simultaneously suppress neuronal survival pathways.

Consistent with some earlier studies (Mattson *et al.*, 1992; Gray & Patel, 1995; Tremblay *et al.*, 2000; Floden *et al.*, 2005), we have shown that A β toxicity in rat primary cortical cultured neurons is partly mediated by excitotoxicity involving glutamatergic NMDA receptor activation. This is supported by two lines of evidence: (i) A β -treatment can significantly increase extracellular glutamate levels in cortical cultured neurons; and (ii) memantine, a low- to moderate-affinity noncompetitive NMDA receptor antagonist, as well as AP-5, a competitive NMDA receptor antagonist, can partially protect cultured neurons against A β -toxicity. Given the evidence that A β peptides can regulate both uptake and release of glutamate from cultured neurons and glial cells (Harris *et al.*, 1996; Noda *et al.*, 1999; Fernandez-Tome *et al.*, 2004), it is possible that enhanced extracellular glutamate levels observed in the present study could be the consequence of increased release and/or decreased uptake of this neurotransmitter. Nevertheless, the importance of elevated glutamate levels in A β -mediated toxicity is indicated by the fact that memantine and AP5, which have been shown to protect neurons in a variety of experimental paradigms involving excitotoxicity (Miszta *et al.*, 1996; Stieg *et al.*, 1999; Tremblay *et al.*, 2000; Rao *et al.*, 2001; Miguel-Hidalgo *et al.*, 2002), can prevent degeneration of cortical cultured neurons. It is of interest to note that memantine did not fully protect neurons against A β toxicity, thus raising the possibility that mechanisms other than NMDA receptor activation may be involved in A β -induced toxicity (Floden *et al.*, 2005). Our results further indicate that pre- or co-treatment with memantine did improve survival of neurons but there was no significant difference in the rate of survival between the two paradigms. It is therefore likely that preconditioning with the drug is not essential for protecting neurons against A β -induced toxicity.

At the cellular level, glutamate has been shown to mediate excitotoxicity by increasing Ca²⁺ influx and consequent stimulation of a cascade of intracellular mechanisms. There is evidence that chronic exposure to glutamate can also lead to stimulation of ERK1/2 and increased phosphorylation of tau protein (Waxman & Lynch, 2005; Wenk *et al.*, 2006). Consistent with these data, our results showed that the protective effect of memantine is mediated by

attenuation of phospho-tau levels and/or expression in A β -treated cortical cultured neurons. This is supported by recent observations which showed that memantine, under experimental paradigms other than A β toxicity, can reverse phosphorylation and accumulation of tau protein by regulating the activity of protein phosphatase-2A (Li *et al.*, 2004; Chohan *et al.*, 2006). Our results, on the other hand, revealed that memantine can decrease phosphorylation of tau protein by reversing the levels of phospho-ERK1/2 and GSK-3 β , the kinases involved in the phosphorylation of tau protein. Additionally, memantine was also found to attenuate the activation of caspase-3 and phospho-CREB levels in A β -treated cultured neurons. However, memantine did not alter conformation or internalization of A β_{1-42} at 10 μ M concentration and it was unable to attenuate A β -induced potentiation of extracellular glutamate levels. Thus, the protective effect of memantine seems to be mediated by attenuation of signaling mechanisms rather than alteration of glutamate levels or regulation of A β conformation or internalization.

Memantine is currently being used for the treatment of moderate to severe AD patients in both Europe and North America. Several clinical studies have shown that this drug can significantly improve cognitive behavioral functions and activities of daily living without major side effects (see Danysz & Parsons, 2003; Sonkusare *et al.*, 2005; Robinson & Keating, 2006; Schmitt *et al.*, 2007). It is likely that memantine, unlike the high-affinity antagonists MK-801 and phen-cyclidine, is able to block the pathological activation of NMDA receptors without affecting their physiological actions required for learning and memory. The relatively fast voltage-dependent unblocking kinetics of the drug prevent it from accumulating in the ion channel and interfering with normal synaptic transmission (Cacabelos *et al.*, 1999; Hynd *et al.*, 2004; Lipton, 2005; Waxman & Lynch, 2005; Wenk *et al.*, 2006). Earlier studies have shown that memantine can restore LTP impairment induced by glutamatergic hyperactivity (Danysz & Parsons, 2003) and can also reverse cognitive behavioral deficits in animal models of AD (Minkeviciene *et al.*, 2004; Van Dam & De Deyn, 2006). Some recent studies have shown that memantine can protect neurons against toxicity at concentrations that do not inhibit LTP or produce learning deficits (Chen *et al.*, 1998; Lipton, 2005; Waxman & Lynch, 2005; Wenk *et al.*, 2006). Our results extended these data by demonstrating for the first time that memantine can protect cortical cultured neurons against A β -induced toxicity by attenuating cellular mechanisms associated with tau phosphorylation. Thus, it is possible that beneficial effects of memantine in AD patients could be related, at least in part, to its ability to protect neurons against A β toxicity by preventing tau phosphorylation. This is supported by a recent study which showed that memantine treatment for 12 months can lead to a decreased level of phosphorylated tau in the cerebrospinal fluid of AD patients (Degerman Gunnarsson *et al.*, 2007). Thus, our results obtained in this study provide new insights into the neuroprotective role of memantine against A β -mediated toxicity and its possible beneficial effects in AD pathology.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phospho-tau accumulation in A β_{1-42} -treated cultured neurons.
Fig. S2. Effects of GSK-3 inhibitor on A β_{1-42} -induced toxicity.

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Abbreviations

AD, Alzheimer's disease; A β , β -amyloid; CREB, cyclic AMP response element binding protein; ECL, enhanced chemiluminescence; EM, electron microscopy; ERK1/2, extracellular signal-related kinase1/2; EthD-1, ethidium homodimer; GSK-3 β , glycogen synthase kinase-3 β ; HBSS, Hanks's balanced salt solution; HFIP, hexafluoroisopropanol; HPLC, high performance liquid chromatography; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide; NMDA, *N*-methyl-D-aspartate; OPA, *o*-phthalaldehyde; PFA, paraformaldehyde; PI-3, phosphoinositide 3; TBS, Tris-buffered saline; THF, tetrahydrofuran; TTBS, TBS with 0.1% Tween-20.

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