Bioactivity of a peptide derived from acetylcholinesterase: involvement of an ivermectin-sensitive site on the alpha 7 nicotinic receptor

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A peptide fragment of 14 amino acids, derived from the C-terminus of acetylcholinesterase (AChE), might underlie the now well-established noncholinergic effects of the enzyme. This peptide is bioactive in a variety of systems including acute (brain slices) and chronic (organotypic culture) preparations of hippocampus, a pivotal area in Alzheimer's disease (AD); invariably, the action of the peptide is mediated specifically via an as yet unknown receptor. In this study, the allosteric alpha 7 agent, ivermectin (IVM), had a modest inhibitory effect, whilst that of the peptide was significantly more marked. However, ivermectin rendered ineffective the toxicity of high doses of the peptide, that is, when the two were co-applied, only the smaller effects of ivermectin were seen. Ivermectin, therefore, is presumably acting at a site that is identical to, or at least strongly interactive with, the normal binding site for AChE-peptide. This observation could have important implications for eventual therapeutic targeting of the action of AChE-peptide, in neurodegeneration.
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Introduction

It is well know that acetylcholinesterase (AChE) has noncholinergic functions in relation to development (Appleyard, 1992; Arendt et al., 1992; Day and Greenfield, 2002; Greenfield, 1991, 1996; Soreq and Seidman, 2001) possibly by enhanced calcium influx (Webb et al., 1996). The parallels between AChE, acting in a nonclassical manner, and actions of APP/β-amyloid (Appleyard et al., 1987; Farlow et al., 1992; Furukawa et al., 1996; Greenfield, 1995; Jones et al., 1995; Milward et al., 1992; Webb et al., 1996; Wu

et al., 1995) have led to the identification of a domain, AEFHRWSSYMVHWK (AChE-peptide), located toward the "C" terminus of AChE, which has a strong homology with APP (Greenfield and Vaux, 2002).

A candidate receptor for the functional effects of noncholinergic AChE is the α7 nicotinic receptor. Indeed, this highly calcium-permeant receptor (Seguela et al., 1993) is transiently expressed in rat brain in parallel with AChE (Broide et al., 1996) and, moreover, strongly binds proteins (Wang et al., 2000a,b) closely related to AChE (Greenfield and Vaux, 2002).

To date, most is known about allosteric modulation on the α4β2 subunit; however, there are also some recent reports of modulation of the α7 nicotinic acetylcholine receptor (nAChR). For example, ivermectin (IVM) acts as an allosteric effector that binds to a specific site on the α7 nAChR: micromolar concentrations of IVM markedly enhance ACh-evoked currents of the neuronal chick and human α7 nAChR in oocytes (Krause et al., 1998). Hence, we have first confirmed that in the hippocampus, IVM does indeed act primarily at alpha-7 receptors, and we have then tested whether the bioactive effects of AChE-peptide could be modified by IVM.

The preparation chosen as most appropriate was one where not only the peptide is effective, but where the noncholinergic actions of AChE have been well-documented: mammalian organotypic cultures (Day and Greenfield, 2002; Holmes et al., 1997; Jones et al., 1995). Since noncholinergic acetylcholinesterase is well known to affect the hippocampus (Appleyard, 1992; Day and Greenfield, 2002; Small et al., 1996) and since this region is so important in Alzheimer's disease (AD), the effects of AChE-peptide on neurite outgrowth were investigated at different concentrations and for different periods of time. These actions were compared with those of "scrambled" peptide sequence HSWRAEVFHKYWSM to control nonspecific bulk peptide effects.

Materials and methods

Materials were obtained as follows: Hank's balanced salt solution, Eagle's MEM, horse serum, poly-D-lysine, chicken plasma, bovine thrombin, glutamine, and mitotic agents were obtained from Sigma-Aldrich Company Ltd (Poole, UK). Fungizone was

obtained from Life Technologies Ltd (Paisley) and ivermectin from Tocris Cookson Ltd (UK). All peptides used were synthesized at the Dyson Perrins laboratory for organic chemistry (Dr. M. Pitkeathley, University of Oxford) using an Applied Biosystems 430A Automated Peptide Synthesiser, utilizing the standard Fmoc protein synthesis methodology. Couplings were achieved using double couple cycles involving two 30-min incubations with a twofold excess of the HBTU-activated form of the amino acid. Any N-termini, which had not reacted and remained after the coupling step, were capped using acetic anhydride and pyridine. The progress of the peptide synthesis was monitored by UV analysis of the dibenzofulvene product released on the cleavage of the transient N-terminal Fmoc protecting group.

The completed peptides were cleaved from the resin and deprotected using trifluoroacetic acid (95% TFA) and an appropriate mixture of the following scavengers (5%): water, ethanedithiol, thioanisole, ethylmethyl sulfide, and triisopropyl silane. After removal of the resin by filtration, the filtrate was concentrated in vacuo, and the crude peptides were precipitated by the addition of diethyl ether. Once crude peptides were received from the Dyson Perrins laboratory, in a lyophilized state, they were purified by reverse phase high-performance liquid chromatography (HPLC), using a dedicated Gilson HPLC system (Mr. K. Pryor, Department of Pharmacology, University of Oxford).

Tissue preparation

Hippocampal organotypic cultures were prepared according to the procedure described by Gahwiler (1981, 1984). Briefly, the hippocampi were removed from brain of 8-day-old Wistar rats (Harlan UK, Oxford, UK), and transverse sections of 400-μm thickness were cut by use of a McIlwain tissue chopper (The Mickle Lab., Engineering Company, Surrey, UK) in a sterile environment, then the slices were separated. Isolated slices were first placed into ice-cold Hank's balanced salt solution supplemented with 5 mg/ml glucose and then mounted on sterile poly-D-lysine-coated coverslips by a chicken plasma/bovine thrombin clot. Once the plasma clot had set, the coverslips were placed in flat-sided tubes with 500 μl of culture medium containing 50% Eagle's MEM, 25% heat-inactivated horse serum, 25% Hanks solution, 6.5 mg/ml glucose, 2 mM glutamine, and mitotic agents to prevent the overproliferation of non-neuronal cells and Fungizone® to reduce the risk of infection. Slices were transferred onto a slowly rotating (0.5 rpm) drum. Cultures were maintained in a humidified incubator with 95% air/5% CO_2 atmosphere at 37°C, and the medium was changed twice a week. Experiments were carried out after 14 DIV.

All experiments were conducted in accordance with the Animals Scientific Procedures Act (1986), approved by Her Majesty's Government Home Office. All efforts were made to minimize the number of animals used and their suffering.

Application of candidate bioactive agents

AChE-peptide and scrambled AChE-peptide (an inverted AChE-peptide) were purified, freeze-dried, and maintained at −20°C. On the day of the treatment, fresh solutions were prepared. The IVM used in all the experiments was composed of both ivermectin B_{1a} (>90%) and B_{1b}. IVM stock solutions were dissolved in 100% dimethylsulfoxide (DMSO) and further diluted in media. The final concentration of DMSO was maximally 0.01%.

For this study, the experimental conditions were supplementing the medium with AChE-peptide at low concentrations (1–0.001 nM), IVM at 1 μM, and co-application of AChE-peptide/IVM for 1 h to 3 days. The cultures treated for 1 h were subjected to a 24-h recovery period before fixation. Cultures that were treated for less than 14 days were maintained in medium and treated at the appropriate times.

For the second set of experiments, hippocampal cultures were incubated in medium with AChE-peptide at a concentration of 10 μM, IVM at concentrations of 1 μM and 100, 10, 1 nM, co-application of AChE-peptide and IVM and control group.

All cultures were maintained for 14 DIV regardless of treatment time.

Analysis of data

On DIV 14, immunohistochemical staining for microtubule-associated protein (MAP-2) was performed using the biotin–avidin–peroxidase method (Hsu et al., 1981). Cultures were fixed in 4% paraformaldehyde and preincubated with PBS containing 10% goat serum. Cells were then incubated with the primary antibody (anti-MAP-2 antibody) (Chemicom International, Ltd, UK) for 24 h at 4°C. Following extensive rinsing with PBS, cells were incubated for 1 h with the second antibody (biotinylated goat-anti-mouse monoclonal antibody) (Novocastra Laboratories Ltd, UK) and treated with 0.3% hydrogen peroxide in 10% methanol to eliminate endogenous peroxidase activity. Finally, cultures were incubated in avidin–biotin complex (ABC elite kit, Novocastra Laboratories Ltd). The peroxidase was visualized with 0.05% diaminobenzidine tetrahydrochloride (DAB) (Sigma, Poole, UK)/0.015% H_2O_2.

Assessments of cell health were evaluated by measuring dendrites outgrowth of pyramidal cells in culture. Dendrites length was measured from cell body to dendrite tip and expressed as a percentage mean ± SEM of a control group consisting of cultures that had been given media only. Morphogenic analysis was by camera Lucida drawings. Statistical analysis was carried out using an ANOVA followed by Tukey post-hoc test for multiple comparisons.
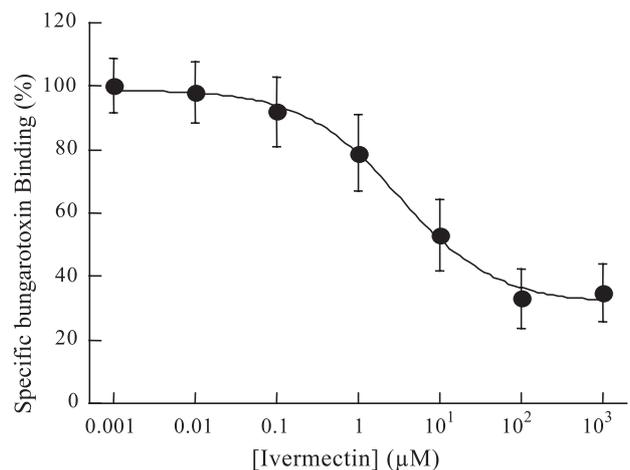

Fig. 1. The effect of IVM on BTX binding on hippocampus in vitro. Concentration-dependent inhibition of ^{125}I-α-BGT binding by IVM. IVM inhibits ^{125}I-α-BGT binding at low micromolar concentrations. Mean data from at least four experiments are presented. Values are expressed as mean ± SEM. The curve shows an approximate EC50 value of 3 μM.
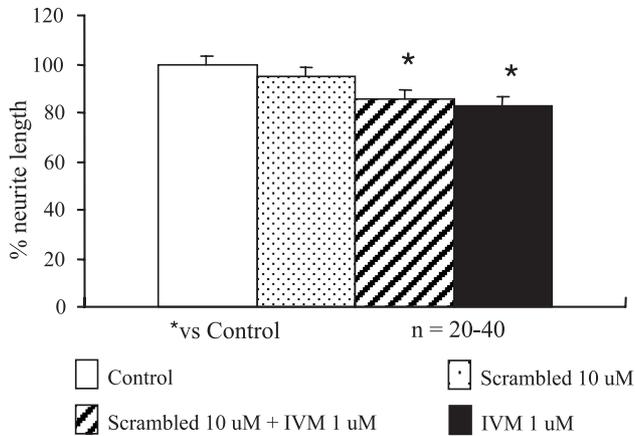
Fig. 2. The effect of co-application of scrambled peptide/IVM on hippocampus in vitro. Organotypic hippocampal cultures were maintained in media supplemented with scrambled peptide (10 μM) in the presence of IVM 1 μM for 14 days and processed for MAP-2 immunohistochemistry. Parameter was neurite outgrowth. Results are expressed as percent mean ± SEM of media-only treated control culture. Statistical analysis was by ANOVA and Tukey multiple comparisons *t* test. *P* < 0.05 was considered as significant.

Assay of binding of ^{125}I-labeled α-bungarotoxin

We verify the selectivity of IVM for α7 nAChR using the binding of ^{125}I-α-bungarotoxin (α-BTX) to suspensions of rat hippocampal membranes. Tissue preparation was based on that previously reported by Wang et al. (2000a,b). Briefly, freshly isolated tissue was homogenized in 50 volumes of ice-cold 10 mM Na⁺-HEPES (pH 7.4) and then centrifuged at 42 000 × *g* for 10 min. The supernatant was discarded and the pellet was washed by resuspending it in 40 volumes of the 10 mM Na⁺-HEPES solution and centrifuging at 42 000 × *g* for 10 min. This washing step was performed three times. Subsequently, the pellet was resuspended into 25 volumes of a buffered incubation medium comprising Na⁺-HEPES buffer (10 mM, pH 7.4), 5 mM MgCl₂, 0.01% bovine serum albumin, a protease inhibitor cocktail, and 100 mM NaCl. Each binding reaction was carried out in tube containing 0.25 ml of the membrane suspension, 0.4 ml of the HEPES-buffered incubation medium, and 0.1 ml of

test compound(s) dissolved in water. Samples were incubated with 10 nM ^{125}I-α-BGT for 2 h at room temperature. Nonspecific binding was assessed in the presence of 2.5 mM nicotine. The binding reaction was terminated by separation of the membrane material from the incubation medium. This was performed using rapid filtration (Brandel Harvester). The samples were washed twice with ice-cold HEPES-buffered solution (10 mM, pH 7.4) and radioactivity determined using standard γ counting.

Results

Characterization of ivermectin: ^{125}I-αBTX binding

To prove the selectivity of IVM for α7 nAChR, we tested the effects of IVM on ^{125}I-αBTX binding on rat hippocampal membranes. The presence of binding sites for ^{125}I-αBTX, a specific ligand of muscle and α7 nAChR subtypes (Lindstrom, 2000), would be consistent with the expression of the α7 nAChR subtypes. As shown in Fig. 1, IVM inhibited ^{125}I-αBTX binding at relatively low micromolar concentrations.

Selectivity of AChE-peptide

The scrambled variant of the peptide had no effect on neurite outgrowth in the hippocampal culture system. There was no significant difference when compared to control cultures (Fig. 2). However, IVM at a tenfold lower concentration, with or without scrambled peptide, did cause a significant reduction in neurite outgrowth (Fig. 2) over 14 days.

Effects of low doses/short exposure of AChE-peptide and ivermectin

Treatment of AChE-peptide at low concentrations (1 nM to 1 μM) for 1 h to 3 days caused a modest increase in MAP-2 positive outgrowth (265.4 ± 8.39 and 268.3 ± 17.43 μm, respectively, *n* = 25–40) when compared to a control group (242.7 ± 5.72 μm, *n* = 40) (Fig. 3). IVM treatment for 1 h did not have any effect on AChE-peptide (1 nM); however, when the incubation time was increased (3 days), IVM (alone or co-applied with AChE-peptide at 1 μM) decreased neurite outgrowth (194.9 ± 8.81 and 191.6 ±
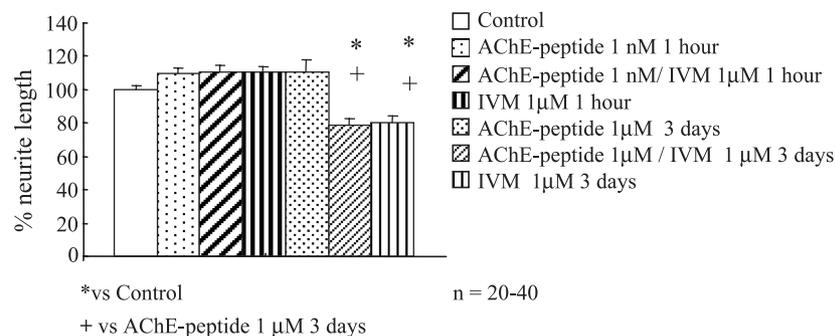

Fig. 3. The effect of co-application of low concentrations of AChE-peptide/IVM on hippocampus in vitro. Hippocampal cell culture were maintained in media supplemented with AChE-peptide (1 nM to 1 μM) in the presence of IVM (1 μM) for 1 h to 3 days and processed for MAP-2 immunohistochemistry. Parameter used was neurite outgrowth. Results are expressed as percent mean ± SEM of media-only treated control culture. Statistical analysis was by ANOVA and Tukey multiple comparisons *t* test. *P* < 0.05 was considered as significant.
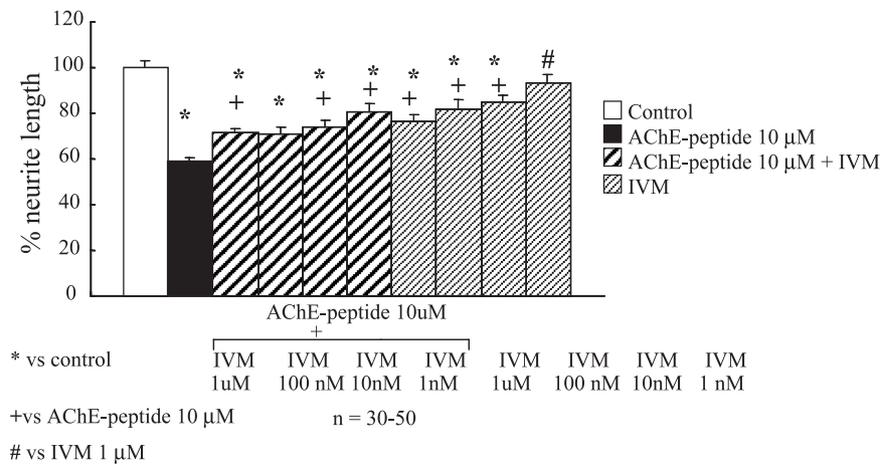
Fig. 4. The effect of co-application of AChE-peptide (10 μM)/IVM on hippocampus in vitro. Hippocampal cell culture were maintained in media supplemented with AChE-peptide (10 μM) in the presence of IVM (1 μM and 100, 10, 1 nM) for 14 days and processed for MAP-2 immunohistochemistry. Parameter used was neurite outgrowth. Results are expressed as percent mean ± SEM of media-only treated control culture. Statistical analysis was by ANOVA and Tukey multiple comparisons *t* test. *P* < 0.05 was considered as significant.

9.05 μm. *P* < 0.05 Tukey multiple comparisons *t* test, *n* = 20–22) when compared to a control group and group treated with AChE-peptide at 1 μM (268.3 ± 17.43 μm) (Fig. 3).

Effects of high doses/long exposure of AChE-peptide and ivermectin

However, when the incubation time and/or the concentrations of AChE-peptide were increased, hippocampal cells became more vulnerable to the toxic action of AChE-peptide. AChE-peptide at a concentration of 10 μM has toxic effects on hippocampal cells in organotypic cultures. Addition of AChE-peptide at this concentration for the entire cultivation period (14 days) significantly decreased neurite outgrowth by 42% (109.2 ± 6.4 μm. *P* < 0.05 Tukey multiple comparisons *t* test, *n* = 34) when compared to a control group (191.6 ± 6.32 μm, *n* = 54) (Fig. 4).

IVM alone had a dose dependent effect on neurite outgrowth, in vitro. IVM at 1 μM caused a decrease on neurite outgrowth when compared to a time-matched medium-only control group (140.77 ± 6.11 μm, *n* = 30). This decrease on neurite outgrowth is less marked as dose of IVM decreases. When co-applied with IVM (at all concentrations tested), the effect of the AChE-peptide made no difference to the more modest effects of IVM. Since the inhibitory effects of IVM were less than that for the peptide, co-application of IVM/AChE-peptide actually induced a net significant increase in neurite outgrowth compared to the effect of AChE-peptide alone (Fig. 4).

Discussion

Procedural considerations

The organotypic culture system was chosen as a model that resembles the in vivo environment as closely as possible, whilst offering the accessibility and reductionism of the in vitro preparation. Some of the advantages of this system include maintenance in a high degree of organotypic organization, cellular differentiation, and tissue-specific cell connections (Gahwiler, 1981, 1984; Caeser

and Aertsen, 1991; Zimmer and Gahwiler, 1984), as well as preservation of local neuronal circuits with the appropriate innervation patterns (Torp et al., 1992). Furthermore, organotypic cultures compared to in vivo models offer the possibility of more ready application of drugs. In addition, neurons in slice culture are still associated with glial cells and are therefore more likely to express their in vivo morphology and characteristics than they would in dissociated neuronal culture. Indeed, organotypic slice cultures of hippocampus still display their characteristic in vivo morphology; they preserve much of the regional differentiation and intrinsic synaptic connectivity of the normal hippocampus in situ and it has been shown that the distribution of specific neurotransmitter phenotypes has a good correlation to the hippocampus in situ (Buchs et al., 1993; Caeser and Aertsen, 1991).

Specificity of ivermectin and of AChE-peptide

This study revealed that IVM binds selectively and with micromolar affinity to α7 nAChR as confirmed using ^{125}I-αBTX binding. IVM caused a reduction of the alpha—αBTX binding. Since IVM is an allosteric effector of the α7 nAChR, it was not expected that it would necessarily cause a detectable variation of the αBTX binding. However, this observation supports the fact that IVM interacts with α7 nAChR expressed in the cultured hippocampus. In addition to its effects on α7 nAChR, IVM has also been shown to exert effects on other members of the ligand-gated ion channel superfamily, such as GluClRs, GABA$_A$Rs, and GlyRs. IVM acts as an allosteric potentiator of glycine-gated currents at low concentrations, and activates GlyR via a novel mechanism (Shan et al., 2001). The high affinity of α7 nAChR for IVM suggests that this receptor has a specific site for this type of pharmacological ligand (Krause et al., 1998).

Bioactivity of AChE-peptide

The scrambled variant of AChE-peptide, used as a control for bulk peptide application for the entire cultivation period, did not have any significant effect on the neurite outgrowth of the hippocampus in vitro. However, AChE-peptide at a comparable concentration in the micromolar range has a selective toxic effect

on hippocampal cultures, and is unlikely to contain a contaminant introduced during peptide synthesis that could account for its actions (Day and Greenfield, 2003b).

AChE-peptide and α7 nAChR

Treatment of hippocampal cultures with AChE-peptide results in a time- and dose-dependent cell death. Low doses of AChE-peptide for short times causes an increase in neurite outgrowth as already reported for "noncholinergic" AChE (Day and Greenfield, 2003a; Holmes et al., 1997; Jones et al., 1995; Munoz et al., 1999), whilst doses midway between the toxic and trophic were toxic if applied for longer periods of time. In fact, AChE-peptide at higher concentrations for 14 days induced a significant decreased in neurite outgrowth and cell death. This dual neurotrophic/neurotoxic actions of AChE-peptide are in agreement with those found by Day and Greenfield (2003a,b) on hippocampal neurons. In this context, studies of a dual action of exogenous AChE have been also reported on neuronal and glial like cells. Both cells exposed to exogenously AChE were affected in a different manner depending on the concentration and incubation time. Low concentrations of AChE induced morphological and metabolic changes in cells. These cells gradually lost their neurite extensions, degenerated, and underwent cell lysis when exposed to higher concentrations (Calderón et al., 1998). Moreover, recent studies, in which the expression of AChE was genetically manipulated, have shown a direct correlation between endogenous AChE content and neurite outgrowth.

In fact, overexpression of AChE resulted in an increase in neurite outgrowth in cell lines, spinal neurons, or retinal cells that paralleled the level of AChE expression. Conversely, when the AChE expression is reduced using antisense techniques, a decreased in neurite length was observed (Bigbee et al., 2000; Brimijoin and Koenigsberger, 1999; Karpel et al., 1996; Koenigsberger et al., 1997; Robitzki et al., 1997; Sternfeld et al., 1998). For instance, overexpression of AChE in rat C6 glioma cells induced process extension from 54 μm at 2 h to 83 μm (Karpel et al., 1996). Likewise, overexpression of AChE in spinal neurons from *Xenopus* embryos resulted in increased neurite length from 88.2 μm in control to 124.0 μm (Sternfeld et al., 1998), in primary dorsal root ganglion neurons, neurite outgrowth increased about two times greater (1444.2 μm) than in control (685.7 μm) μm (Bigbee et al., 2000), and in neuroblastoma cells (N1E.115 cell lines), overexpressing AChE extended more and longer neurites (140% f control) than control or wild-type cells (Koenigsberger et al., 1997). Similar, in Sharma's results, AChE-overexpressing N1E.115 cell lines show a 250% increase in AChE expression whereas the AChE-underexpressing cell line has only 60% of the AChE levels present in the wild-type cells, and this level of AChE directly correlates with the extent of neurite outgrowth (Sharma et al., 2001). In contrast, neurite outgrowth in cells with a reduced AChE expression was reduced by 40–50% in dorsal root ganglion neurons (Bigbee et al., 1999, 2000) and by 75% of control in neuroblastoma cells (Koenigsberger et al., 1997). This direct involvement of AChE in neurite outgrowth has been established to be through a mechanism that is independent of its catalytic activity (Layer et al., 1993; Sharma et al., 2001; Sternfeld et al., 1998). Indeed, it has been suggested that AChE modulates neurites adhesion and these changes in adhesion may lead to enhance axonal outgrowth (Sharma et al., 2001). On the other hand, it has also demonstrated a role for

AChE in neurite outgrowth by applying anticholinesterases agents to cell cultures. These results indicate that the level of AChE inhibition closely correlated with the extent of decreased outgrowth. Bigbee et al. (1999) found that the AChE inhibitors BW284c51 or physostigmine have a dose-dependent reduction in neurite outgrowth of dorsal root ganglia neurons prepared from E-15 rat embryos (from 98% of control at 10^{-7} M to 29.4% of control at 10^{-4} M or from 87.7% of control at 10^{-6} M to 47.4% of control at 10^{-5} M, respectively).

AChE-peptide/ivermectin interactions

Following co-application of AChE-peptide/IVM, no differences could be observed on neurite outgrowth when compared with the IVM effect alone. If the substances were working at independent sites, a summation of inhibitory responses, or at least a "floor" effect, should have been observed. However, the inefficacy of peptide in the presence of IVM suggests that IVM is able to bind the α7 nAChR at the same site, or highly related site as AChE-peptide, thereby blocking the complexing of the AChE-peptide to the receptor, even in micromolar range. The present data suggest that the AChE-peptide-induced toxicity involves α7-nAChR function. However, it should be noted that IVM is an allosteric modulator and therefore does not per se activate the receptors. Thus, effects observed by IVM incubation must be based on the modulation of otherwise active receptors.

The current results suggests that IVM can bind selectively to a specific site on α7 nAChR, which is closely interactive with, or indeed identical to, the binding site of AChE-peptide on this receptor. Since both the α7 nAChR (Guan et al., 2002; Guerguiev et al., 2000; Jessen et al., 2001; Nagele et al., 2002; Papke et al., 2000; Rusted et al., 2000; Wang et al., 2000a,b; Wevers et al., 2000) as well as noncholinergic AChE (Burghaus et al., 2003; Geula and Mesulam, 1995; Greenfield, 1996; Munoz and Inestrosa, 1999; Smith and Cuello, 1984; Webb et al., 1996) have been implicated in neurodegenerative processes, this finding may be of relevance for refining possible novel drug targets for AD.

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