A peptide derived from acetylcholinesterase is a pivotal signalling molecule in neurodegeneration

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Abstract

It is now widely accepted that acetylcholinesterase (AChE) also displays non-cholinergic functions, completely independent of cholinergic transmission. Indeed, AChE has been implicated in a variety of trophic and toxic actions in a range of different systems. However, it is still uncertain what part of the AChE molecule may be responsible for these actions, and indeed via what receptor. Recent work has identified a peptide towards the C-terminus of the AChE molecule that appears to have very similar effects to non-cholinergic AChE itself. This action is to enhance calcium entry, in acute and chronic preparations across a trophic–toxic spectrum, depending on concentration applied and/or duration of exposure.
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'Non-cholinergic' AChE, in particular, the AChE–peptide therefore is not only important in development, but in neurodegenerative diseases where the final common path is believed to be one of excitoxicity. AChE triggers the influx of calcium and subsequent activation of various second messenger pathways, leading to apoptosis. Tolerance to calcium will vary as a factor of age, and hence developmental mechanisms, in which AChE–peptide may be pivotal, might therefore be, at normal concentrations, nonetheless toxic in the adult brain. In addition, the net effects of AChE–peptide will either be trophic or toxic depending on exposure time and dose. The receptor via which calcium is elevated appears primarily to be the nicotinic alpha-7 receptor, where the peptide acts at an allosteric site. Once activated, secondary calcium ionophores are also triggered, including the L-type voltage-dependent calcium channel and the NMDA receptor.

This mechanism has wider implications for function and dysfunction. More specifically, expression of the peptide and, indeed, its receptor could be triggered during generation of excessive free-radicals, such as might occur in neurodegeneration. We have shown enhanced calcium influx into glia following free-radical insult such as might occur in stroke and neurodegenerative diseases. Glial cells are already known to induce neurogenesis, although the signalling mechanism has not been identified. Since stem cells express AChE when they commit to the neuronal cell-line, it is possible that one way in which this could be achieved in the adult brain would be via release of AChE by glial cells. Most recent work in our lab is showing that exposure of glial cells to hypoxic conditions results, after 24 h, in enhanced release of AChE to enhanced levels mRNA for R-T, but not T-AChE. In summary, non-cholinergic AChE, more specifically the peptide toward its C-terminal region, might therefore be an important mechanism, not just in development, but more generally in neurogenesis, and its use and abuse by the adult brain.

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1. The background: neurodegeneration and 'non-cholinergic' AChE

Alzheimer's disease (AD), Parkinson's disease (PD), and Motor neuron disease (MND), are the three most common neurodegenerative disorders. Unfortunately, their frequency is increasing due in part to an increased life expectancy, where diseases of older age will inevitably come to the fore. A further factor that cannot be ignored, is the improved prognosis for non-CNS disorders resulting from current biomedical advances in diagnosis, medication and surgical techniques. Yet despite the urgent need to develop earlier diagnosis, improved treatment, and even a cure for degenerative disorders, the underlying neurochemical mechanisms have proved elusive.

One familiar and well-established idea, is that for AD at least, the key problem is the formation of amyloid plaques, derived from abnormal cleavage of amyloid precursor protein (APP). However, whilst the association of amyloid accumulation with AD is without doubt, its role as a simple, an actual cause, is less obvious. Although amyloid accumulation into fibrils will disrupt neuronal membranes, such a process on is own, without any other constraining factors, would not account for the regional and indeed neuronal selectivity which characterise neurodegeneration. It seems more likely that although fibril toxicity might aid and abet the degenerative process, a more specific mechanism is responsible for the characteristic, highly selective progressive neuronal death.

Another well known idea, that has indeed inspired the rationale for the current treatment of early stage AD, is the 'Cholinergic Hypothesis' [25] that asserts that the primary cause is a deficiency in the transmitter acetylcholine (Ach), due to the death of critical, cholinergic populations of neurons within the brain. Hence, enhancing endogenous Ach levels with an acetylcholinesterase (AChE) inhibitor such as Aricept, the current treatment of choice, should prove therapeutic. However, one immediate concern, is that abnormal transmitter levels are usually a reflection of the problem, not the problem itself. Hence, the deficiency in dopamine and acetylcholine that traditionally characterize, respectively, PD and AD are simply the result of the death of key neuronal populations. Hence, increasing the levels of these substances is only temporarily alleviating the ongoing symptoms, – not arresting the all – important cell death. A more serious difficulty still with the Cholinergic Hypothesis is that whilst, unlike the Amyloid Hypothesis, it respects the cell selectivity that characterises
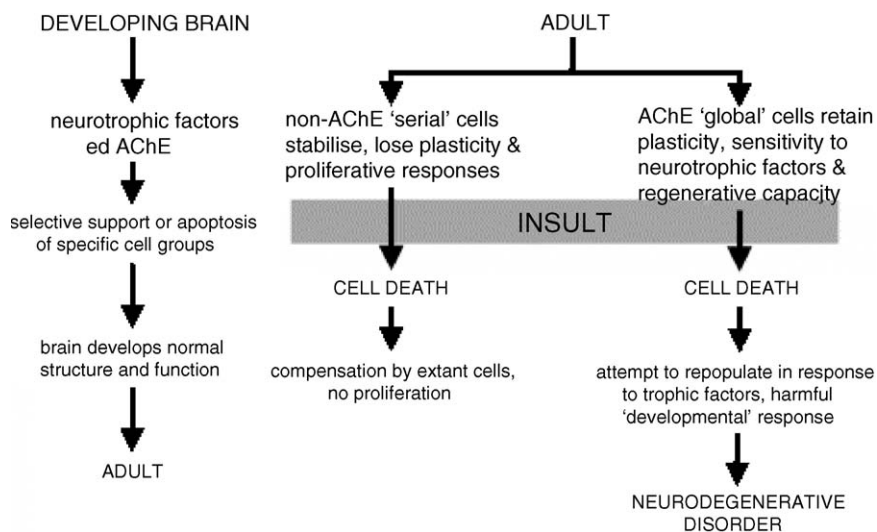

Fig. 1. Scheme for selective degeneration of certain groups of neurones in Alzheimer's, Parkinson's and motor neurone disease. In the developing brain most neuronal groups will transiently express neurotrophic factors such as AChE, which will ensure the development of specific cell circuitry that characterises the adult brain. Once mature, the majority of these serial circuits will no longer express AChE and, indeed, lose much of their plasticity and proliferative responses. If there is an insult, the cell death that ensues will ensure a functional compensation that characterises, in many cases, recovery or partial recovery in stroke. However, the global AChE-containing neurones, because they have retained their ability to regenerate and their sensitivity to trophic factors, will respond to a local insult in a different fashion. In this case, there will be an exuberant axonal regeneration in an attempt to repopulate leading to enhanced release of AChE. AChE, acting in a non-cholinergic capacity, or a fragment derived from AChE, will then trigger calcium entry, both within the local global cell population as well as on to target serial cells of hippocampus and cortex. Whereas, a certain degree of calcium would be beneficial in development, the mature cell is less able to handle intracellular calcium levels which therefore become toxic. The death of the cells will then constitute a further insult, leading to a pernicious cycle of events, neurodegeneration, from [14].

neurodegeneration—there is nonetheless a mismatch between the neurons that are indeed vulnerable, and the cholinergic systems in the brain. Not all cholinergic neurons are generically affected in AD, nor are the neurons that are actually lost, in any case, exclusively cholinergic. Once again therefore, although the loss of certain cholinergic neurons may add to the pathophysiological landscape, the ensuing drop in brain Ach, as witnessed by the relative long-term inefficacy of Aricept, is not a sufficient explanation for AD.

It is still a puzzle why particular neurons, and not others in the brain, are particularly vulnerable to neurodegeneration. However, a clue might come from the observation, often overlooked, that PD, AD, although presenting with different clinical profiles, may have a common underlying pathology [14]. In all cases, neurodegeneration could be attributed to the slow death of cells forming a central hub in the brain, and extending from the spinal cord to the mid-brain. This group of cells were originally referred to as the 'isodendritic core'

[22], but have been more recently referred to as 'Global' neurons [27], due to their diffuse projections from the more basic regions of the brain, to the 'higher' centres. Interestingly enough, these neurons are fundamentally different from their counterparts elsewhere in the CNS, in diverse characteristics ranging from foetal provenance to electrophysiological and biochemical properties: most significantly they differ from other neurons in that they have retained their capacity for axonal regeneration and proliferation [27].

We have proposed [14], that this persistent developmental mechanism might account for the underlying, as yet unidentified, cause of PD, AD and MND: the specific disease identity and the issue of any co-pathology will be a consequence of the degree of respective damage to substantia nigra, basal forebrain and/or motor-neurons, all of which are encompassed in this region. Co-pathologies [1,5,12,18,22] would occur when there was more extensive damage: indeed, additional nuclei within this area (the locus coeruleus and the Raphe nuclei) are also
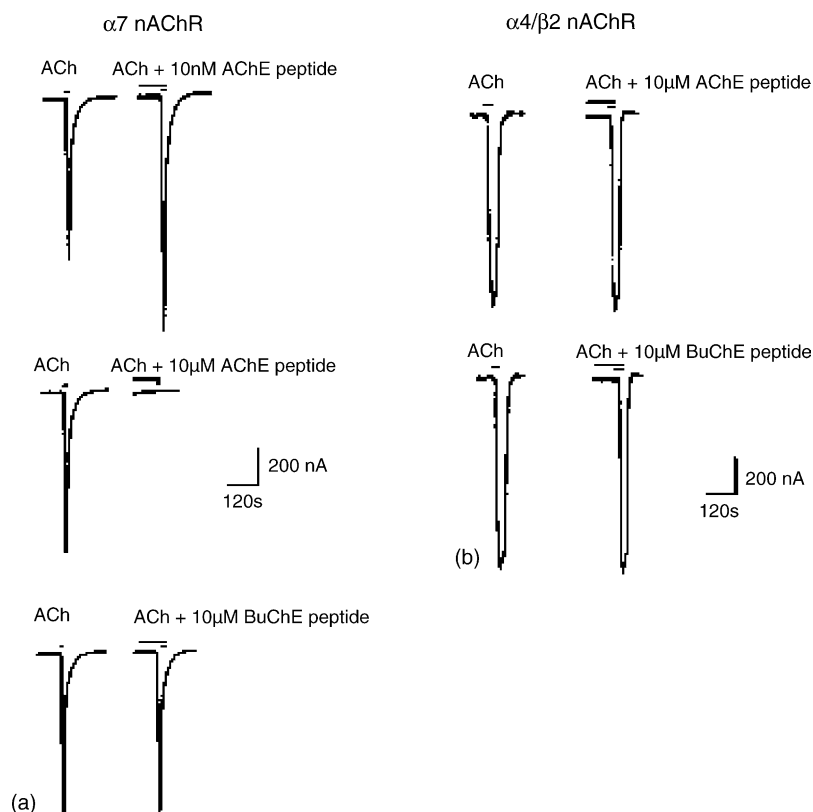

Fig. 2. The effects of AChE–peptide on the function of human $\alpha7$ and $\alpha4/\beta2$ nAChRs in *Xenopus* oocytes. (a) Current responses of a human $\alpha7$ nAChR-expressing oocyte to 100 μm Ach (EC$_{50}$ concentration) before and during co-application of 10 nm (top trace), 10 μm (middle trace) AChE–peptide or 10 μm BuChE–peptide (bottom trace). Note application of AChE–peptide alone (longer bar) has no effect. (b) Current responses of $\alpha7$ nAChR-expressing oocytes to 30 μm Ach (EC$_{50}$ concentration) before and during application of 10 μm AChE–peptide (top trace) or 10 μm peptide (bottom trace) from [15].
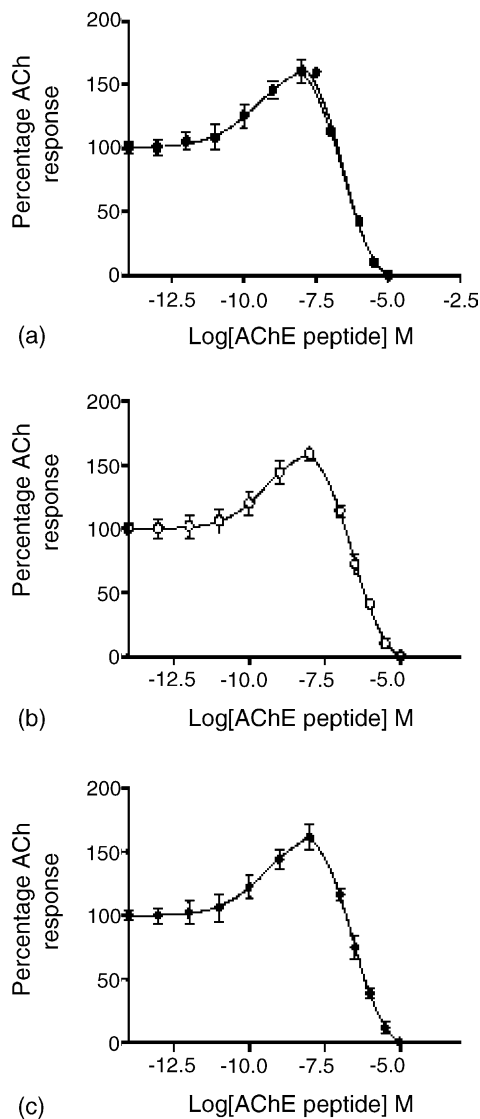
Fig. 3. The effects of AChE–peptide on the concentration–response curve of Ach at human α7 nAChRs expressed in *Xenopus* oocytes. (a) The effects of AChE–peptide on EC_{50} Ach-induced current responses in human α7 nAch-R-expressing oocytes were plotted as a percentage of the response to ACh alone (mean ± S.E.M., 10 oocytes). (b) One group of oocytes was incubated in 10 μm BAPTA/AM for 1 h prior to recordings. In (c), oocytes were continuously superfused with Ba^{2+}-Ringer solution. Data were fitted as indicated in Materials and methods from [15].

Fig. 4. The effects of AChE–peptide on electrophysiological responses in the hippocampal slice in vitro. (a) Application of μm AChE–peptide had no effect on the baseline fEPSP recorded in the CA1, but augmented the response to STP stimulation (grey arrow, 10 pulses, 50 Hz) into a long-lasting potentiation. The degree of potentiation was measured relative to LTP induced at the end of the experiment by 100 Hz tetanic stimulation (black arrow, 100 pulses). The effect of AChE–peptide was not mimicked by the application of 1 μm BuChE–peptide: $n = 3$. (b) AChE–peptide (1 μm) produced a selective potentiation of the fEPSP in the presence of 50 μm D-AP5, which was inhibited by 100 nm α-BgTx. The degree of potentiation was measured relative to LTP induced at the end of the experiment by 100 Hz tetanic stimulation (black arrow, 100 pulses). Application of 1 μm BuChE–peptide had no effect: $n = 3$; (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, from [15].

affected in both AD and PD. I suggest [14] that, following an insult for whatever reason, be it genetic predisposition, head injury, toxicity, or stroke, Global neurons will employ their special ability to regenerate in an attempt to compensate: however, in doing so, unlike all other neurons in the rest of the brain, they will set in train a process that is potentially lethal. A final trigger for neurodegeneration is well known to be excessive calcium
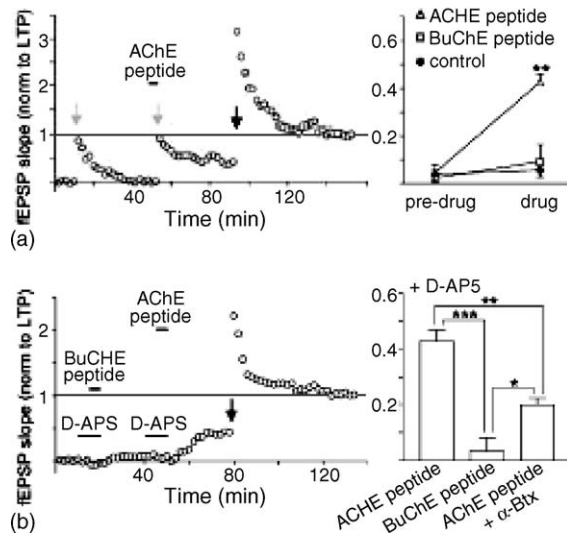
entry: ([6,7,10]); however, mature neurons can withstand far more calcium than mature ones [11]. Accordingly, trophic agents might therefore prove highly toxic if aberrantly activated in the inappropriate landscape of the adult brain.

Interestingly enough, all Global neurons, irrespective of the transmitter they respectively employ, contain AChE [14]. Moreover, it appears that this AChE can act independent of its traditional catalytic action, and instead serve in an alternative capacity as neurotrophic agent. AChE, although primarily familiar as the enzyme that hydrolyses ACh, is now well-established as having a non-enzymatic role within the brain [24]. Indeed, evidence for this notion came initially from the finding (see [13]), that in particular the key area lost in PD, the substantia nigra, contains large amounts of AChE, but small amounts of acetylcholine and its synthesizing enzyme, choline acetyltransferase. Moreover, within the substantia nigra the dopaminegic neurons which are lost in PD not only contain, but secrete, AChE in response to

physiological events. In turn, AChE can modulate these neurons [17,26] independent of its normal enzymatic function. Specifically, we have shown that AChE, operating in a non-cholinergic capacity, can enhance neurite outgrowth and improve cell survival, by means of activating calcium influx [7]. Hence, 'non-cholinergic' AChE could be the critical factor in defining the neurons vulnerable to neurodegeration: the Global neuron population that has retained the protein into maturity will employ it, when damaged, in an attempt to regenerate. But in doing so, the ensuing result will be to cause levels of calcium to enter the neurons that are inappropriate for the age of the brain, and excitotoxicity will result: there will be a pernicious cycle therefore that repeats over and over, that we recognize as neurodegeneration (see Fig. 1).

This mechanism could apply not only to AD and PD, but to MND too. Not only do motor neurons form part of the Global cell population, but they secrete AChE [21], which in turn has a non-cholinergic, trophic action

[2]. Meanwhile, vulnerable motor neurons express auto-antibodies to AChE, whilst MND itself can present as a co-pathology with AD and/or PD [19]. However, the next crucial question is: how can the AChE molecule trigger such changes independent of its catalytic site?

2. The molecule: a 14 amino-acid peptide

We have identified a 14 amino acid peptide 'AChE–peptide' having the sequence: AEFHRWSSYMVHWK, and corresponding to amino-acid residues 535–548, at the C-terminus of AChE [14]. Recent work in our laboratory has shown that AChE–peptide is indeed bioactive: it enhances calcium entry into neurons [4], as well as exerting a trophic/toxic action via calcium entry, accordingly to duration of exposure and concentration of peptide to neurons [8,9]. The next step has been to identify a candidate receptor.
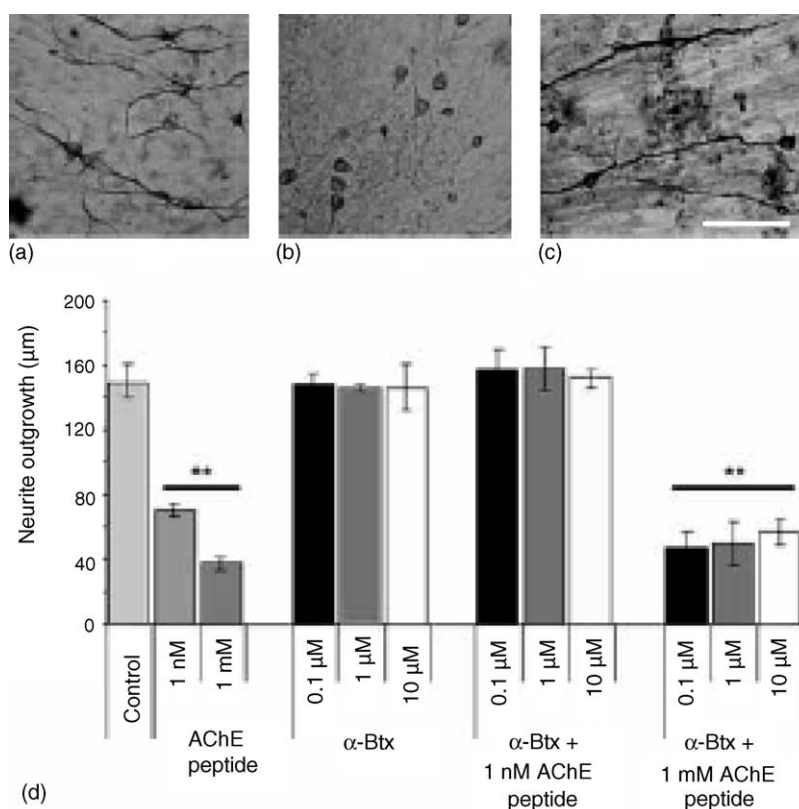

Fig. 5. The effect of nicotinic ACh receptor antagonism on in vitro AChE–peptide induced toxicity in rat hippocampal organotypic cultures. Cultures were maintained in serum-free medium for 14 days and then processed for MAP-2 immunochemistry. Representative photomicrographs of organotypic hippocampal cultures: (a) control; (b) treated with 1 nm AChE–peptide for 14 days; and (c) treated with 1 nm AChE–peptide and 10 μm α-BgTx for 14 days (scale bar = 50 μm). (d) Quantification of effects of AChE–peptide and antagonism by α-BgTx (10 μm). Neurite outgrowth was measured by selected cells in a non-baised manner and using camera Lucida drawings. Experiments were repeated a minimum of three times with separate culture groups; $n = 131$–134; (**) $p < 0.01$, from [15].

3. The receptor: the α-7 nicotinic acetylcholine receptor

The α-7 receptor is a potent calcium ionophore pivotal in neural development [3,23] and neurodegeneration [20]. Moreover, there is a close correspondence during development between the transient appearance of AChE in certain brain regions, with a parallel and similarly transient expression of mRNA for the alpha-7 receptor [3]. In three different in vitro preparations, 'AChE–peptide' appears to be bioactive at this receptor in a ligand-specific and concentration-dependent manner [15]: first, it modulates the effect of acetylcholine on *Xenopus* oocytes transfected with human $\alpha7$, but not $\alpha4/\beta2$, nAChR (Fig. 2). Secondly, in organotypic cultures of rat hippocampus, chronic peptide exposure attenuates neurite outgrowth: this effect is selectively blocked by the α7 nAChR antagonists, α-bungarotoxin (α-BgTx) and methyllycaconitine (Fig. 3). Thirdly, in recordings from CA1 neurons in guinea-pig hippocampal slices, it modulates synaptic plasticity in a α-BgTx sensitive manner (Fig. 4). Both a scrambled variant, and the analogous peptide from butyrylcholinesterase, are ineffective in all three paradigms. Hence, we have concluded [15] that AChE–peptide binds at an allosteric modulatory site on the α-7 nictonic receptor.

This idea is currently being confirmed more directly by binding studies on washed membranes from mouse brain. Using the Rapid Filtration Binding Technique we have obtained pilot data that the non-cholinergic action of AChE is operating by specific interaction of this peptide sequence with high affinity binding sites in the mammalian brain. Critically, this binding is displaced by alpha-BgTx, whilst conversely unlabelled peptide displaces the radiolabelled toxin (Fig. 5).

4. The mechanism: a trophic–toxic axis

Once AChE–peptide has modulated the alpha-7 receptor to cause more calcium to enter the neuron, there will be a cascade of consequences. Whilst in the physiological situation of a developing neuron, appropriate processes will be set in train for cell growth and stabilization, in the mature neuron, the calcium influx will increase still further due to activation of voltage-dependent calcium channels and indeed to fulfilling the voltage requirement for activation of the NMDA receptor: we know that these additional channels are activated as the toxic effects of AChE–peptide are sensitive to selective antagonism of these two further types of calcium channel. Moreover, it is possible to continue to dissect pharmacologically the subsequent sequence of
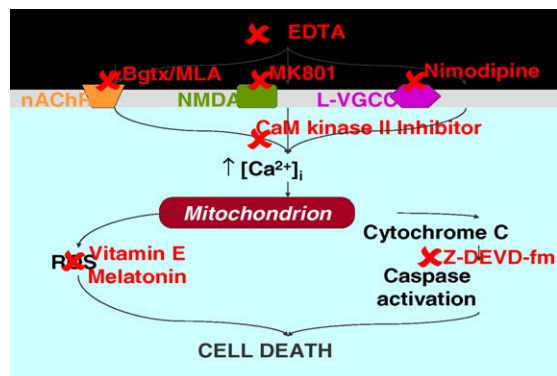

Fig. 6. Schematic representation of a potential biochemical pathway for cell death induced by AChE–peptide. Activation of α-7 nAChR induced membrane depolarization leading to the activation of the NMDA receptor. Excessive activation of NMDA receptor (NMDA) induces massive calcium influx and abnormal elevations in intracellular calcium ($[Ca^{2+}]_i$). This in turn causes a decrease in ATP synthesis and the opening of the mitochondrial permeation pore. Mitochondrial dysfunction elicits a further reduction in intracellular ATP pools, an increase in free radical generation, cytochrome e release followed by caspase-3 activation, and ultimately cell death via apoptosis, X inhibition, L-VGCC L-type voltage-gated calcium channel, extracellular calcium $[Ca^{2+}]_e$, MK-801 dizocilpine, αBgtx α-bungarotoxin, NLA methyllycaconitine, CaM calmodulin, ROS reactive oxygen species. Crosses show the identity and site of the activity of various agents that can block the effect, from [8,9,15].

events (Fig. 6), to see how initial enhanced activation of the alpha-7 receptor by AChE–peptide might result in toxicity when the original trophic action occurred out of context, either because the exposure time to the peptide was prolonged and/or the levels of peptide were too high and/or the neurons were unable to tolerate the levels of calcium readily used in developing neurons.

5. The applications: a novel surrogate marker?

One interesting application of this work, is that AChE–peptide, or its analogue, could be used as a 'surrogate marker' to monitor the course of neurodegeneration in each individual patient. Such a marker is currently much sought after since it would have three major benefits: firstly, if the individual and their carers knew their particular detailed future over the forthcoming years, for example, how long before being in a wheel chair, or needing specialised care, then they could plan their lives more effectively; secondly, if the test could reveal those in the early stage of the disease before the symptoms were apparent, then current, palliative medication could be started earlier and the short-term prognosis improved; thirdly, if such a marker ensured that the patient could effectively be their own 'control' by comparing their
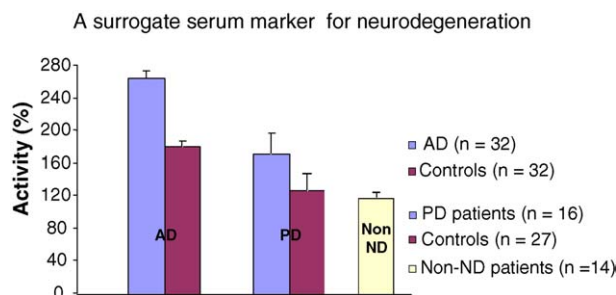
Fig. 7. Preliminary data showing elevated activity using novel assay in samples taken from Alzheimer patients, compared to match controls. In the smaller sample of patients with Parkinson-like disorder and neurodegeneration again, there is a tendency to increase compared to controls, as well as patients presenting with non-neurodegenerative neurological disorders.

scores on the assay each month, then the costs and timing of clinical trials would be dramatically reduced, and the costs of developing eventual new therapies concomitantly improved.

According to our hypothesis [14], in patients suffering from AD, PD and indeed MND, there will be elevated levels of AChE–peptide. The first step has therefore been to develop an assay for AChE–peptide. Using a novel assay detection system we have observed that indeed, in blood samples taken from patients with probable and conformed MND from the clinic of Professor Chris Shaw, that levels of activity were indeed significantly greater with respect to controls [16]. Meanwhile, preliminary data from much smaller groups of AD and PD patients, so far, show a similar trend from other neurological, but non-neurodegenerative conditions and, indeed, controls (Fig. 7).

Fig. 7 shows the effect of serum or plasma from patients with Parkinson's disease on the activity of G_1-AChE in comparison to disease and non-disease controls.

6. Conclusions

A novel strategy for combating neurodegeneration, would be to exploit two parallel approaches. (1) *A stabilising medication that arrests further loss of brain cells*. Although in itself not presenting a cure, if such medication was given in the early stages of neurodegeneration, it would be a huge improvement over the current prospects that a patient faces. If the model suggested here proves valid, i.e. inappropriate modulation of the alpha-7 allosteric site by AChE–peptide, then it could be adopted as a novel target for developing new drugs that activated only the AChE–peptide binding site, and thus still allowed physiological amounts of calcium

into the cell. Moreover, if indeed it is this system that is pivotal to triggering neurodegeneration, then side-effects would be minimal as only discrete sites in the brain would be affected: indeed it could mean that neuron loss itself was actually arrested. However, were there to be (2) *a rapid, low-cost, and relatively painless screen* available to anyone over the age of 60, or at particular risk of neurodegeneration, then any individual identified as presymptomatic, could be started immediately on this stabilising therapy. The data reported here suggests a means for developing just such a screen for neurodegeneration.

Although the model described here based on a peptide derived from AChE is far from being a magic bullet 'cure' that would reverse all the ravages of even early stage neurodegeneration, the combined exploitation in both (1) and (2) (above) of this novel signaling system could lead to a much better treatment for PD, AD and MND: by identifying cell death before symptoms onset, and blocking further neuronal loss, ideally *the symptoms would then never appear*.

Acknowledgements

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