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High-resolution spatio-temporal bioactivity of a novel peptide revealed by optical imaging in rat orbitofrontal cortex *in vitro*: Possible implications for neurodegenerative diseases

Antoine-Scott Badin*, John Eraifej, Susan Greenfield

Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, United Kingdom

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ABSTRACT

Acetylcholinesterase (AChE) is now well known to have a secondary, non-enzymatic function independent of cholinergic transmission. In the last decade, the part of the molecule responsible for this action has been identified, i.e. a 14 amino acid peptide fragment ('T14'), deriving from the C-terminus of AChE: this peptide has been shown to be bioactive in a range of preparations and to act at an allosteric site on α_7 nicotinic acetylcholine receptors (α_7-nAChRs). Of particular significance is the finding that AChE-related peptides trigger calcium-induced neurotoxicity that may be pivotal in the process of neurodegenerative diseases, such as Alzheimer's. However to date all studies have been performed on isolated cell preparations. The aim of this study was therefore to characterise the bioactivity of T14 on meso-scale *in vitro* cortical networks ('neuronal assemblies') from rat brain slices containing orbitofrontal cortex. Local field potential (LFP) recordings showed that the T14 peptide has a selective, holistic action on cortical networks in a modulatory biphasic manner i.e. predisposing excitation at concentrations of up to 1 μM, after which the trend is reversed in favour of inhibition at higher doses (>1 μM). By contrast, a scrambled variant of the T14 peptide sequence (S14), showed no significant changes in neuronal activation. Optical imaging using voltage-sensitive dyes (VSDI) corroborated the electrophysiological findings and also provided further insight into the spatial dynamics of the effects of the peptide: T14 application had a facilitatory effect i.e. increased the time-course of activation at sub-micromolar concentrations only (700 nM) without significantly affecting the spread of evoked assemblies. Moreover: co-applying T14 with the α_7-nAChR competitive antagonist methyllycaconitine (MLA) produced inhibition in activation synchrony not seen with either agent on their own, suggesting an additive inhibitory effect. In conclusion, the T14 peptide derived from AChE produced a dose-dependent biphasic modulation of cortical networks activity dependent on the α_7-nAChR: these findings should thus provide a more comprehensive insight into the immediate actions of a novel bioactive agent of high potential relevance to neurodegenerative disorders such as Alzheimer's disease.

1. Introduction

Over the past three decades, there have been no effective new treatments for neurodegenerative diseases, notably Alzheimer's disease (AD). Drugs traditionally used to tackle the symptoms of AD include AChE inhibitors such as Galanthamine and Tacrine as well as NMDA receptor antagonists such as Memantine (Pohanka, 2011): however such treatments are widely reported to have limited clinically efficiency (Scarpini et al., 2003). This lack of progress is most likely to be due to the lack of understanding of the basic mechanisms that underpin these diseases. One possibility may be that neurodegeneration is an aberrant form of development, with the pivotal signalling molecule being a 14 amino acid peptide derived from the C-terminus of AChE identified as the salient fragment for the non-cholinergic function of AChE (Greenfield and Vaux, 2002; Greenfield et al., 2008). AChE, and hence this peptide ('T14'), is expressed in all central nervous system (CNS) neurons prone to neurodegeneration, independent of cholinergic transmission: it has already been shown to be bioactive in a range of preparations (Bon and Greenfield, 2003; Day and Greenfield, 2004; Zbarsky et al., 2004). Previous research has indeed found that T14 application induces a dose-dependent calcium entry via α_7-nicotinic acetylcholine receptors (α_7-nAChRs) (Greenfield et al., 2004, 2008; Webb et al., 1996). Indeed, it has been established

* Corresponding author. Tel.: +44 01865 271626.
E-mail address: scott.badin@seh.ox.ac.uk (A.-S. Badin).

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that, independent of any cholinergic mechanisms, T14 modulates the gating dynamics of α_7-nAChRs, allowing greater Ca^{2+} currents to enter neurons. Thereby presenting a new therapeutic possibility: intercepting the actions of C-terminus AChE peptides at α_7-nAChRs before sufficient neurodegeneration has taken place to trigger the emergence of symptoms.

Although two early electrophysiological studies suggested a bioactivity of the peptide (Bon and Greenfield, 2003; Greenfield et al., 2004), most of the more recent subsequent research has been undertaken in cell cultures (Bond and Greenfield, 2007; Bond et al., 2006; Onganer et al., 2006) and tissue cultures (Day and Greenfield, 2004; Emmett and Greenfield, 2004; Zbarsky et al., 2004). Moreover, an additional drawback of these approaches has been the protracted time course of the effect studied. In order to study the bioactivity of the peptide therefore on a more physiological spatial and temporal scale, i.e. with a resolution of micrometres (μm) and milliseconds (ms), the aim of this study was to monitor the immediate effects of the peptide on integral cortical networks, induced by T14 application. Such an optical imaging approach has already been shown to provide unparallelled comprehensive data about the dynamics of cortical networks in relation to bioactive compounds (Collins et al., 2007; Devonshire et al., 2010a, 2010b).

This study, however, is the first of its kind to utilise combined techniques of electrophysiology and VSDI to investigate the acute effect of T14 on cortical networks: the more established and familiar technique of field potentials provide a frame of reference for comparing the observations with optical imaging. In both types of experiments the effects of T14 were compared with those of a scrambled variant (S14) and also tested in the presence of a competitive α_7-nAChR antagonist, methyllycaconitine (MLA).

2. Methods

2.1. Brain slice preparation

Male Wistar rats (35–40 day old) were anaesthetised using isoflurane: 15 mL 100% w/w isoflurane was applied to the cotton bed at the bottom of an anaesthesia chamber (glass box 20 × 15 × 15 cm) where rats were then placed for ~45 s until anaesthesia was complete. The hind paw of each anaesthetised rat was pinched to check for appropriate depth of anaesthesia. Once the anaesthesia was confirmed, rats were quickly decapitated before immersing the brain in oxygenated ice-cold artificial cerebrospinal fluid (aCSF in mmol: 120 NaCl, 5 KCl, 20 $NaHCO_3$, 2.4 $CaCl_2$, 2 $MgSO_4$, 1.2 KH_2PO_4, 10 glucose, 6.7 HEPES salt and 3.3 HEPES acid; pH: 7.1) for 4 min, the time taken to cut the brain into slices. Coronal slices (400 μm thick) were cut from a block of brain containing prefrontal cortex (PFC; slices were taken between +12.20 and +10.70 mm Interaural and +3.20 and +1.70 mm Bregma) using a Vibratome (Leica VT1000S) and transferred to a bubbler pot containing aCSF at room temperature ('recording' aCSF in mmol: 124 NaCl, 3.7 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgSO_4$, 1.3 KH_2PO_4 and 10 glucose; pH: 7.1), which was identical to that which was used during electrophysiological recordings and VSDI. Slices were left in oxygenated (95% O_2–5% CO_2) 'recording' aCSF to recuperate for at least 2 h before preparing them for electrophysiology, and 1 h before VSD staining.

2.2. Drug preparation & application

Acetylcholinesterase (AChE) C-terminus 14 amino acid peptide (T14; sequence: 'N' – AEFHRWSSYMVHWK) was custom synthesised and purchased from Genosphere Biotechnologies (Paris, France) at >99% purity, while the scrambled variant (S14; sequence: 'N' – HSWRAEVFHKYWSM), used as a positive control in this study, was custom synthesised and purchased from AnaSpec (San Jose, CA, USA) at >90% purity. All peptides were synthesised using the fmoc method, purified with HPLC and analysed using mass spectrometry. Methyllycaconitine citrate salt hydrate (MLA; Sigma–Aldrich, Saint Louis, MO, USA), the selective α_7-nAChR antagonist, was purchased from Sigma–Aldrich. All drug stock solutions were prepared prior to the start of experiments in frozen aliquots. For production of drug perfusion solutions, stock solution aliquots were thawed and added to 'recording' aCSF as appropriate and bath applied at a constant rate of 2.5 mL per min perfusion using a Minipulse 3 pump (Gilson Scientific Ltd, Bedfordshire, UK). Perfusion conditions were 32 min in duration (35 averaged frames per perfusion condition, Fig. 1C and D).

2.3. Electrophysiology

Field potential recordings (fEPSP) were acquired using GC150F-10 glass capillaries (Harvard Apparatus, Kent, UK) recording electrodes filled with 2 M NaCl solution containing 2% Pontamine sky blue 5BX dye (BDH Chemicals Ltd., Poole, England) for the electrode to be visible under the 4.5× magnification microscope (Micro Instruments Ltd, Oxford, UK). Layer I–II/III were identified with respect to distance from the pial surface and position within the ventral part of the dorsal agranular insular (AId_2) cortex, Layer V–VIa were accepted to be 500–750 μm from the position of the stimulating electrode, deep within the cortex (Van Eden and Uylings, 1985). Concentric bipolar stimulating electrodes (Frederick Haer & Co., ME, USA), with impedance (measured at 1000 Hz): 500 kΩ, were positioned within Layer II/III (since that is approximately where thalamo-cortical afferents project to stimulate cortical networks). Recording electrodes, on the other hand, were placed within Layer V–VIa, approximately 600 μm from the stimulating electrode in accordance with cytoarchitectonic studies performed in the rat PFC (Van De Werd and Uylings, 2008) in order to record the transduced, integrated neuronal response after 4–5 ms following initial stimulation; as a result, fEPSP traces showed response deflections nicely segregated from their respective stimulation artefact.

fEPSPs were amplified using an IR-283 Amplifier (Neuro Data Instruments Corp., PA, USA) coupled to a Micro 1401 mk II acquisition system (CED Ltd, Cambridge, UK) and displayed in Signal software (CED Ltd, Cambridge, UK) then saved onto the disk for off-line analysis. The IR-281 Amp was used to amplify field potential signals 1000×, so all fEPSP graphs are shown with units $\times 10^{-3}$ V. Single stimulations (14 s inter-stimulus interval – ISI) were applied to Layer II/III and the evoked responses recorded from Layer V–VIa pyramidal cell bodies with perfusion solutions being held at 30 ± 1.5 °C by a TC-202A Temperature Controller (Digitimer Research Instruments, Hertfordshire, UK) (Van De Werd and Uylings, 2008).

2.4. VSD setup

Slices were placed in a dark, high humidity chamber filled with aCSF bubbling with 95% O_2–5% CO_2. Once there, the dye solution (4% 0.2 mM styryl dye pyridinium 4-[2-[6-(dibutylamino)-2-naphthalenyl]-ethenyl]-1-(3-sulfopropyl)hydroxide (Di-4-ANEPPS, Invitrogen, Paisley, UK) (Tominaga et al., 2000) in aCSF 48%, fetal bovine serum 48%, DMSO 3.5% and cremophore EL 0.4%) was applied to the slices for 20–25 min before being transferred to an aCSF bubbler pot (room temperature, 22 ± 1.5 °C) for 1 h to wash off excess dye and recover. When starting VSD recordings, slices were placed in the recording bath on a small piece of filter paper to keep slice alive and was weighed down appropriately using a home-made plastic grid placed atop the slice. Stimulating electrodes were placed in the same location as in electrophysiology recordings (Layer II/III, ~80 μm from pial surface) with the same ISI and temperature control. For acquiring of VSD data, 16-bit images were recorded with 1 ms resolution with a digital camera (Brain Vision MiCAM Ultima R3-V20 Master) with Ultima 2004/08 imaging software (Brain Vision) coupled to Spike 2 V6.0 (CED Ltd, Cambridge, UK) which was used to trigger stimulations with respect to appropriate ISI. Light was generated using an Osram halogen xenophot 64634 HLX EFR Display/Optic lamp and was filtered to emit green (530 ± 10 nm) light using a MHF-G150LR (Moritex Corporation) coupled to MiCAM Ultima ultra-fast imaging system and filtered the emitted fluorescence through a >590 nm high-pass filter as described previously (Collins et al., 2007; Devonshire et al., 2010a, 2010b).

2.5. Data analysis and statistics

Electrophysiology experiments produced a total of 140 data traces (frames) per drug condition (32 min, 14 s ISI). Stimulations produced a large artefact which was quite distinct and separate from the region of interest of the response trace (see Supplemental Fig. 1). Using MatLab (v7.9.0.529; The MathWorks, Inc., USA), every 4 frames were averaged together to produce a total of 35 averaged frames per drug condition. The lowest point of the negative trace deflection between 4 and 7 ms after stimulation was measured for every averaged trace and plotted on axis of 'Response Amplitude' (y) vs 'Averaged Frames' (x) to produce the final electrophysiology trend graphs seen in Fig. 1 (see Supplemental Fig. 1 for methods).

VSDI produced 4 × 4 mm (100 × 100 pixels) 2-dimensional images from which critical data were extracted such as the time-course of activation and intensity of the overall elicited signal. For each VSDI experiment, each snapshot's data between 2 and 15 ms after stimulation, encapsulating the peak response (see Supplemental Fig. 1), had their parameters measured and averaged for each drug condition (total of 76 snapshots per drug condition). Such data were then compiled to produce detailed, quantitative, graphs of the extent of activation intensity as well as qualitative 'space-time' maps allowing to measure the effects of drug treatment on the spatio-temporal activation patterns of elicited neuronal assembly activation. For each experiment, the averaged data of each perfusion epoch were plotted in order to gain better knowledge of the extent to which activation intensity and time-course were affected by drug treatment. All statistical tests (paired bidirectional Analysis of Variance – ANOVA) were performed using Mathematica 8 (Wolfram Research, USA). For all statistical tests $P < 0.05$ was considered significant; data are expressed as mean ± S.E.M.