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(II) Physiological profiling of an endogenous peptide in the basal forebrain: Age-related bioactivity and blockade with a novel modulator

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ABSTRACT

Previous studies have suggested that neurodegeneration is an aberrant form of development, mediated by a novel peptide from the C-terminus of acetylcholinesterase (AChE). Using voltage-sensitive dye imaging we have investigated the effects of a synthetic version of this peptide in the *in vitro* rat basal forebrain, a key site of degeneration in Alzheimer's disease. The brain slice preparation enables direct visualisation in real-time of sub-second meso-scale neuronal coalitions ('Neuronal Assemblies') that serve as a powerful index of brain functional activity. Here we show that (1) assemblies are site-specific in their activity profile with the cortex displaying a significantly more extensive network activity than the sub-cortical basal forebrain; (2) there is an age-dependency, in both cortical and sub-cortical sites, with the younger brain (p14 rats) exhibiting more conspicuous assemblies over space and time compared to their older counterparts (p35-40 rats). (3) AChE-derived peptide significantly modulates the dynamics of neuronal assemblies in the basal forebrain of the p14 rat with the degree of modulation negatively correlated with age, (4) the differential in assembly size with age parallels the level of endogenous peptide in the brain, which also declines with maturity, and (5) this effect is completely reversed by a cyclised variant of AChE-peptide, 'NBP14'. These observations are attributed to an enhanced calcium entry that, according to developmental stage, could be either trophic or toxic, and as such may provide insight into the basic neurodegenerative process as well as an eventual therapeutic intervention.

1. Introduction

A peptide derived from the C-terminus of acetylcholinesterase (AChE) has a bioactivity independent of cholinergic transmission: it enhances calcium entry via an allosteric site on alpha-7 acetylcholine receptors (α_7-nAChR) (Greenfield, 2013), which in turn results in trophic-toxic effects in organotypic cell cultures (Day and Greenfield, 2004) and neuronal cell lines (Garcia-Rates et al., 2013), longer term proliferation of α_7-nAChRs (Bond et al., 2009), and enhancement of calcium potentials in mammalian brain slices (Bon and Greenfield, 2003), leading to enhanced long-term potentiation (Greenfield et al., 2004). Moreover, the trophic action of AChE-peptide can also be observed at the more complex level of 'meso-scale' brain operations, where it modulates sub-second, large-scale neuronal coalitions ('neuronal assemblies') (Gerstein et al., 1989) in the cortex via α_7-nAChRs (Badin et al., 2013), thereby having an eventual effect on plasticity via calcium influx (Broide and Leslie, 1999).

One suggestion for the underlying mechanism of neurodegeneration is an aberrantly activated form of development, triggered by levels of calcium influx inappropriate in the context of the more mature brain (Greenfield, 2013). Accordingly, the initial goal of this study was to see whether neuronal assemblies could be evoked in the basal forebrain, a key site of degeneration in Alzheimer's disease (Auld et al., 2002; Coyle et al., 1983), and if so,

Abbreviations: AChE, acetylcholinesterase; nAChR, nicotinic acetylcholine receptor; AI, prefrontal agranular insular cortex; AID, dorsal agranular insular cortex; AIV, ventral agranular insular cortex; aCSF, artificial cerebrospinal fluid; Aβ, amyloid Beta; BF, basal forebrain; Di-4-ANEPPS, 4-(2-[6-(Dibutylamino)-2-naphthalenyl] ethenyl)-1-(3-sulfopropyl)pyridinium inner salt; ELISA, enzyme-linked immunesorbent assay; fEPSP, excitatory post-synaptic potentials as recorded using field potentials; HDB, horizontal limb diagonal band; ISI, inter-stimulus interval; LO, lateral orbital frontal cortex; MS, medial septal nucleus; MWCO, molecular weight cut-off; NO, nitric oxide; PBS, phosphate buffer saline; ROI, region of interest; S1BF, primary somatosensory cortex; SEM, standard error of the mean; VDB, vertical limb diagonal band; VSDI, voltage-sensitive dye imaging.

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whether any effects of the peptide might be age-dependent. We therefore investigated the possible modulatory effects of a synthetic version of AChE-peptide as a function of both brain-region and age using optical imaging with voltage-sensitive dyes, which enable a real-time visualisation of neuronal dynamics (Tominaga et al., 2000), and extracellular electrophysiology (field potentials).

Whilst the active sequence of AChE-peptide can be attributed to a specific 14 amino acid sequence originating from the C-terminus tail of AChE (T14) (Greenfield, 2013), exogenous AChE-peptide treatment in investigations from our lab has more recently involved a 30 amino acid peptide (T30) which includes the active T14 motif: the larger T30 is less likely to form fibrils when in solution, thereby possessing a higher stability and greater efficacy than T14 (Bond et al., 2009). Hence the T30 peptide was used throughout this study.

Given that the proposed mechanism of peptide-induced excitotoxicity would be of great relevance to furthering the development of effective drugs for halting the neurodegenerative process, we also tested whether a molecular variant would act as an antagonist, i.e. a cyclised variant of the active motif of AChE-peptide: 'NBP14' (Greenfield, 2015). The practice of cyclisation of compounds to improve their stability is not new (Goodwin et al., 2012) and has been exploited previously as a therapeutic strategy (Haworth et al., 1999; Hess et al., 2008). However, to the best of our knowledge, cyclisation of a peptide to act as a blocker of its endogenous, linear counterpart in relation to neurodegenerative disorders is unprecedented.

In rat brain slices, effects of exogenous peptide (T30) on assembly dynamics in basal forebrain were investigated and contrasted with those in the cortex and at different ages (p14 and p35-40), and compared with levels of endogenous AChE-peptide. Any effects of the peptide, which may contribute to the processes of neurodegeneration, were then tested for blockade by a cyclised variant of the AChE-peptide, NBP14.

2. Materials and methods

2.1. Preparation of rat brain homogenates for measuring AChE-peptide levels

The samples were prepared as follows: the brain sample was weighed before being placed in a dounce (tissue grinder) and 1 μL of PBS was added per 1 mg of brain material. The brain sample was homogenised by using the "loose" plunger. The "tight" plunger was used to further homogenise the material. The homogenised sample was collected in 2 mL Eppendorfs and centrifuged for 15 min at 13,000 g, 4 °C. Once the centrifugation was finished, the supernatant was collected into 0.5 mL 30 KDa MWCO filters. The samples were filtered by centrifuging through the filter for 30 min at 13,000 g, 4 °C and protease inhibitor cocktail (Roche complete PIC 04693116001) was added to the filtrate. This was used for the ELISA for T14 peptide. Unfiltered rat brain homogenate supernatant was used to determine the concentration of protein of the initial sample using the Pierce assay and subsequently ELISA determination using T14 antibody as described in (Garcia-Rates S., Morrill P., Pottiez G., Badin A-S., Tormo-Garcia C., Heffner C. and Greenfield SA. (I) Pharmacological profiling of a novel modulator of the α7 nicotinic receptor: blockade of a toxic peptide increased in Alzheimer brain.).

2.2. Preparation of brain slices and ex-vivo recordings

Coronal rat brain slices were prepared according to the procedure described in (Badin et al., 2013) but this time containing either agranular insular cortex, primary somatosensory cortex or basal forebrain (+0.70 and −0.26 millimetres (mm) from bregma (Paxinos and Watson, 1998)). Extracellular electrophysiology (field potentials) and optical imaging using voltage-sensitive dyes was then performed as previously described (Badin et al., 2013).

2.3. Preparation of exogenous peptides

Acetylcholinesterase (AChE) C-terminus 30 amino acid peptide (T30; sequence: 'N' – KAEFHRWSSYMVHWKNQFDHYSKQDRCSDL), the 15 amino acid inert part of the T30 (T15; sequence: 'N' – NQFDHYSKQDRCSDL) as well as the cyclic version of the active 14 amino acid region of T30 (NBP14; sequence: c[AEFHRWSSYMVHWK]; 'c[]' = cyclic, N-terminal to C-terminal lactam) were all prepared as described in (Garcia-Rates S., Morrill P., Pottiez G., Badin A-S., Tormo-Garcia C., Heffner C. and Greenfield SA. (I) Pharmacological profiling of a novel modulator of the α7 nicotinic receptor: blockade of a toxic peptide increased in Alzheimer brain.). When starting an experiment, aliquots were thawed and added to 'recording' aCSF as necessary, which was then perfused into the bath at a constant rate of 2.0 mL/min using a Minipulse 3 peristaltic pump (Gilson Scientific Ltd., Bedfordshire, UK). Perfusion conditions were 25 min in duration.

In the present investigation, the term 'AChE-peptide' refers to any peptide derived from the C-terminus of AChE and which has bioactivity at α7 nicotinic acetylecholine receptors, 2 different preparations of this peptide have previously been used: a 14 amino acid version (T14) (Badin et al., 2013) and a 30 amino acid version (T30, used here). Even though these two peptides act via the same mechanism of action, the T14 peptide has been found to come out of solution and create fibrils, hence why the T30 is preferred in the present investigation (Bond et al., 2009).

2.4. Data analysis and statistics

Experiments were carried out with simultaneous extracellular electrophysiology (field potentials, fEPSP) and VSDI monitoring of evoked population activity (See Fig. 1D i). Field potential records produced 53 data frames (800 ms in length each) per perfusion condition (25 min total – 10 min of acclimatisation followed by 15 min of recording; 28 s ISI). Electrical stimulations elicited a large artefact which lasted 2–3 ms immediately after stimulation, whilst the fEPSP population activity trace deflection peak occurred 5–6 ms after initial stimulation. Field potentials data from each experiment were excluded only if 1) the magnitude of recorded fEPSP signal was too small (maximum deflection peak <2 standard deviations from baseline level) equating to a low signal-to-noise ratio for that particular experiment, or if 2) the fEPSP deflection was not easily discernible from the stimulation artefact, making it unclear where the stimulation artefact finished and where the population activity fEPSP deflection started. For each individual experiment, of the 53 data frames recorded per perfusion epoch (raw data), every 4 frames were averaged together to give a total of 13 'mean' frames per condition, using MatLab (v8.2.0.701; The Mathworks Inc., USA). The maximum point, whether due to a positive or negative deflection, between 4 and 7 ms was measured for every 'mean' trace and plotted as a trend graph on axes of response amplitude (y-axis) vs mean frame number (x-axis), as shown in Fig. 7.

VSDI data were recorded in 4 × 4 mm 2-dimensional images, equivalent to 100 × 100 pixels – each pixel being 40 × 40 micrometres (μm), from which critical data were extracted. As opposed to electrophysiology, VSDI data was not gathered throughout the experimental run, but in fact was recorded in discrete periods of time 15 min long (recording epochs, see Fig. 7). The ISI between stimulations was the same as for electrophysiology (28 s) and therefore, every recording epoch consisted of 32 successive