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RESEARCH ARTICLES

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Bioactivity of a peptide derived from acetylcholinesterase in hippocampal organotypic cultures

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Abstract While the molecular basis underlying the non-classical actions of acetylcholinesterase (AChE) is presently unknown, a candidate peptide sequence located at the C-terminus of AChE (AChE-peptide) has recently been identified. This study explored the bioactivity of synthetic AChE-peptide using in vitro organotypic cultures of rat hippocampus. Neurotrophic effects, detected as increased neurite outgrowth from MAP-immunopositive neurones, were apparent using 1 h exposure to 1–10 nM AChE-peptide. As exposure time increased, cell death occurred as indicated by TdT-mediated dUTP biotin nick-end labelling (TUNEL). This process was accelerated at higher AChE-peptide concentrations, with lactate dehydrogenase (LDH) efflux observed following prolonged exposure to 1–10 μM AChE-peptide. Apoptotic cells were detected by Hoechst 33342 staining following 24 h application of 10 nM AChE-peptide. However, propidium iodide reactivity revealed a simultaneous loss of membrane integrity indicative of necrosis, suggesting that AChE-peptide induces cell death via a continuum of apoptotic and necrotic processes. Prolonged exposure to AChE-peptide also resulted in a concentration-dependent reduction in neurite outgrowth from MAP2-positive neurons, although immunohistochemical studies provided some evidence of differential responsiveness in GABAergic, cholinergic and somatostatin neurones. In addition, bioactivity was sequence specific since a scrambled AChE-peptide analogue, as well as the corresponding BuChE-peptide, was ineffective. In conclusion, the bioactivity associated with the AChE-peptide sequence may account for the non-cholinergic actions of AChE, whilst its neurotrophic-apoptotic-necrotic spectrum of action may be involved in the aetiology of neurodegenerative disorders such as Alzheimer's disease.

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Introduction

The disparity of acetylcholinesterase (AChE: EC 3.1.1.7) distribution relative to its normal substrate, acetylcholine (ACh; Silver 1974), together with the existence of a secreted form (Greenfield 1984), has prompted the notion that the enzyme may have non-classical actions (see Greenfield 1996, Soreq and Seidman 2001). Indeed, it is well established that AChE has non-cholinergic functions related to neuronal development that are insensitive to classic catalytic cholinesterase inhibitors (Layer et al. 1993; Chan and Quik 1993; Dupree and Bigbee 1994; Small et al. 1995; Holmes et al. 1996). These developmental, trophic functions of AChE may be related to enhanced calcium entry (Greenfield 1996; Webb et al. 1996; Day and Greenfield 2002), a key driver in the process of neuronal maturation (Mattson 1999). In contrast, AChE may instead act as a neurotoxic factor in mature systems that are susceptible to sustained increases in calcium (Eimerl and Schramm 1994; Calderón et al. 1998). Such opposing roles have been suggested for other substances: Dickie et al. (1996) showed that NMDA is neurotrophic at low concentrations and toxic at higher concentrations. Furthermore, Koh and Choi (1995) found that neurotrophins (BDNF, NT-3 and NT-4/5) may protect against apoptotic cell death but actually enhance necrotic-like cell death.

Interestingly, functional parallels exist between the non-classical actions of AChE and amyloid precursor protein (APP) in terms of neurotrophic activity (Greenfield and Vaux 2002): accordingly, a region at the C-terminus of AChE with structural and sequence homology to beta-amyloid has recently been described (Greenfield and Vaux 2002). This 14 amino acid sequence (AEFHRWSSYMVHWK) is encoded by exon 6 of the AChE gene, a region unrelated to catalytic function, which instead

modulates process extensions in neuronal and glioma cells (Karpel et al. 1996; Sternfeld et al. 1998).

The aim of this study was to explore whether AChE-peptide could be bioactive by testing a synthetic analogue using in vitro organotypic cultures, a model in which AChE exerts non-cholinergic actions (Jones et al. 1995; Holmes et al. 1996; Day and Greenfield 2002). Potential bioactivity was investigated over a wide peptide concentration range for varying exposure periods. Neurite outgrowth from immunohistochemically defined neurones was determined as a quantitative marker for neurotrophic activity; neurons were stained for microtubule associated peptide (MAP2) as a generic neuronal marker with cell selectivity evaluated in defined cell populations using transmitter specific antibodies [glutamate decarboxylase (GAD), choline acetyltransferase (ChAT) and somatostatin]. In addition, both quantitative [TdT-mediated dUTP biotin nick-end labelling (TUNEL) and lactate dehydrogenase (LDH)] and qualitative (Hoechst 33342 and propidium iodide) markers of apoptosis and necrosis were used to determine neurotoxic potential. Staurosporine, a phospholipid/calcium-dependent protein kinase inhibitor, was used as a benchmark in neurotoxicity studies. Peptide specificity was confirmed using a synthetic scrambled variant of AChE-peptide, as well as a synthetic peptide comprising the analogous amino acid sequence from butyrylcholinesterase (BuChE).

Materials and methods

Organotypic hippocampal cultures were prepared as detailed elsewhere (Gähwiler 1981, 1984). In brief, postnatal day 7 Wistar rats (Harlan Olac, Oxford, UK) were killed by halothane overdose, and the brain removed. Hippocampal slices (400 μm) were prepared using a McIlwain tissue chopper and embedded on a plasma clot. Cultures were maintained on a roller drum at 37°C. All treatments were applied in serum-free medium [Dulbecco's minimal essential media (DMEM) supplemented with B-27] containing antimitotic agents (uridine and cytosine β-D-furanoside; both at 1 μM; Sigma-Aldrich Co., Poole, UK) to prevent proliferation of non-neuronal cells, and fungizone (Life Technologies Ltd., Paisley) to reduce the risk of infection.

Relevant peptides were synthesised (Dr. M. Pitkeathley, Dyson Perrins Laboratory for Organic Chemistry, University of Oxford) using an Applied Biosystems 430A automated peptide synthesiser with standard Fmoc protein synthesis methodology. AChE-peptide and variants thereof were purified by HPLC (>95%) (Mr. K. Pryor, Department of Pharmacology, University of Oxford), freeze-dried and maintained in aliquots (5 mM) at −20°C. On treatment days, fresh solutions were prepared [1 mM in phosphate buffered saline (PBS)] and serially diluted to the required concentration in fresh filtered serum-free medium.

Cultures were treated with synthetic AChE-peptide, scrambled AChE-peptide (HSWRAEVFHKYWSM), or the homologous peptide derived from BuChE (AGFHRWNNYMMDWK) over a wide concentration range (1 nM–10 μM). Cultures were fed biweekly with serum-free medium, supplemented with peptide as required. Regardless of treatment duration (1–336 h), all cultures were maintained for 336 h (i.e. peptides were applied at the appropriate interval prior to termination of a 336-h total culture period). Cultures treated for 1 h were subjected to a 24-h recovery period prior to fixation.

Immunohistochemistry

AChE-peptide bioactivity was evaluated in defined cell populations using the biotin-avidin peroxidase immunohistochemical method. The following antibodies were used: microtubule associated peptide (MAP2; 1:1,500 dilution; Chemicon International) as a non-specific neuronal marker, glutamic acid decarboxylase (GAD; 1:300 dilution; Chemicon International) to detect GABA-positive cells, choline acetyltransferase (ChAT; 1:1,000 dilution; Roche Diagnostics) to detect cholinergic cells and an anti-somatostatin antibody (1:1,000 dilution; Chemicon International) to detect somatostatin containing neurones. Cultures were fixed with 4% paraformaldehyde for 1 h at room temperature. After washing, primary antibodies (100 μl) were applied to each culture for 24 h at 4°C. Following extensive washing, cultures were then exposed to the secondary antibody (1:300 dilution; goat anti-rabbit; Novocastra Labs., UK) before immunoreactivity was detected using an ABC kit (Vector Labs., Peterborough, UK) according to the manufacturer's instructions. Camera lucida drawings were used to determine neurite outgrowth in the defined cell populations. A non-bias method involving a grid (100 μm^2) superimposed over an image of the culture was used. Neurite outgrowth from immunopositive neurones in every 5th square of the grid was measured using an eye piece graticule attached to a Nikon Diaphot inverted microscope.

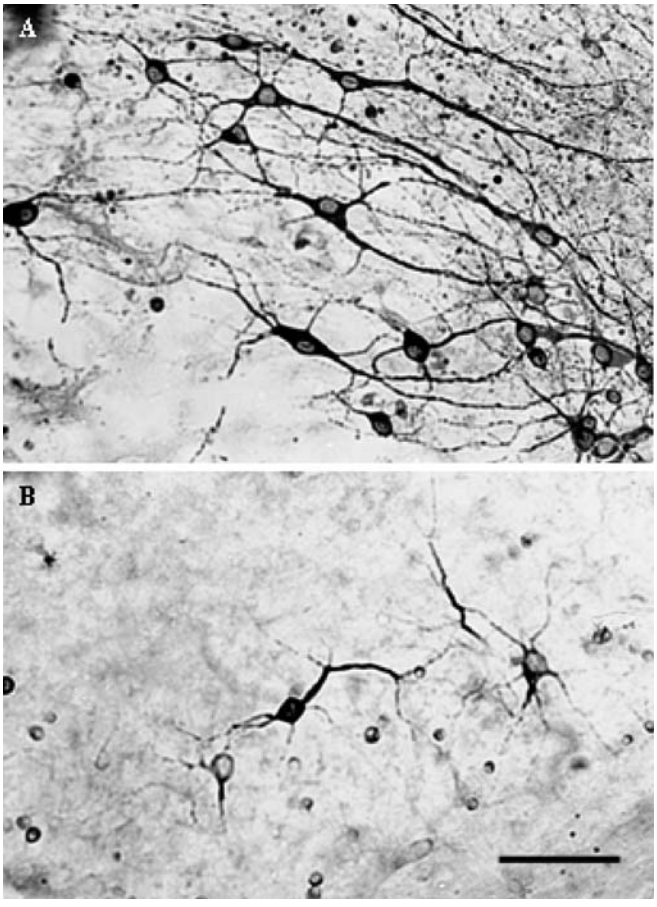

Fig. 1A, B MAP2 immunochemistry of representative hippocampal organotypic cultures grown in serum-free conditions for 14 days. Cultures treated with AChE-peptide (10 μM) showed a marked decrease in neurite outgrowth **(B)** as compared to control cultures **(A)**. *Scale bar* 50 μm

TdT-mediated dUTP biotin nick-end labelling

Nuclear DNA fragmentation was detected by labelling newly formed free ends of DNA using TdT-mediated dUTP biotin nick-end labelling (TUNEL), according to the manufacturer's instructions (CN Biosciences UK). In brief, slice cultures are fixed with 4% paraformaldehyde for 1 h at room temperature, and then incubated with biotinylated dUTP in TPT buffer for 1 h at 37°C. Non-specific binding was blocked with 1 μM bovine serum albumin (BSA). TUNEL staining was visualised using 0.05% 3, 3'-diaminobenzidine tetrahydrochloride (DAB). Sister cultures treated with 100 μM staurosporine for 24 h were used as a positive control; negative controls omitted TdT. Cell counts were performed on dehydrated, DePex mounted slices using a non-bias method involving a grid (100 μm^2) superimposed over an image of the culture; the number of TUNEL-positive cells in every 5th square on the grid was counted.

Lactate dehydrogenase efflux

Membrane integrity was monitored by measuring lactate dehydrogenase (LDH) efflux according to the manufacturer's instructions (Sigma-Aldrich Co., Poole, UK). Briefly, the culture medium was removed after 336 h and placed into a 96-well plate. The sample start reagent (mmol/l; NADH 0.194, pyruvate 16.2 in phosphate buffer, pH 7.5) was added, and colourimetric intensity was determined at 340 nm after 3 min. Sister cultures exposed to 100 μM staurosporine for 24 h acted as a control; staurosporine had no effect on LDH release from organotypic hippocampal cultures (data not shown).

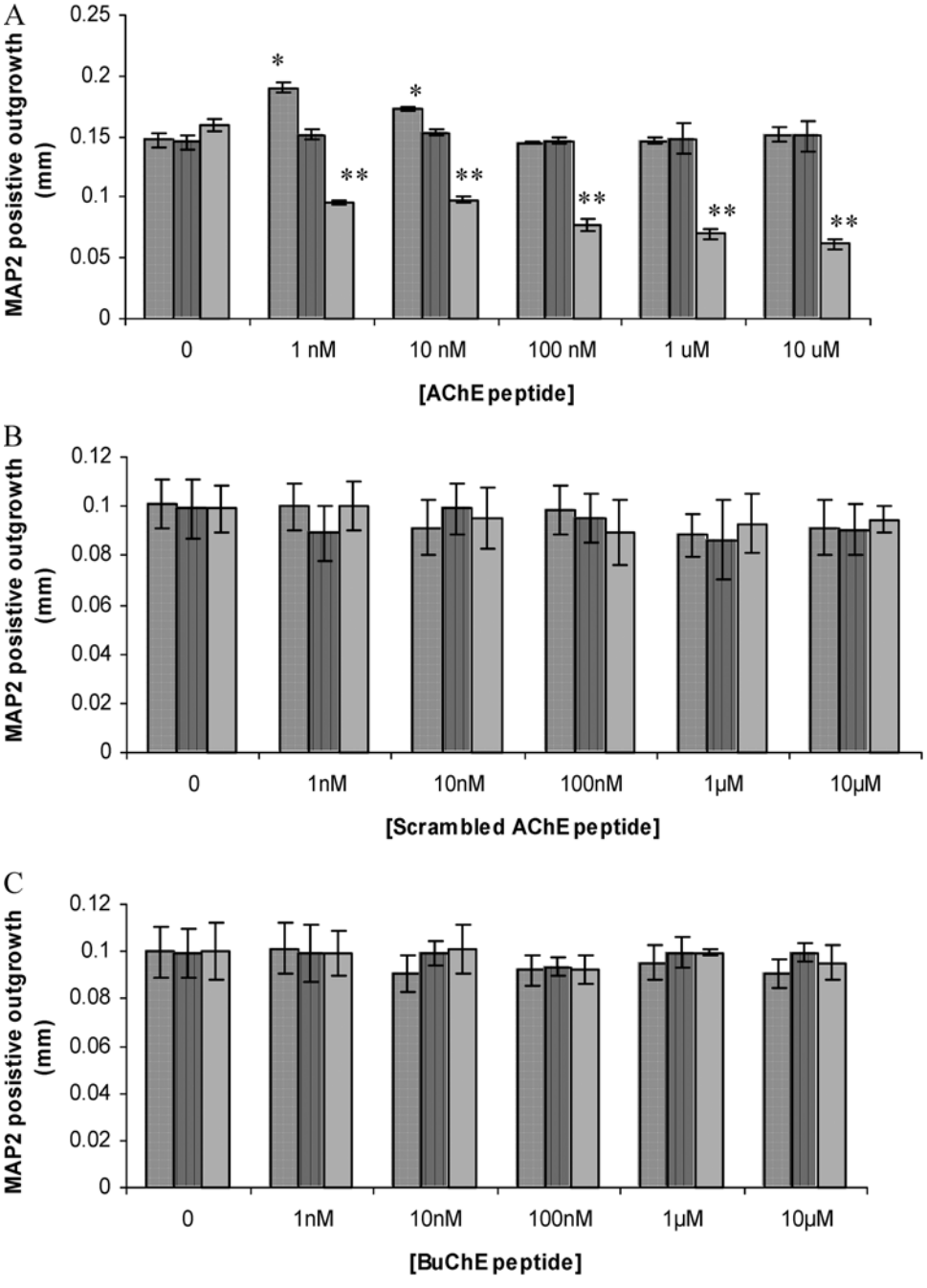

Fig. 2 Effect of AChE-peptide **(A)**, scrambled AChE-peptide **(B)** and BuChE-peptide **(C)** on neurite outgrowth from MAP2-immunopositive neurones. Organotypic hippocampal cultures were exposed to the peptide variants for 1, 24 and 336 h. Following MAP2 immunostaining, neurite outgrowth was determined using a non-biased method; neurite outgrowth was measured in every 5th square of a 100-μm^2 grid superimposed over an image of the culture using an eyepiece graticule attached to a Nikon Diaphot inverted microscope. Results are expressed as mean neurite outgrowth (mm) ± SEM. Statistical analysis used ANOVA followed by the Dunnett's multiple comparisons *t*-test (**$P<0.01$; *$P<0.05$; $n=160$). Key: *medium grey columns* 1 h exposure, *dark grey columns* 24 h exposure, *light grey columns* 336 h exposure

Hoescht 33342

Cell staining by Hoechst 33342, a DNA binding dye, was used to visualise chromatin condensation in apoptotic cells. Following fixation of cultures in 4% paraformaldehyde for 1 h at room temperature, Hoescht 33342 (0.5 μg/ml; Molecular Probes) was added for 15 min at room temperature. Representative photomicrographs were immediately captured using a Nikon Diaphot inverted fluorescent microscope equipped with a DAPI filter (460±20 nm).

Propidium iodide

DNA binding of the cell impermeant dye, propidium iodide, was used as a microscopic marker of cell membrane integrity. Propidium iodide (Cambridge Bioscience, UK) was prepared from a 1-mg/ml stock in serum-free medium at a final concentration of 4.6 μg/ml. Following fixation in 4% paraformaldehyde for 1 h at room temperature, cultures were incubated with propidium iodide for 1 h at 37°C. Representative photomicrographs were captured immediately using a Nikon Diaphot inverted fluorescent microscope equipped with a rhodamine filter (abs 536/em 617).

Analysis

Each treatment group included cultures from at least three different litters. For LDH and TUNEL, data are derived from 13–18 independent cultures. Hoechst 33342 and propidium iodide data are representative data similar to that observed in at least 14–19 independent cultures. Results were expressed as percentage mean ± SEM. Statistical analysis was by ANOVA followed by a Dunnett's multiple comparison *t*-test.

Results

Sequence specificity of AChE-peptide bioactivity

Synthetic AChE-peptide, a scrambled AChE-peptide variant and the equivalent peptide sequence derived from butyrylcholinesterase (BuChE) were used to assess sequence specificity. Organotypic hippocampal cultures were exposed to peptides for various incubation periods and neurite outgrowth was measured from MAP2-immunopositive cells; MAP-positive neurones in control cultures exhibited a neurite length of 146.9±5.6 μm (Fig. 1A; mean ± SEM; n=160).

Acute 1 h incubation with low concentrations of AChE-peptide (1 and 10 nM) promoted neurite outgrowth (Fig. 2A; 190.4±6.8 μm and 173.5±5.2 μm respectively, P<0.01; Dunnett's multiple comparisons *t*-test; n=150). This effect was not observed at higher concentrations of AChE-peptide (100 nM–10 μM; Fig. 2A), following 24 h exposure to any concentration of AChE-peptide (Fig. 2A), or with any concentration of either scrambled AChE-peptide or the BuChE-peptide analogue (Fig. 2B, C).

In contrast, prolonged 336-h exposure to AChE-peptide (1 nM–10 μM) reduced neurite outgrowth in a concentration related manner (Fig. 2A). Neurite outgrowth was maximally reduced by 64% following incubation with 10 μM AChE-peptide (Fig. 1B). Neither scrambled AChE-peptide nor the BuChE-peptide homologue reduced neurite outgrowth at any concentration tested (Fig. 2B, C).

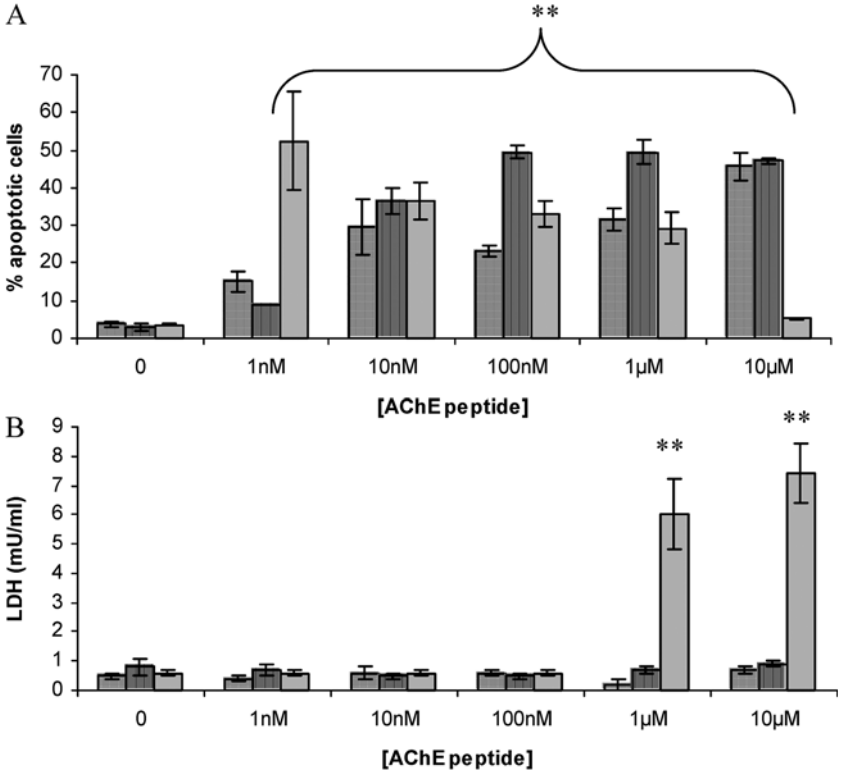

Fig. 3A, B Effect of AChE-peptide on cell viability in organotypic hippocampal cultures. Cells were maintained in serum-free media supplemented with AChE-peptide (1 nM–10 μM) for 1–336 h, or with 100 μM staurosporine for 24 h. Cell viability was assessed by TUNEL **(A)** and LDH release **(B)**. Results are mean ± SEM, with TUNEL data expressed as the % of the number of staurosporine-positive cells, and LDH as enzyme activity in culture supernatants. Statistical comparisons consisted of ANOVA followed by Dunnett's multiple comparison *t*-test (**P<0.01; *P<0.05). Key: *medium grey columns* 1 h exposure, *dark grey columns* 24 h exposure, *light grey columns* 336 h exposure

Toxic action of AChE-peptide

TUNEL staining was used to detect DNA fragmentation and, compared with staurosporine, a known apoptotic stimulus (Prince and Oreland 1997; Yuste et al. 2002). The lowest concentration of AChE-peptide (1 nM) increased the number of TUNEL-positive neurones, but only following prolonged 336-h exposure to AChE-peptide (Fig. 3A); ~50% of the staurosporine-susceptible cell population was affected. At higher concentrations (10 nM–1 μM), AChE-peptide accelerated cell death, with TUNEL-positive neurones detected at all timepoints (Fig. 3A). The maximum number of TUNEL-positive neurones was detected following a 24-h exposure to 10 nM–1 μM AChE-peptide. The number of TUNEL-positive neurones was similar to that observed following 336-h exposure to 1 nM AChE-peptide. Equivalent numbers of TUNEL-positive neurones were detected following 1- and 24-h exposure to 10 μM AChE-peptide, whereas the number of TUNEL-positive neurones was reduced following 336-h exposure (Fig. 3A).

Lactate dehydrogenase (LDH) release into the culture medium was also monitored as a marker for cell lysis. A significant increase in LDH release was only detected following exposure to 1 or 10 μM AChE for 336 h (Fig. 3B).

Characterisation of AChE-peptide mediated toxicity

Neuronal nuclei were visualised using Hoechst 33342, a cell permeant fluorescent dye that binds to chromatin. Chromatin aggregation was apparent in numerous neurones following incubation of 10 nM AChE peptide for 24 h (Fig. 4A). Similar profiles were noted after 1- and 336-h exposure, and at other peptide concentrations (data not shown). Chromatin condensation was not observed in untreated cultures (Fig. 4B).

Cell membrane integrity was monitored using propidium iodide (PI), a fluorescent probe that is impermeant in viable cells. Although a small number of PI-positive neurones were detected in untreated cultures (Fig. 5A), the number of cells increased dramatically following exposure to 10 nM AChE-peptide for 1–72 h (Fig. 5B, C). In contrast, a relatively small number of neurones were PI-positive following 336 h exposure to 10 nM AChE-peptide (Fig. 5D).

Cell specificity of AChE-peptide bioactivity

To determine whether AChE-peptide bioactivity was cell specific, neurite outgrowth was measured in immunochemically identified neuronal populations using antibodies to visualise GABA (GAD positive), cholinergic (ChAT positive) and somatostatin neurones. While the acute neurite promoting effect of low AChE-peptide concentrations noted using the generic MAP2 method was not observed, a concentration dependent reduction in neurite outgrowth was observed following an extended 336-h exposure to AChE-peptide in each neuronal population. Neurite outgrowth was maximally reduced following incubation with 10 μM AChE-peptide by 69%, 82% and 56% in somatostatin-, ChAT- and GAD-immunopositive cells respectively (Fig. 6A–C).

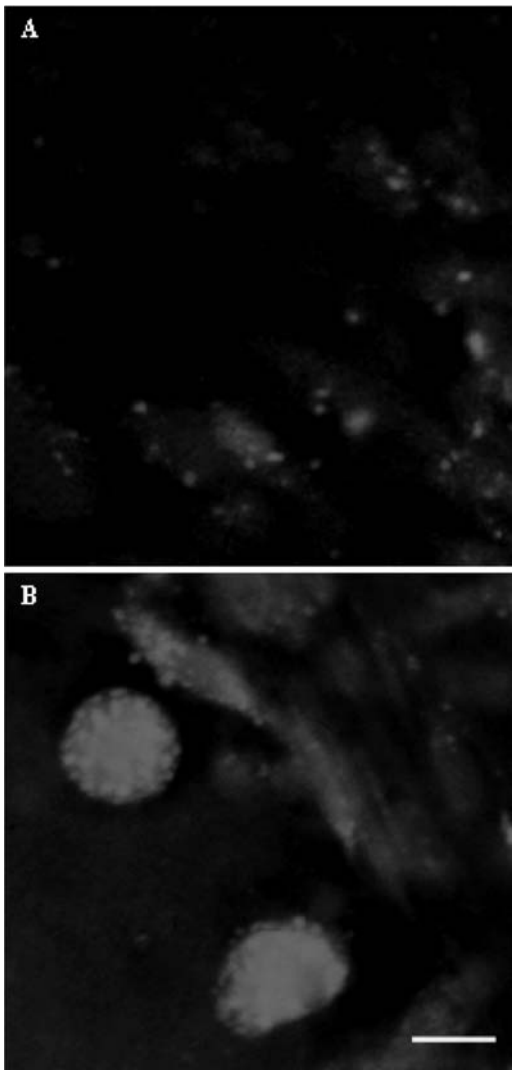

Fig. 4 Effect of 24-h exposure to 10 nM AChE-peptide **(A)** or vehicle **(B)** on chromatin condensation visualised using Hoescht 33342. *Scale bar* 1 μm

Discussion

Procedural considerations

The aim of this study was to investigate the bioactivity of a synthetic peptide derived from the C-terminus region of AChE using in vitro hippocampal organotypic cultures. The structural integrity of organotypic cultures allows the study of defined neuronal populations in a relatively physiological environment, and the robust nature of this model allows both acute and subchronic studies of

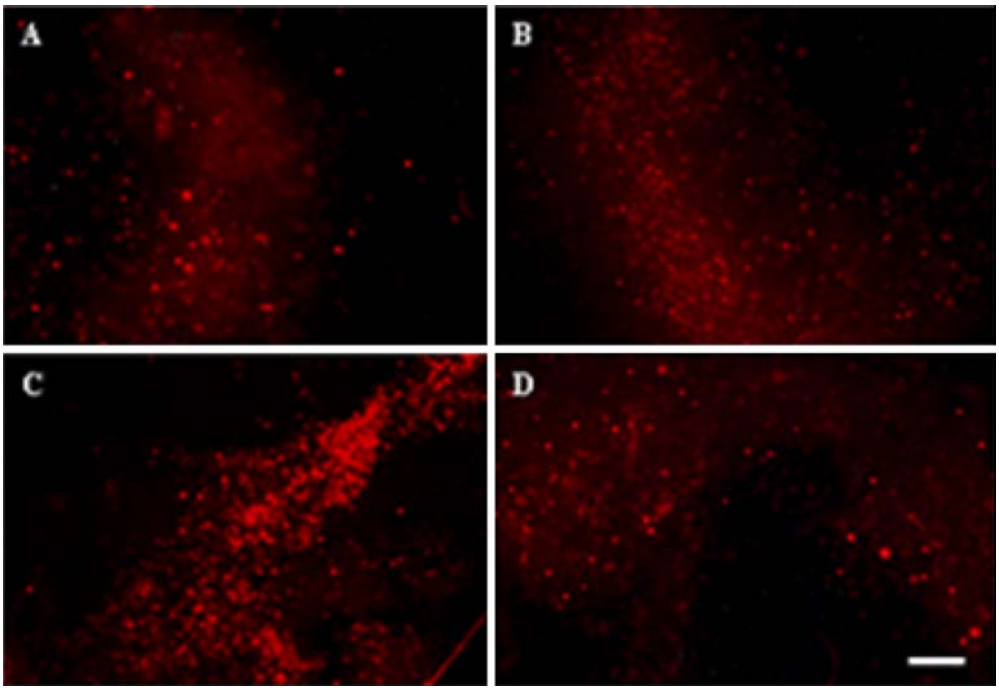

Fig. 5A–D Effect of AChE-peptide on cell membrane permeability visualised using propidium iodide. **A** represents a control culture maintained for 14 days and can be directly compared to application of 10 nM AChE-peptide for 1 h **(B)**, 24 h **(C)** and 336 h **(D)**. *Scale bar* 10 μm

neuronal viability. Neurite outgrowth from immunohistochemically defined neurones was used as a quantitative marker of neurotrophic activity, the most established non-classical action of AChE (Greenfield 1996; Holmes et al. 1996; Day and Greenfield 2002). In addition, TUNEL staining and LDH efflux were measured as quantitative measures of cell death. Since the specificity of TUNEL as an apoptotic marker has been questioned (Negoescu et al. 1998; Wolvekamp et al. 1998), further qualitative studies were performed to visualise two key characteristics of the cell death processes; Hoechst 33342 was used to detect chromatin condensation associated with apoptosis, and propidium iodide used to detect the loss of membrane integrity characteristic of necrosis.

Neurotrophic actions of AChE-peptide

A neurotrophic action of highly purified synthetic AChE-peptide, detected as an increase in neurite outgrowth from MAP-positive neurones, was observed using low peptide concentrations (1–10 nM) and short exposure periods (1 h): this effect is reminiscent of the established effect of exogenous AChE in organotypic cultures (Holmes et al. 1996; Day and Greenfield 2002). Increased neurite outgrowth was not observed after exposure to a scrambled AChE-peptide variant or the BuChE-peptide homologue, suggesting that a contaminant introduced during peptide synthesis is unlikely to be involved. Interestingly, morphological data from immunohistochemically identified cell types suggest that neurotrophic effects are not manifest in GABA, ACh or somatostatin containing hippocampal interneurones, indicating that a distinct neuronal population is involved. While the predominant glutamatergic neuronal population in the hippocampus is the most likely candidate, a lack of commercially available antibodies capable of detecting this neuronal population precludes a direct confirmation of this assumption. The effect detected in MAP-positive neurones may therefore underestimate the true extent of neurotrophism because unresponsive neuronal populations will tend to reduce the net positive effect detected using a generic neuronal stain.

Neurotoxic actions of AChE-peptide

In contrast to the acute neurotrophic effects seen at low AChE-peptide concentrations, a marked neurotoxic response was noted as peptide concentration and/or exposure time increased. Prolonged (336-h) exposure to 1 nM AChE-peptide induced apoptosis-like cell death, as determined by TUNEL and Hoechst 33342 staining. Conversely, prolonged exposure to micromolar AChE-peptide induced necrotic cell death, as defined by LDH efflux. While TUNEL data suggest that apoptotic cell death is accelerated at an intermediate 10 nM AChE-peptide concentration, propidium iodide stained neurones, indicative of necrosis, were observed in the same cultures even in the absence of LDH release. One explanation is that a relatively small population of cells is susceptible to necrotic cell death at intermediate AChE-peptide concentrations, precluding detection using the less sensitive LDH readout. In contrast, a more severe necrotic insult delivered by prolonged exposure to higher peptide concentrations results in detectable LDH efflux. This pattern of AChE-peptide mediated cell death suggests a continuum of neurotoxic susceptibility ranging from a predominantly apoptosis-like process at low peptide concentrations to a primarily necrotic process at higher concentrations. At intermediate concentrations, AChE-

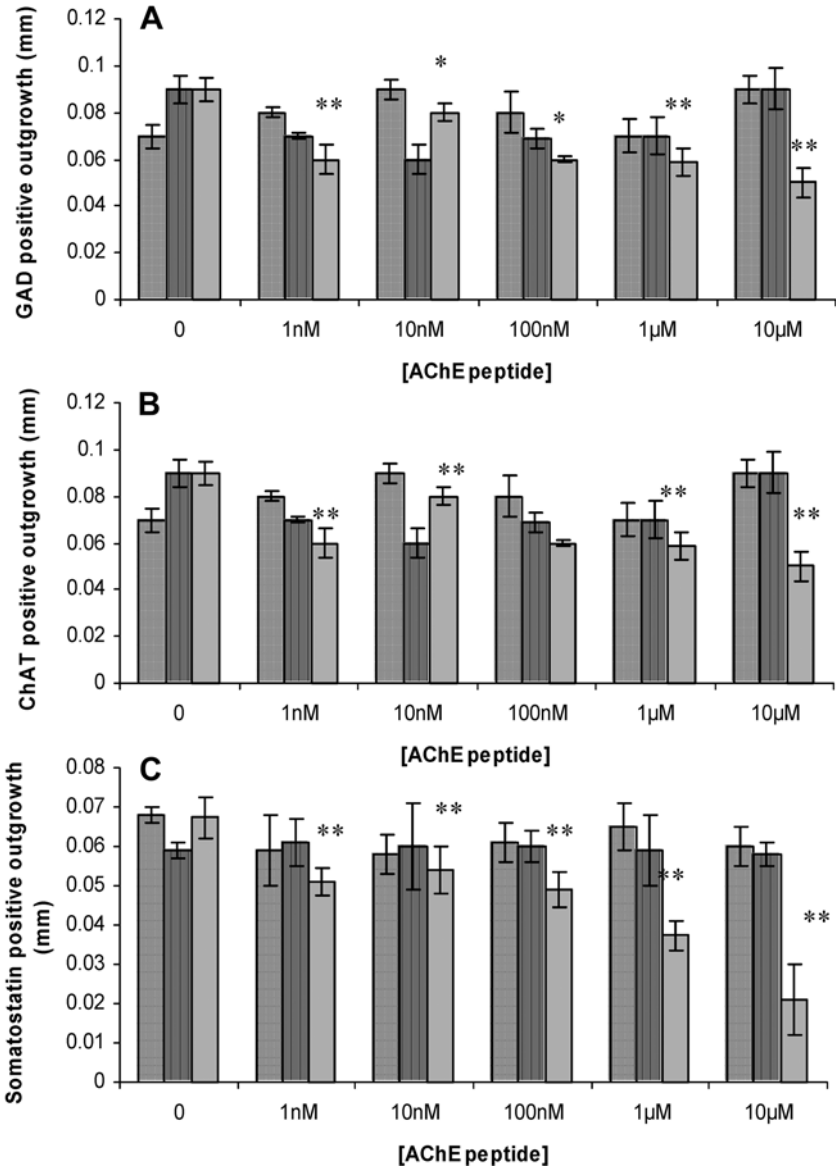

Fig. 6 Effect of AChE-peptide on neurite outgrowth from GABA (GAD-immunopositive; **A**), cholinergic (ChAT-immunopositive; **B**) and somatostatin-immunopositive **(C)** neurones. Organotypic hippocampal cultures were incubated with various concentrations of AChE-peptide for 1, 24 and 336 h. Following transmitter specific immunostaining, neurite outgrowth was determined using a non-biased method; neurite outgrowth was measured in every 5th square of a 100-μm^2grid superimposed over an image of the culture using an eyepiece graticule attached to a Nikon Diaphot inverted microscope. Results are expressed as mean neurite outgrowth (mm) ± SEM. Statistical analysis used ANOVA followed by the Dunnett's multiple comparisons *t*-test (***P*<0.01; **P*<0.05; *n*=160). Key: *medium grey columns* 1 h exposure, *dark grey columns* 24 h exposure, *light grey columns* 336 h exposure

peptide induced neurotoxicity is likely to involve simultaneous processes of apoptotic and necrotic cell death, the precise proportions of each being determined by peptide concentration and exposure period.

Interestingly, NMDA toxicity is also associated with a continuum of apoptotic and/or necrotic processes depending on the severity of the insult (Bonfoco et al. 1995; Sakaguchi et al. 1997, 1999). Similarly, β-amyloid, a peptide associated with Alzheimer's disease with close sequence homology to AChE-peptide (Greenfield and Vaux 2002), induces cell death either by apoptosis or necrosis depending on concentration and/or exposure duration (Iversen et al. 1995; Kienlen-Campard et al. 2002). The early and late phases of β-amyloid toxicity are also similar to those seen for NMDA toxicity (Abe and Kimura 1996).

Mechanism of AChE-peptide action

Morphological data demonstrate that neurite outgrowth from both generic MAP-positive neurones, and specific populations of immunohistochemically identified hippocampal interneurones, is inhibited by prolonged exposure to AChE-peptide. Since there were cell-specific variations in the degree of sensitivity to AChE peptide, it seems unlikely that the effect was artefactual, or caused by the mere presence of bulk peptide: this deduction is supported by the observation that neither scrambled AChE-peptide nor the BuChE-peptide analogue reduced neurite outgrowth, and suggest that a specific biological mechanism underlies the bioactivity of AChE-peptide in this culture model.

It seems likely that bioactivity involves enhanced calcium entry: a similar continuum of calcium-induced trophic-toxic actions can be seen with increasing concentrations of NMDA (Dickie et al. 1996) or with increasing

age as calcium tolerance decreases (Eimerl and Schramm 1994). Indeed, AChE-peptide may modulate the α7 nicotinic acetylcholine receptor (α7 nAChR) function, since this receptor is transiently co-expressed during development with AChE (Broide et al. 1997), and neurotoxic potential (Berger et al. 1998; Lukas et al. 2001).

Relevance of AChE-peptide bioactivity

Neurotrophic actions of exogenous AChE have been demonstrated (Jones et al. 1995; Holmes et al. 1996; Day and Greenfield 2002), whilst reports of toxic actions in neuronal and glia-like cultures have also appeared (Calderón et al. 1998). The present data suggest that a specific peptide sequence resident in AChE-peptide is bioactive, according to dose and exposure time, along a spectrum of neurotrophism to apoptosis to necrosis. Since bioactivity is noted at very low concentrations of AChE-peptide, this phenomenon is likely to represent a pathophysiological process that can be manifest in vivo. Neurodegeneration has previously been proposed to represent an aberrant form of development based on an age-dependent loss of calcium tolerance (Greenfield 1996, Greenfield and Vaux 2002). Since, as shown here, AChE-peptide has an appropriately graded action depending on the paradigm in which it is applied, it could potentially be a pivotal mechanism in the aetiology of neurodegeneration, and hence as a novel pharmaceutical target.

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