α7-Nicotinic acetylcholine receptors mediate an $A\beta_{1-42}$-induced increase in the level of acetylcholinesterase in primary cortical neurones

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Abstract

The β-amyloid protein (Aβ) is the major protein component of amyloid plaques found in the Alzheimer brain. Although there is a loss of acetylcholinesterase (AChE) from both cholinergic and non-cholinergic neurones in the brain of Alzheimer patients, the level of AChE is increased around amyloid plaques. Previous studies using P19 cells in culture and transgenic mice which overexpress human Aβ have suggested that this increase may be due to a direct action of Aβ on AChE expression in cells adjacent to amyloid plaques. The aim of the present study was to examine the mechanism by which Aβ increases levels of AChE in primary cortical neurones. $A\beta_{1-42}$ was more potent than $A\beta_{1-40}$ in its ability to increase AChE in primary cortical neurones. The increase in AChE was unrelated to the toxic effects of the Aβ peptides. The effect of $A\beta_{1-42}$ on AChE was blocked by inhibitors of α7 nicotinic acetylcholine receptors (α7 nAChRs) as well as by inhibitors of L- or N-type voltage-dependent calcium channels (VDCCs), whereas agonists of α7 nAChRs (choline, nicotine) increased the level of AChE. The results demonstrate that the effect of $A\beta_{1-42}$ on AChE is due to an agonist effect of $A\beta_{1-42}$ on the α7 nAChR.

Keywords: acetylcholinesterase, Alzheimer's disease, amyloid, cholinergic, nicotinic.
J. Neurochem. (2004) **88,** 1186–1193.

The pathology of Alzheimer's disease (AD) is characterized by extensive neuronal and synaptic loss as well as by the presence of amyloid plaques and neurofibrillary tangles in the brain (Selkoe 1994; Soto *et al*. 1994). The β-amyloid protein (Aβ) is the major protein component of the amyloid plaques (Masters *et al*. 1985) and overproduction of Aβ is considered to be a key step in the pathogenesis of AD (Evin *et al*. 1994; Small *et al*. 2001).

Overall, the level of acetylcholinesterase (AChE) is decreased in the brain of AD patients (Atack *et al*. 1983; Fishman *et al*. 1986). However, AChE is increased around amyloid plaques and in neurofibrillary tangle-bearing neurones early in the process of amyloid deposition (Mesulam 1986; Geula and Mesulam 1989). This local increase in AChE may be due to a direct action of Aβ on AChE. Sberna *et al*. (1997) found that Aβ peptides increase AChE levels in neuronally differentiated P19 cells. This effect was mediated by L-type voltage-dependent calcium channels (L-VDCC) as the Aβ-induced increase in AChE in P19 cells was blocked by L-VDCC antagonists (Sberna *et al*. 1997). More recent studies have shown that Aβ can increase AChE in primary cortical cells (Sáez-Valero *et al*. 2003), and increased AChE has also been observed in the brains of transgenic mice that express human amyloid protein precursor (APP) or a

Received June 3, 2003; revised manuscript received October 24, 2003; accepted October 28, 2003.

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Abbreviations used: Aβ, β-amyloid protein; AChE, acetylcholinesterase; AD, Alzheimer's disease; AFP, atomic force microscopy; APP, amyloid protein precursor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; DNase, deoxyribonuclease; FBS, fetal bovine serum; HS, horse serum; MLA, methyllycaconitine citrate; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 (4-sulfophenyl)-2H-tetrazolium]; nAChR, nicotinic acetylcholine receptor; PBS, phosphate-buffered saline; SBTI, soybean trypsin inhibitor; VDCC, voltage-dependent calcium channel.

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C-terminal fragment of human APP containing the Aβ sequence (Sberna *et al.* 1998; Fodero *et al.* 2002).

Interestingly, not all forms of AChE are increased by Aβ. Several isoforms of AChE which can be distinguished by their different molecular weights and hydrodynamic properties have been identified (Taylor and Radic 1994). The human brain contains both amphiphilic (membrane-associated) and non-amphiphilic (soluble) forms, mostly globular tetramers (G4), light globular species (dimers, G2, and monomers, G1) and trace amounts of asymmetric (A12) isoforms (Brimijoin 1983; Massoulié *et al.* 1993). Aβ induces expression of a monomeric amphiphilic ($G1^a$) isoform (Sberna *et al.* 1998; Sáez-Valero and Small 2001; Sáez-Valero *et al.* 2003). This isoform can be distinguished from other AChE isoforms by its unusual glycosylation pattern (Sáez-Valero *et al.* 1999). In APP CT100 transgenic mice, this $G1^a$ isoform is increased over the levels in background strain controls (Sberna *et al.* 1998). The abnormally glycosylated isoform of AChE (Glyc-AChE) is also elevated in the brain and CSF of patients with AD (Sáez-Valero *et al.* 1997, 2000; Sberna *et al.* 1998). Therefore, Glyc-AChE may be a useful diagnostic marker of AD.

Several studies indicate that Aβ can bind to nicotinic acetylcholine receptors (nAChRs). We first demonstrated that an Aβ peptide ($A\beta_{25-35}$) can bind to the nAChR on adrenal chromaffin cells and inhibit nicotine-stimulated catecholamine secretion (Cheung *et al.* 1993). The action of $A\beta_{25-35}$ on adrenal chromaffin cell nAChRs is complex, as the peptide also inhibits receptor desensitization (Cheung *et al.* 1993). More recent studies suggest that full-length Aβ (particularly $A\beta_{1-42}$) can bind to the α7 subtype of nAChR. For example, $A\beta_{1-42}$ co-immunoprecipitates with the α7 nAChR in samples from post-mortem AD hippocampus, and α7 nAChR antagonists compete for $A\beta_{1-42}$ binding to heterologously expressed α7 nAChR (Wang *et al.* 2000a). $A\beta_{1-42}$ has been variously reported to inhibit (Liu *et al.* 2001; Pettit *et al.* 2001; Tozaki *et al.* 2002) or stimulate (Dineley *et al.* 2001, 2002; Wang *et al.* 2003) α7 nAChRs. The action of Aβ on α7 nAChRs is of particular interest for AD because of the potential involvement of these receptors in synaptic plasticity and memory mechanisms (Broide and Leslie 1999).

The aim of the present study was to examine the mechanism by which Aβ increases AChE in primary cortical neurones. We show that $A\beta_{1-42}$ is more potent than $A\beta_{1-40}$ in its ability to increase AChE and that the $A\beta_{1-42}$-induced increase in AChE in primary cortical neurones is mediated by a direct agonist effect of $A\beta_{1-42}$ on α7 nAChRs.

Materials and methods

Materials

Trypsin, soybean trypsin inhibitor (SBTI), deoxyribonuclease I (DNase), poly-D-lysine, nifedipine, diltiazem hydrochloride, ω-agatoxin IVA, ω-contoxin GVIA (–)-cytisine (+/–)-epibatidine hydrochloride (–)-nicotine, choline chloride, α-bungarotoxin (α-btx), methyllycaconitine citrate (MLA) and mecamylamine hydrochloride were all obtained from Sigma-Aldrich Pty Ltd (Seven Hills, Australia). The CellTitre 96 AQueous One Solution Cell Proliferation Assay kit was from Promega Corporation (Annandale, Australia). Culture plates were purchased from Nunc (Naperville, IL, USA). Dulbecco's modified Eagle's medium (DMEM), B27 media supplement and gentamycin were purchased from Gibco Life Technologies (Mulgrave, Victoria, Australia). Muscovite mica for atomic force microscopy (AFM) was purchased from NT-MDT (Zelenograd, Russia). UltraClear centrifuge tubes (8 × 49 mm) were purchased from Beckman Coulter Aust. Pty Ltd (Gladesville, Australia). Aβ peptides were synthesized and purified as previously described (Mok *et al.* 2002). Stock solutions of peptides [10 mg/mL in dimethyl sulphoxide (DMSO)] were stored at −80°C in 20 µL aliquots until required. If necessary, peptides were 'aged' by diluting stock solutions to 0.5 mg/mL with sterile distilled water and incubating at 37°C in 5% CO_2 for 5 days. 'Ageing' the Aβ peptides by incubation for 5 days was previously found to increase the proportion of oligomeric species (Postuma *et al.* 2000).

Culture of mouse cortical neurones

Primary cortical neurones were prepared by dissecting cortices from the brains of embryonic day 14 (E14) C57/BL6 wild-type mice. First, the meninges were removed and then the cortices dissected out and digested in 0.025% trypsin in Krebs' buffer (7.25 g/L NaCl, 0.4 g/L KCl, 0.14 g/L $NaH_2PO_4.H_2O$, 2.6 g/L D-glucose, 0.01 g/L phenol red, 5.94 g/L HEPES, pH 7.4) for 15–20 min at 37°C before the addition of SBTI/DNase buffer. Tissue was triturated, cell debris allowed to settle and a single cell suspension collected. The suspension was centrifuged in the Allegra™ 21R Beckman Coulter Centrifuge at 720 *g* for 5 min at room temperature. Cells were suspended in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 10% (v/v) horse serum (HS), 50 µg/mL gentamycin, 2 mM glutamine, 25 mM KCl and 5 g/L glucose. High-density cortical neurone cultures were plated at a density of 550 000 cells/cm^2 onto poly-D-lysine (5 µg/mL)-coated 12-well plates. This method produced cultures which were 95% pure for neurones (White *et al.* 1999). Cultures were maintained at 37°C in 5% CO_2.

Incubation of cells with Aβ peptides or drugs

Five days after plating cells, the culture medium was replaced with DMEM containing B27 media supplement, 50 µg/mL gentamycin, 2 mM glutamine, 25 mM KCl and 5 g/L glucose. Drugs or peptides were added at this stage, to the 5-day-old cortical neurones cultures, which were then incubated for a further 2.5 days at 37°C in an atmosphere containing 5% CO_2 before assaying for AChE activity. Unless otherwise indicated the final concentration of peptides was 10 µM. Control incubations (lacking peptide) contained the same amount of DMSO (vehicle) as those incubations containing peptide.

Determination of cellular AChE activity

Culture medium was removed and cells were rinsed twice with phosphate-buffered saline (PBS). Cells were scraped into 1 mL of ice-cold homogenization buffer (50 mM Tris–HCl, 1 M NaCl and 50 mM $MgCl_2$, pH 7.4 containing 1%, w/v, Triton X-100). The cells were sonicated on ice for 20 min and then centrifuged at 100 000 *g*

at 4°C in a Beckman L8–80 M ultracentrifuge using a 70.1 Ti rotor for 1 h and the supernatant fraction collected and AChE activity determined using a colorimetric assay employing acetylthiocholine as substrate (Sáez-Valero *et al.* 1999). AChE activity was calculated in mUnits (μmol acetylthiocholine hydrolysed/min/mg of protein at 22°C). The protein concentration of the supernatant fraction was determined using the bicinchoninic acid method with bovine serum albumin as standard (Smith *et al.* 1985). The percentage increase in specific AChE activity (AChE activity/mg protein) for each treatment group over the untreated mean control value was then determined. For incubations that did not contain any drugs or peptides, the specific activity of AChE was routinely about 1.0 mUnit/mg.

Measurement of cell viability

Cellular viability was measured using the CellTitre 96 AQueous One Solution Cell Proliferation Assay kit. After 2.5 day treatment, 10 μL of Aqueous One solution containing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium] (MTS) was added to 100 μL of sample in 96-well plate and allowed to incubate for 2 h at 37°C in 5% CO_2. The absorbance of the samples was then read at 560 nm using the Wallac plate reader. Toxicity was calculated as the percentage decrease in absorbance in the treatment group containing Aβ peptide compared with the mean untreated control value.

SDS–PAGE

Samples 10 μL were analysed on 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) gels using a Tris–tricine buffer system over 1.5 h (Fuller *et al.* 1995). After electrophoresis, Aβ was detected by western blotting. Protein was electrophoretically transferred from the gel onto nitrocellulose at a current of 300 milliamperes (mA) overnight. Membranes were then blocked with 0.5% (w/v) casein in PBS, pH 7.4 for 1 h at room temperature. The blocking solution was replaced with primary monoclonal mouse antibody, WO2 (1 : 50 dilution in blocking solution) and incubated with gentle agitation for 2 h at room temperature. Blots were then probed with a secondary rabbit polyclonal anti-mouse IgG antibody conjugated to horseradish peroxidase (1 : 5000 dilution in blocking solution; Amersham Pharmacia Biotech, Sydney, NSW, Australia) with gentle agitation for 1 h and then developed by the ECL detection system.

Atomic force microscopy

Aβ 1–42 (5 μL) solutions were applied to a substrate (mica) for one min, which was then rinsed twice with 100 μL of distilled water, before being dried with a stream of nitrogen for one min. Atominc force microscopy (AFM) imaging was performed by Nanoscope IV (Digital Instrument, Santa Barbara, CA, USA), using tapping mode in air and silicon cantilevers (model TESP, DI) operating at frequencies of the 300–400 kHz.

Results

Primary neurones were isolated from mouse brain cortical tissue and the cells cultured for 5 days, after which various Aβ peptides ($A\beta_{1-40}$, $A\beta_{1-42}$, $A\beta_{17-40}$, $A\beta_{1-28}$, and $A\beta_{29-42}$) were added to the culture medium to yield a final concentration of 10 μM. The cells were then incubated for a further 2.5 days. The culture medium was then removed, and the amount of AChE activity and the protein content of the cells measured. The specific AChE activity (AChE activity per mg protein) was then calculated and the percentage increase in specific AChE activity relative to the untreated control group determined (Fig. 1a).

No effect on total cell AChE activity was seen after incubation with $A\beta_{1-40}$ or $A\beta_{1-42}$ that were freshly prepared from stock solutions in DMSO. However, when $A\beta_{1-42}$ was first 'aged' by incubation at 37°C for 5 days in distilled water prior to addition into cell culture, the peptide induced a significant increase (30–35%) in the level of AChE activity. Lower concentrations of $A\beta_{1-42}$ were not found to produce any significant effect on AChE levels (control incubation: AChE-specific activity = 1.11 ± 0.06 mUnits/mg $n = 6$; 1 μM $A\beta_{1-42}$ incubation: AchE-specific activity = 1.19 + 0.05 mUnits/mg, $n = 5$, $p > 0.05$, Student's *t*-test). Several N- and C-terminal analogues of Aβ were also tested for their effect on AChE activity. However, no increase in activity was seen.

To examine the possibility that the increase in AChE was a consequence of Aβ-induced neurotoxicity, the neurotoxicity of the Aβ peptides was examined using an MTS assay of

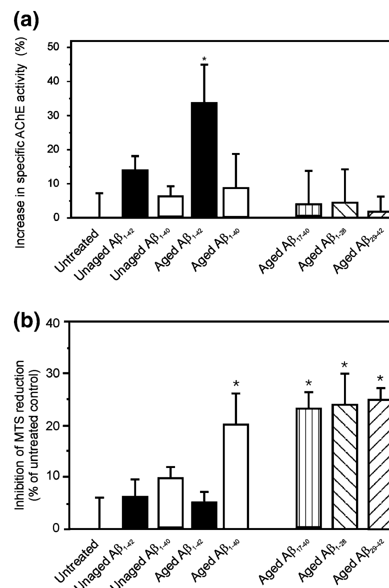

Fig. 1 Effect of Aβ peptides on AChE activity in mouse primary cortical neurones. Cells were grown for 5 days and then treated for a further 2.5 days in the presence of various Aβ peptides (10 μM) before being assayed for AChE activity (a) or for viability using an MTS assay (b). In (a), data are expressed as percentage increase in specific AChE activity over control incubations lacking peptide. In (b), the percentage decrease in absorbance at 560 nm compared with the mean control value is shown. Values are means ± SEM values ($n = 3$). *Significantly different from untreated group ($p < 0.05$), as assessed by Student's *t*-test.

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mitochondrial function (Fig. 1b). No clear correlation was found between the effects of Aβ peptides on oxidative metabolism and their effects of AChE levels. Although $A\beta_{1-40}$, $A\beta_{17-40}$, $A\beta_{1-28}$ and $A\beta_{29-42}$ were all found to have some toxic effect, these peptides were without effect on AChE activity. Although aged $A\beta_{1-42}$ increased AChE levels, it did not produce a significant toxic effect on the cells. This lack of statistically significant effect on toxicity may have been due to the relatively small sample number ($n = 3$).

The process of 'ageing' has been shown in many studies to cause an increase in the concentration of higher molecular weight Aβ aggregates which can be analysed by SDS–PAGE (e.g. see Postuma *et al.* 2000). The effect of ageing on the state of aggregation of $A\beta_{1-42}$ was examined both by SDS–PAGE and by atomic force microscopy (AFM) (Fig. 2). Unaged $A\beta_{1-42}$ migrated on SDS–PAGE mostly as a monomer with a relative molecular mass of 4 kDa (Fig. 2a). A small amount of dimer (8 kDa) was observed. However, after ageing for 5 days, the amount of monomer was lower and several bands of higher molecular weight were seen migrating at approximately $M_r = 8$ kDa (dimer), 12 kDa (trimer) and 16 kDa (tetramer). Higher molecular weight aggregates of 30–200 kDa were also seen in the aged sample.

AFM experiments confirmed that the peptide was more aggregated after ageing. The preparation of unaged peptide exhibited no fibrillar aggregates (Fig. 2b). The peptide appeared globular in structure with sizes based on the images estimated to be between 10 and 25 nm. In contrast, the aged material contained a large number of fibrillar or protofibrillar aggregates. The width of fibrils was estimated to be typically 25–35 nm. It should be noted that in AFM, estimates of size based on image are typically overestimated by 30–50% due to tip/sample convolution.

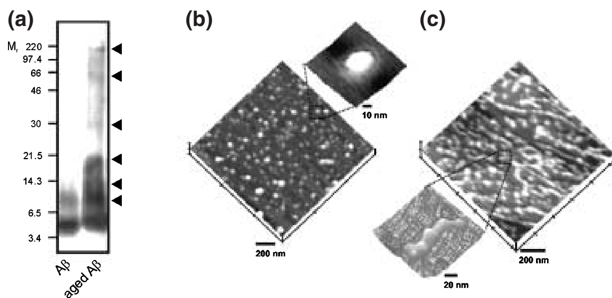

Fig. 2 Analysis of $A\beta_{1-42}$ aggregation by SDS–PAGE and by AFM. Analysis of unaged and 5-day-aged Aβ1–42 by 15% Tris–tricine PAGE (a). To visualize Aβ, protein was electrophoretically transferred to nitrocellulose which was then stained with a monoclonal antibody (WO2). Arrowheads show positions of oligomeric forms of Aβ which are increased after ageing. Figure also shows topographic AFM images of $A\beta_{1-42}$ unaged (b) or aged for 5 days (c), obtained using tapping mode in air. (b inset; 100 nm × 100 nm) shows a typical globular structure. (c inset; 200 nm × 200 nm) shows a typical protofibril structure seen after ageing.

Effect of VDCC inhibitors

Previous studies have demonstrated that the expression of AChE is regulated by L-type VDCCs (Luo *et al.* 1996; Sberna *et al.* 1997). Therefore, the role of VDCCs in mediating the effect of $A\beta_{1-42}$ on AChE in primary cortical neurones was examined. Inhibitors of L-, N-, and P- or Q-type VDCCs were tested for their effects on $A\beta_{1-42}$-induced increase in AChE activity (Figs 3a and b). Similar to our observations in P19 cells, inhibitors of L-type VDCCs (diltiazem, nifedipine) blocked the $A\beta_{1-42}$-induced increase, whereas the P- and Q-type VDCC inhibitor ω-agatoxin IVA did not block the $A\beta_{1-42}$-mediated increase. However, unlike our previous study, the effect of $A\beta_{1-42}$ was also decreased in the presence of an N-type VDCC inhibitor, although there was no significant increase in AChE activity in the presence of ω-conotoxin GVIA.

Effect of nAChR agonists and inhibitors on AChE activity

As Aβ peptides bind to the α7 nAChR, we examined the possibility that the effect of $A\beta_{1-42}$ on AChE was mediated via a nAChR. The ability of nAChR agonists and antagonists

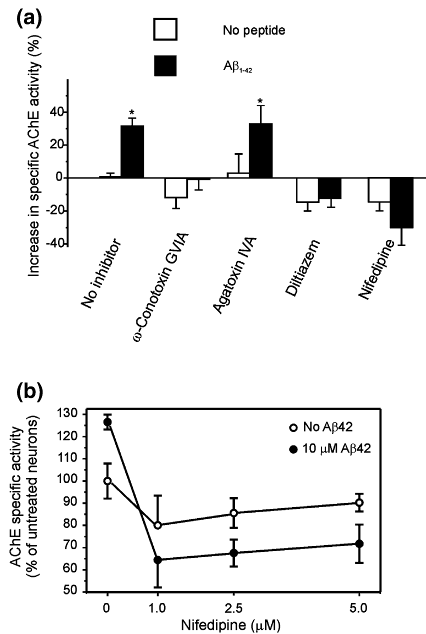

Fig. 3 Effects of VDCC antagonists on the ability of $A\beta_{1-42}$ to increase AChE activity. (a) Cells were incubated in the presence or absence of aged $A\beta_{1-42}$ (10 μM), ω-agatoxin IVA (50 nM), nifedipine (2.5 μM), diltiazem (1 μM) and ω-conotoxin GVIA (1 μM). *Significantly different from the control incubations lacking $A\beta_{1-42}$ and VDCC inhibitor as assessed by a Student's *t*-test ($p < 0.05$). Data are expressed as a percentage increase in specific AChE activity over control incubations and represent means ± SEM ($n = 3$). (b) Concentration dependence of the effect of nifedipine on AChE specific activity. Data are expressed as means ± SEM ($n = 3$).

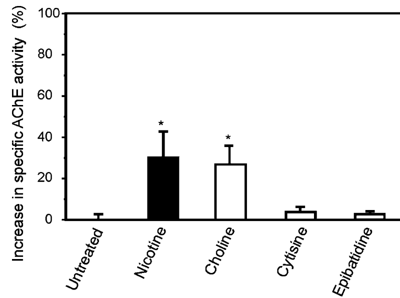

Fig. 4 Effect of chronic (2.5-day) treatment with nAChR agonists on levels of AChE in primary cortical neurones. Data are expressed as a percentage increase in specific AChE activity over control incubations and represent means ± SEM ($n = 3$). The concentration of agonists was nicotine (10 μM), choline (500 μM), cytisine (20 μM) and epibatidine (2 μM). *Significantly different from the control incubations as assessed by a Student's *t*-test ($p < 0.05$).

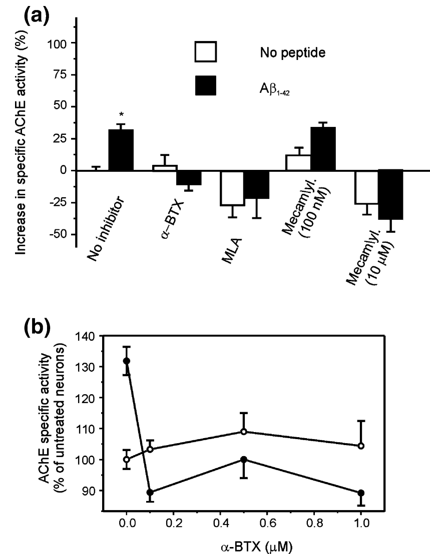

Fig. 5 Effect of nAChR antagonists on $A\beta_{1-42}$-induced increase in AChE activity. (a) Cells were incubated in the presence or absence of aged $A\beta_{1-42}$ (10 μM), α-bungarotoxin (α-BTX) (1 μM) and MLA (1 μM) or mecamylamine for 2.5 days. Data are expressed as a percentage increase in specific AChE activity over control incubations and represent means ± SEM ($n = 3$). *Significantly different from the control incubations as assessed by a Student's *t*-test ($p < 0.05$). Mecamyl. = mecamylamine. (b) Concentration dependence of the effect of α-BTX on AchE-specific activity.

to alter the level of AChE in primary cortical neurones was examined. Chronic treatment (over 2.5 days) of cells with choline (500 μM) a selective agonist of α7 nAChRs (EC_{50} = 1.6 mM; Papke *et al.* 1996; Alkondon *et al.* 1997) or the non-specific agonist nicotine (10 μM) significantly increased AChE activity (Fig. 4). In contrast, the nAChR agonists epibatidine (2 μM) and cytisine (20 μM), which exhibit high binding affinity for α4β2 nAChRs (Gerzanich *et al.* 1995; Xiao *et al.* 1998), but which have little activity on α7 nAChRs, did not significantly alter AChE levels in the cortical neuronal cultures.

Inhibitors of α7 nAChRs were found to block the effect of $A\beta_{1-42}$ on AChE (Fig. 5a). The α7-selective nAChR antagonists, α-bungarotoxin (1 μM α-BTX), methyllcaconitine (100 nM MLA; Ward *et al.* 1990; LopeZ *et al.* 1998), and the less specific nAChR antagonist mecamylamine (10 μM), significantly inhibited the $A\beta_{1-42}$-induced increase in AChE activity. However, when mecamylamine was used at a lower concentration (100 nM), which is more selective for other classes of nAChR and which does not inhibit the α7 nAChR (Chavez-Noriega *et al.* 1997), no effect on Aβ-induced AChE activity was observed. The specificity of the effect for the α7 nAChR was further supported by the finding that concentrations of α-BTX as low as 100 nM produced complete inhibition of $A\beta_{1-42}$-mediated increase in AChE levels (Fig. 5b).

Discussion

Previous studies have shown that Aβ can increase levels of AChE in P19 cells and in primary cortical cells (Sberna *et al.* 1997; Sáez-Valero *et al.* 2003). The present study demonstrates that the $A\beta_{1-42}$-induced increase in AChE in primary cortical neurones is mediated by an action on α7 nAChRs. Chronic treatment over 2.5 days with agonists of the α7 nAChR (nicotine and choline), but not agonists selective for other nAChRs (epibatidine, cytisine), mimicked the effect of $A\beta_{1-42}$ on AChE, while α7 selective antagonists (α-bungarotoxin, MLA) inhibited the $A\beta_{1-42}$-mediated increase in AChE. Mecamylamine, a less specific antagonist, had no effect on $A\beta_{1-42}$-mediated increase in AChE activity at concentrations (100 nM), which are not active on α7 nAChRs (Chavez-Noriega *et al.* 1997). Although at a higher concentration (10 μM), inhibition was observed.

Previous studies have suggested that Aβ can increase AChE as a consequence of Aβ-induced oxidative stress (Melo *et al.* 2003). However, our studies suggest that this is not the mechanism by which AChE is increased by $A\beta_{1-42}$ in cortical neurones. Other Aβ peptides, some of which were equally toxic as $A\beta_{1-42}$ (e.g. $A\beta_{1-40}$, $A\beta_{17-40}$, $A\beta_{1-28}$) and $A\beta_{29-42}$), did not have any effect on AChE levels. Similarly, studies by Soreq and co-workers have described a novel read-through variant of AChE which is increased under conditions of stress and may be associated with neurodegeneration (Grisaru *et al.* 1999). However, the Aβ-induced isoform in our studies is unlikely to be identical to this read-through isoform. The isoform of AChE which is increased by Aβ is amphiphilic in character (Sberna *et al.* 1998; Sáez-Valero *et al.* 1999, 2000), whereas the read-through isoform is non-amphiphilic (Grisaru *et al.* 1999).

The action of Aβ on nAChRs may be quite complex. Our results demonstrate that $A\beta_{1-42}$ can act as an agonist of the

α7 nAChR, and this conclusion was also reached in two separate studies (Dineley *et al.* 2001, 2002). However, several groups (Liu *et al.* 2001; Pettit *et al.* 2001; Tozaki *et al.* 2002; Grassi *et al.* 2003; Lee and Wang 2003) have reported that $A\beta_{1-42}$ can inhibit α7 nAChRs. This apparent conflict may be explained by the studies of Dineley *et al.* (2002) in *Xenopus* oocytes, which have shown that low concentrations of $A\beta_{1-42}$ can activate the α7 nAChR, whereas high concentrations are desensitizing. Therefore, the degree of receptor activation or inhibition may depend upon the extent to which the receptor is saturated with $A\beta_{1-42}$ and the local concentration of acetylcholine. In the absence of acetylcholine, $A\beta_{1-42}$ would be expected to stimulate the receptor. However, pretreatment of cells with high concentrations of peptide would also be expected to inhibit acetylcholine stimulation of the receptor.

However, there are clear differences between the results we have obtained in our study and those obtained by other groups studying α7 nAChR activation by Aβ. Previous studies have reported that low picomolar concentrations of $A\beta_{1-42}$ can stimulate the α7 nAChR and that monomeric Aβ is more potent than oligomeric Aβ (Wang *et al.* 2000a, 2000b). In contrast, we have not found any significant effect at concentrations of Aβ lower than 10 μM (i.e. 1 μM). Furthermore, our studies very clearly indicate that ageing $A\beta_{1-42}$ by incubation for 5 days is necessary for the effect on AChE levels. However, after ageing, the level of Aβ monomer, as assessed by SDS–PAGE, was lower and the concentration of aggregated Aβ was greater.

One explanation for this difference is that the effect on AChE occurs only after chronic treatment with Aβ. Acute stimulation of the receptor under conditions in which desensitization would have been minimal (low concentrations of agonist over a time course of a few minutes) was not found to influence AChE levels (data not shown), possibly because short time periods do not provide sufficient time for changes in the level of total AChE activity. Under normal conditions, the α7 nAChR is rapidly desensitized. Therefore, for an effect on AChE to be seen, the α7 nAChR would have to be chronically stimulated and it is conceivable that for chronic receptor stimulation, high Aβ concentrations may be needed.

In addition to this, the significance of picomolar affinity interactions between Aβ and the α7 receptor is very unclear. The concentration of $A\beta_{1-42}$ in the cerebrospinal fluid of normal individuals is approximately 100 picomolar (Ida *et al.* 1996). Assuming a similar (or greater) concentration for Aβ in the brain, this would imply that the α7 nAChR is chronically stimulated by Aβ under normal conditions. This would be a rather surprising finding, if true. However, if the α7 nAChR is influenced only by high concentrations of Aβ *in vivo*, then increased AChE expression would only be expected in association with amyloid deposits, similar to that previously reported (Mesulam 1986; Geula and Mesulam 1989; Sáez-Valero *et al.* 1999).

Our results provide an explanation for the increase in AChE activity that is seen around amyloid plaques (Friede 1965; Perry 1980; Brashear *et al.* 1988; Mesulam and Geula 1988; Gomez-Ramos *et al.* 1992) in the AD brain. We propose that the build-up of Aβ in the AD brain activates α7 nAChRs, which in turn induces increased AChE expression. Indeed, α7 nAChRs are increased in APP transgenic mice (Apelt *et al.* 2002; Bednar *et al.* 2002) as well as in the AD brain, particularly around amyloid plaques (Perry *et al.* 1995, 2000; Wevers *et al.* 1999). Interestingly, both AChE and α7 nAChR are increased very early in the APP SW Tg2576 transgenic mouse model of AD (Dineley *et al.* 2001; Fodero *et al.* 2002), suggesting that the levels of both proteins may be co-ordinately regulated.

Our previous studies using P19 cells indicated that the Aβ-induced increase in AChE is mediated by calcium influx. Our results in the present study support this hypothesis. Following α7 nAChR stimulation, intracellular calcium may increase as a direct consequence of entry of calcium through the receptor, or by membrane depolarization, which may result in calcium influx through VDCCs. In support of this idea, Luo *et al.* (1994, 1996, 1999) have found that influx of calcium through L-type VDCCs is associated with increased expression of AChE in myocytes.

However, some caution is needed in interpreting the results of these experiments, as inhibitors of VDCCs may also act on nAChRs. For example, Villarroya *et al.* (1997) reported that inhibitors of L-type VDCCs at a concentration of 3 μM could partially inhibit the nAChR on bovine chromaffin cells. Similarly, Herrero *et al.* (1999) reported that the L-type VDCC inhibitor diltiazem can inhibit nAChRs expressed in *Xenopus* oocytes, although the α7 nAChR was very poorly inhibited by diltiazem. Given the lack of specificity of some VDCC antagonists, it is possible that at least some of the inhibitory effect of nifedipine and diltiazem observed in the present study could be due to a direct inhibition of the α7 nAChR. Nonetheless, the observation that the effect of $A\beta_{1-42}$ on AChE was completely blocked even by low concentrations (1 μM) of L-type VDCC antagonists argues in favour of the view that their action is mediated via the calcium channel.

Interestingly, the mean level of AChE was slightly lower in the $A\beta_{1-42}$ treatment group in the presence of either nifedipine (Fig. 3b) or α-bungarotoxin (Fig. 5b). This effect could be due to a non-specific (i.e. toxic) action of Aβ which decreases the level of AChE, which is entirely separate from the effect of Aβ on AChE mediated by α7 nAChRs. It is possible that by inhibiting the α7 nAChR-mediated mechanism, the other mechanism was revealed. Although $A\beta_{1-42}$ was not found to be very toxic in an MTS assay (Fig. 1b), it is possible that the level of AChE may be a more sensitive indicator of a toxic effect. Indeed, the overall loss of AChE which occurs in the AD brain (independent of the increased AChE around amyloid plaques) may be a consequence of the

some of the neurotoxic effects of amyloid (Small *et al*. 1996).

The finding that $A\beta_{1-42}$ can act on α7 nAChRs has implications for some of the cognitive changes that occur in AD. The production of $A\beta_{1-42}$ is closely associated with the pathogenesis of AD (Small *et al*. 2001) and the α7 nAChR is involved in synaptic plasticity in the brain (Broide and Leslie 1999), which is important for memory formation. Therefore, blocking the action of $A\beta_{1-42}$ on α7 nAChRs could have value in the treatment of AD.

Acknowledgements

This work was supported by grants to DHS from the National Health and Medical Research Council of Australia.

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