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Selective enhancement of the activity of C-terminally truncated, but not intact, acetylcholinesterase

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Abstract

Acetylcholinesterase (AChE) is one of the fastest enzymes approaching the catalytic limit of enzyme activity. The enzyme is involved in the terminal breakdown of the neurotransmitter acetylcholine, but non-enzymatic roles have also been described for the entire AChE molecule and its isolated C-terminal sequences. These non-cholinergic functions have been attributed to both the developmental and degenerative situation: the major form of AChE present in these conditions is monomeric. Moreover, AChE has been shown to lose its typical characteristic of substrate inhibition in both development and degeneration. This study characterizes a form of AChE truncated after amino acid 548 (T548-AChE), whose truncation site is homologue to that of a physiological form of T-AChE detected in fetal bovine serum that has lost its C-terminal moiety supposedly due to proteolytic cleavage. Peptide sequences covered by this C-terminal sequence have been shown to be crucially involved in both developmental and degenerative mechanisms *in vitro*. Numerous studies have addressed the structure–function relationship of the AChE C-terminus with T548-AChE representing one of the most frequently studied forms of truncated AChE. In this study, we provide new insight into the understanding of the functional characteristics that T548-AChE acquires in solution: T548-AChE is incubated with agents of varying net charge and molecular weight. Together with kinetic studies and an analysis of different molecular forms and aggregation states of T548-AChE, we show that the enzymatic activity of T548-AChE, an enzyme verging at its catalytic limit is, nonetheless, apparently enhanced by up to 800%. We demonstrate, first, how the activity of T548-AChE can be enhanced through agents that contain highly positive charged moieties. Moreover, the un-competitive mechanism of activity enhancement most likely involves the peripheral anionic site of AChE that is reflected in delayed substrate inhibition being observed for activity enhanced T548-AChE. The data provides evidence towards a mechanistic and functional link between the form of AChE unique to both development and degeneration and a C-terminal peptide of T-AChE acting under those conditions.

Keywords: C-terminal peptide, degeneration, development, enzyme activity enhancement, monomeric acetylcholinesterase, substrate inhibition.

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The primary role of acetylcholinesterase (AChE) is to terminate neurotransmission by fast hydrolysis of its substrate acetylcholine (ACh) at cholinergic synapses and neuromuscular junctions (Massoulie *et al.* 1993a,b; Taylor and Radic 1994). However, it has long been acknowledged that AChE also displays a large set of non-catalytical functions (Greenfield 1984; Small *et al.* 1996; Soreq and Seidman 2001). These non-enzymatic functions include, amongst others, roles in neuritogenesis and synaptogenesis (Soreq and Seidman 2001; Paraoanu *et al.* 2006). More recently, these functions have also been attributed to isolated fragments of the AChE C-terminus (Grisaru *et al.* 2001; Bon and Greenfield 2003; Day and Greenfield 2003; Pick *et al.* 2006).

The AChE gene contains six exons with three of these exons (E2, E3, and E4) encoding for the core polypeptide consisting of 543 amino acids (aa) that is common to all different enzyme variants with exon E1 being non-coding (Soreq and Seidman 2001). The major difference in molecular structure between these distinct AChE splice variants

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Abbreviations used: aa, amino acid; ACh, acetylcholine; AChE, acetylcholinesterase; ATC, acetylthiocholine; MW, molecular weight; PAS, peripheral anionic site; R-AChE, readthrough form of AChE; T548-AChE, T-AChE truncated at aa 548; T-AChE, tailed form of AChE.

can be ascribed to the C-terminus of the molecule. In particular, the tailed form of AChE (T-AChE) results from splicing of E4 through to E6 with the translation of this mRNA leading to the C-terminal extension of the core by a 40 aa residue peptide that contains a cysteine (Cys580) that has been postulated to favor dimerization (Massoulie *et al.* 2005). On the other hand, splicing from E4 through Intron 4 to E5 gives rise to the extension of the AChE C-terminus over the common core of only 26 aa that lack cysteine (Li *et al.* 1991), thus, suggesting that the so-called readthrough AChE (R-AChE) is destined to remain monomeric. While the form of AChE predominantly found in the central nervous system is T-AChE (Fernandez *et al.* 1996), other forms of AChE come into play in dysfunction, e.g., R-AChE is crucially involved in stress responses [see for review, Pick *et al.* (2006)].

Based on the suggestion that the C-terminal sequences of both R- and T-AChE exert non-cholinergic biological functions (Greenfield and Vaux 2002; Greenfield 2005; Dori and Soreq 2006), a vast number of studies have been undertaken to directly investigate their role (Karpel *et al.* 1994, 1996; Grisaru *et al.* 2001; Deutsch *et al.* 2002; Perry *et al.* 2002; Belbeoc'h *et al.* 2003, 2004; Bon *et al.* 2004; Massoulie *et al.* 2005; Xie *et al.* 2005; Dori and Soreq 2006; Grisaru *et al.* 2006; Pick *et al.* 2006). Similarly, T-AChE devoid of its C-terminus has been analyzed so as to further understand the functional and structural role of this carboxylic fragment for full-length T-AChE: It has been impossible to obtain a crystal structure for monomeric T-AChE because of the presence of the dimerization-favoring cysteine in the C-terminal region of T-AChE. Therefore, the sequence of T-AChE was truncated after aa 548 (hence, named T548-AChE) in order to obtain a stable monomer (Marchot *et al.* 1996) that has been used in a wide range of studies (Sternfeld *et al.* 1998; Morel *et al.* 2001; De Jaco *et al.* 2002; Kovarik *et al.* 2003; Whyte and Greenfield 2003; Kovarik *et al.* 2004; Bond *et al.* 2006; Bourne *et al.* 2006).

It is of particular importance that T548-AChE is the only form of AChE that can be used to study the purely monomeric form of T-AChE in solution: a specific monomeric AChE isoform appears to play a crucial role in neurodegeneration (Arendt *et al.* 1992) as well as development (Moreno *et al.* 1998), physiological situations where AChE apparently loses its characteristic feature of substrate inhibition. However, while intense research has been undertaken to, first, elucidate the structure of this particular form of AChE and, second, identify the mechanism underlying its loss of substrate inhibition, we still need to pinpoint this mechanism.

The aim of this study is to characterize the functions of T548-AChE in solution. We, first, investigated the sensitivity of T548-AChE activity to increasing concentrations of detergent. Second, we explored the impact of charge and molecular weight of the enhancing agents on the dimension of activity enhancement. Third, enzyme kinetics studies were undertaken to elucidate the mechanism of action of the enhancing agents on the T548-AChE molecule: we assessed if the enhancing agents act (un-)competitively on AChE, e.g., on its peripheral anionic site (PAS). Fourth, T548-AChE was assessed for substrate inhibition. Finally, different molecular forms were studied for activity enhancement.

We present evidence towards a mechanism of action of this activity enhancement that, apparently, involves up to a fivefold increase in reaction rate that is, most likely, associated with an interaction of the enhancer with the PAS of AChE. This extraordinary feature may have important implications in analytical studies (Tarrab-Hazdai *et al.* 1984; Lassiter *et al.* 2003). Furthermore, we strongly believe that the here described findings about synthetic monomeric T-AChE provide clues about the physico-chemical characteristics of the physiological monomer and the reason for its central role in development and neurodegeneration.

Materials and methods

Materials

All chemical substances including full-length T-AChE, some peptides (as indicated in Table 1), as well as buffer salts and Triton X-100 were obtained from Sigma–Aldrich (Gillingham, Dorset, UK), while most of the peptides were obtained from the American Peptide Company (Sunnyvale, CA, USA), Anachem (Luton, Bedfordshire, UK), Cambridge Biosciences (Cambridge, UK) or Genosphere (Paris, France). Nonidet NP40 was obtained from BDH Chemicals (Poole, Dorset, UK). T548-AChE was a kind gift from Palmer Taylor (Department of Pharmacology, University of California, San Diego, CA, USA). The specific activities of T548-AChE and full-length T-AChE are indicated as 2.206 U/mg (Marchot *et al.* 1996) and 349 U/mg (Sigma–Aldrich, Gillingham, Dorset, UK), respectively. For the purity of both T-AChE forms see Fig. 1.

Staining for proteins and AChE activity in gels

For Coomassie staining preparations of both full-length and T548-AChE in Laemmli-buffer (Laemmli 1970) were loaded onto 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for molecular weight (MW) separation. Coomassie staining was performed by fixing and staining for 3 h (2-propanol 25%, acetic acid 10%, Coomassie blue 0.025%). The gel was destained for 2 h changing the solution every 30 min (2-propanol 10%, acetic acid 10%). AChE activity was assessed in non-reducing gels following the method described by Koelle (1951) as modified by Chubb and colleague (Chubb and Smith 1975). Briefly, after pre-incubation in wash solution (24 g/100mL Na_2SO_4, 70 mmol/L sodium maleate, pH 6.5) the gel was incubated in substrate containing buffer [wash solution supplemented by $CuSO_4$ 4 mmol/L, glycine 20 mmol/L, $MgCl_2$ 3 mmol/L and acetylthiocholine (ATC) 4 mmol/L]. After washing in wash buffer, followed by

Table 1 Peptides used in the activity enhancement studies

No	Sequence	Name, species	MW	Net charge	s
1	ADSGEGDFLAEGGGVR	Fibrinopeptide A, human	1537	−3.5	A
2	AEFHRWSSYMVHWK	full-length T-AChE (586–599), human	1864	0.54	C
3	AGCKNFFWKTFTSC	Somatostatin, human	1640	0.99	B
4	ASHLGLAR	Anaphylatoxin C3a fragment 70–77, human	824	0.53	B
5	CGYGPKKKRKVGG		1378	4.19	A
6	QHSDRAEVFHKYHGED		1955	−2.43	D
7	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	Amyloid β 1–42, human	4514	−3.45	D
8	DDDDD		594	−5.5	A
9	DLWQK	Uremic Pentapeptide, human	689	−0.5	A
10	DSDPR	Human IgE pentapeptide HEPP, human	589	−1.5	A
11	DVVDADEYLIPQ	Epidermal growth factor receptor peptide (985–996), human	1376	−4.51	A
12	GEQRKDVYVQLYL	Thymopoietin II fragment 29–41, human	1611	−0.53	B
13	GRGDS		491	−0.5	A
14	HFRWGKPVGKKRRPVKVYP	ACTH (adrenocorticotropic hormone) (6–24), human	2336	6.46	A
15	IIGLM	β-Amyloid (31–35), human	546	−0.5	A
16	KEEAE	[Lys^{23}]-Thymosin α_1 fragment 23–27	605	−2.51	B
17	KKKKK		659	4.42	B
18	MQWNSTAFHQT	[Ala127] Hepatitis B virus pre-S Region (120–131)	1350	−0.47	A
19	PLSRTLSVAAKK		1270	2.47	B
20	PVGKKRRPVKVYPNVAENESAEAFPLEF	ACTH (adrenocorticotropic hormone) (12–39), rat	3173	0.44	A
21	QRPRLSHKGPMPF	Apelin-13, human, bovine	1551	2.51	A
22	RERMS	ß-Amyloid/A4 protein precursor (APP) (328–332), human	678	0.5	A
23	RKDVY	Thymopentin, human	680	0.5	A
24	RLRFH	b-Bag cell factor, human	728	1	A
25	RPPGF	Bradykinin (1–5), human	573	0.5	A
26	RYLPT	Proctolin, invertebrate	649	0.5	A
27	SAEEYEYPS	Cholecystokinin flanking peptide, non-sulfated	1074	−3.52	A
28	SSEVAGEGDGDSMGHEDLY	Preproenkephalin B (186–204), human	1955	−6.48	A
29	SYSMEHFRWGKPVGKKR	ACTH (adrenocorticotropic hormone) (1–17), human	2094	3.47	A
30	SYSMEHFRWGKPVGKKRRPVKVYP	ACTH (adrenocorticotropic hormone) (1–24), human	2934	5.45	A
31	TRSAW	Parathyroid hormone related peptide (107–111), human	620	0.5	A
32	TTYADFIASGRTGRRNAIHD	cAMP dependent PK inhibitor (5–24), human	2223	0.52	A
33	VPDPR		583	−0.5	A
34	WKAEFHRWSSYMVHWKNQFDHYSKQDRSSDL	[Cys611Ser] full-length T-AChE (584–614)	4000	−0.7	E
35	YGGFM	Met-Enkephalin, human	574	−0.5	A
36	YGGFMTLFKNAIIKNAYKKGE	b-Endorphin (1–5) + (16–31), human	2394	2.42	A
37	YGGFMTSEKSQTPLVTLFKNAIIKNAH	b-Endorphin (1–27), camel, bovine, ovine	2996	1.47	A
38	YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE	b-Endorphin, human	3465	2.4	A
39	YIGSR	Laminin (929–933), mouse	595	0.5	A
40	YPFPG	b-Casomorphin (1–5), bovine	580	−0.5	A
41	YRGDS	Y-R-G-D-S	597	−0.5	A
42	YRVRFLAKENVTQDAEDNC	CD36 peptide P (93–110), human	2271	−1.76	A

A number code is assigned to every peptide that is referred to in Fig. 3. This table lists the sequence, name and species of origin, molecular weight (MW) in Dalton, net charge at pH 8 and supplier source (s) of the different peptides (A, American Peptide Company; B, Sigma–Aldrich; C, Anachem; D, Cambridge Biosciences; E, Genosphere).

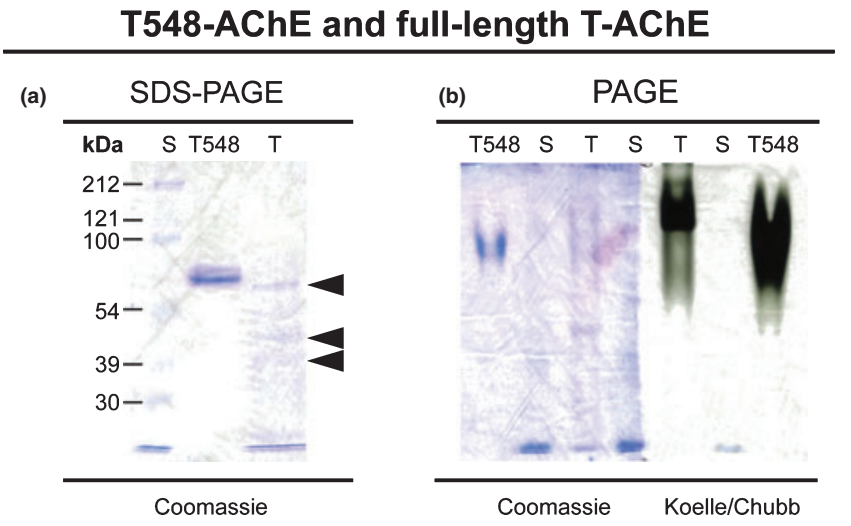


Fig. 1 Determination of purity of T548-AChE and full-length T-AChE in SDS–PAGE and non-reducing PAGE. (a) Coomassie stained 8% SDS–PAGE gel of T548-AChE (T548) and full-length T-AChE (T) to detect for proteins: a single band is obtained for T548-AChE (T548, middle lane), while three bands are detected for T-AChE (T, rightmost lane, black arrowheads). MW standards (S) were loaded in the left-most lane; the specific MWs in kDa are indicated on the left of the panel. (b) 7% PAGE gel for T548-AChE (T548) and full-length T-AChE (T). Left panel: Coomassie stain for T548-AChE (T548, leftmost lane) and full-length T-AChE (T, third lane from the left). One fairly contained band is obtained for T548-AChE, while the full-length T-AChE band is stretched out. Right panel: Koelle stain for AChE activity to detect for the protein bands from the left that correspond to AChE (Koelle 1951; Chubb and Smith 1975). Dark green signals are obtained for both full-length T-AChE (T, third lane from the right) and T548-AChE (T548, rightmost lane). MW standards (S) were loaded onto lanes 2, 4, and 6. 0.3 μg protein were loaded per lane.

pure water the gel was incubated in saturated aqueous solution of dithio-oxamide.

Determination of AChE activity enhancement

The activity enhancement of AChE was tested using the method of Ellman and colleagues with minor modifications (Ellman *et al.* 1961). Briefly, agents (i.e., peptides, detergents) were serially diluted in Ellman buffer as necessary (for specific concentrations see figures 2 and 3). Thereafter, incubation of AChE was performed: 10 μL of diluted agent and 10 μL buffer were added to 80 μL of either T548-AChE or full-length T-AChE of known activity; in parallel, a sample of 10 μL diluted agent in 90 μL Ellman buffer was prepared (to assess the activity of the agent alone) as well as a sample containing 80 μL AChE and 20 μL buffer (to assess the activity of AChE alone). 25 μL of each sample prepared were then added to a 96-well microtitre plate (Barloworld Scientific, Stone, Staffordshire, UK), in triplicate. 175 μL of Ellman buffer were then added to each well. Absorbance was read at 405 nm over a 20 min time-span using a Molecular Devices plate reader (Alpha Laboratories Ltd., Eastleigh, Hampshire, UK). Analysis was performed using the equation below to detect the change in AChE activity following incubation (inc.) with the various agents: the relative change in AChE activity (act.) was obtained using the following formula: $|[(\text{act. after inc.}) - (\text{act. before inc.})]/(\text{act. before inc.})|*100$, with 'act. after inc.' representing the activity of AChE incubated with agent, while 'act. before inc.' indicates the sum of the activities of agent and AChE prepared separately in buffer. The concentration of T548-AChE employed in the relevant experiments is 64.4 nmol/L, unless otherwise stated. Note that where activity enhancement of T548-AChE versus that of full-length T-AChE is assessed, it is referred to the same initial non-boosted activity of both forms (see figure legends 2 and 7). Otherwise, results are displayed as relative increase in activity with the activity of non-boosted T548-AChE, therefore, being equal to zero, thus, implying the statistical relevance of the data.

Determination of net-charge at pH 8

The net charge of all peptides studied was estimated using the peptide charge calculator as published on http://www.usm.maine.edu/~rhodes/Goodies/PeptChg.xls.

Sucrose density-gradient centrifugation

T548-AChE or full-length T-AChE were layered onto 5–40% sucrose gradients. Centrifugation was carried out in an ultracentrifuge with a TLA 100.4 fixed angle rotor (Beckman Coulter, Fullerton, CA, USA) for 19 h at 4°C and 100 000 *g*. After centrifugation, the material was analyzed in 80 μL fractions by carefully pipetting off the top of the gradient: the 80 μL fractions were either mixed with 20 μL Ellman buffer or an equal amount of 5% Triton X-100 (final concentration 1%) to determine the activity enhancement as described above.

Inhibition of AChE activity and enzyme kinetics measurements

Enzyme activity was measured in the absence and presence of Triton X-100 (0.5% final concentration) as a function of substrate concentration. ATC iodide was used in concentrations ranging from 0.3125 to 20 mmol/L. Lineweaver-Burk plots of the reciprocal of the rate of reaction versus the reciprocal of substrate concentration

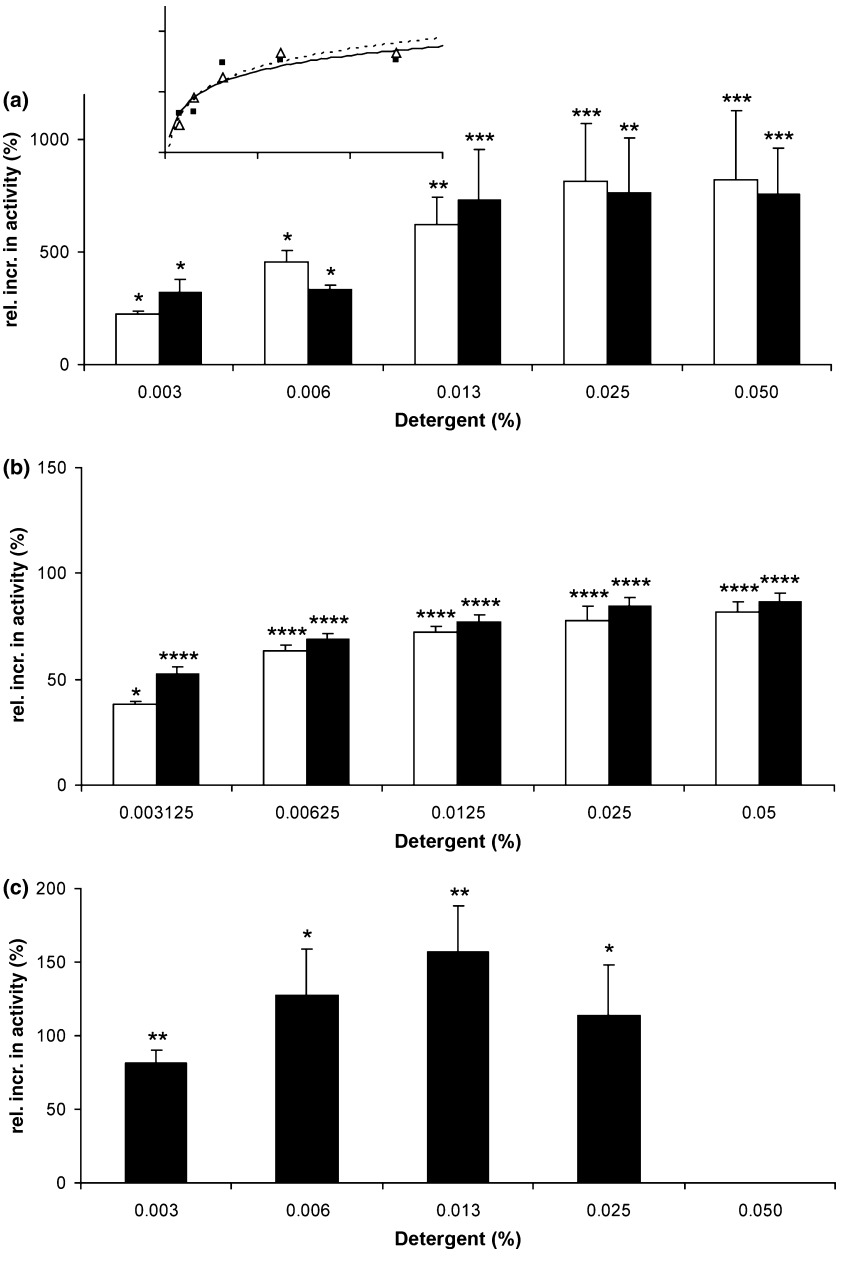


Fig. 2 Incubation of T548-AChE and full-length T-AChE with various detergents. (a) The non-ionic detergents Triton X-100 (white bars) and Nonidet NP40 (black bars) enhance T548-AChE activity up to 800%. A clear ceiling effect is observed at final detergent concentrations above 0.0013% (insert). Absolute activity of non-boosted T548-AChE: 41.55 ± 12.72 mU/mL. (B) The activity of full-length T-AChE is increased by only maximally 80% after incubation with the non-ionic detergents Triton X-100 (white bars) and Nonidet NP40 (black bars). Absolute activity of non-boosted full-length T-AChE: 40.77 ± 2.17 mU/mL. (c). The cationic detergent benzalkonium chloride enhances T548-AChE activity up to twofold. Beyond the critical micelle concentration of 0.02% (i.e., at the experimental final concentration of 0.002%) the enhancing qualities of the detergent decrease rapidly. Absolute activity of non-boosted T548-AChE: 40.61 ± 4.40 mU/mL. Detergent concentrations indicated on the *x*-axis refer to final concentrations. AChE activity enhancement is calculated as outlined in Materials and methods and displayed as relative increase in activity, with the activity of non-boosted T548-AChE, therefore, being equal to zero. Statistical analysis of absolute non-boosted versus absolute enhanced T548-AChE activity reveals a highly significant activity increase for every single data point as reported in the figure (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$). Results are displayed as the mean of three independent experiments. Error bars reflect standard error.

were used to determine the quality of enzyme enhancement or inhibition, i.e., to understand if the enhancement observed was competitive or un-competitive. K_M and V_{max} values were determined from the *x*- and *y*-intercept, respectively.

Results

Both non-ionic and cationic detergents enhance T548-AChE activity

The activity of T548-AChE is increasingly enhanced by rising concentrations of the non-ionic detergents Triton X-100 and Nonidet NP40 (Fig. 2a). Moreover, a clear ceiling effect for enzyme activity enhancement is observed when concentrations of Triton X-100 or Nonidet NP40 beyond 0.0013% are used (Fig. 2a, insert). In contrast, while showing some activity enhancement, full-length T-AChE activity is not affected in this highly significant manner by incubation with detergent (Fig. 2b). Also cationic detergents, such as benzalkonium chloride enhance T548-AChE activity up to twofold (Fig. 2c).

T548-AChE activity enhancement depends upon amount, charge and size of the enhancing agent

A selection of peptides was employed to assess their ability to enhance T548-AChE activity (see alphabetically ordered Table 1 for the sequence, MW, and net charge at pH 8 of

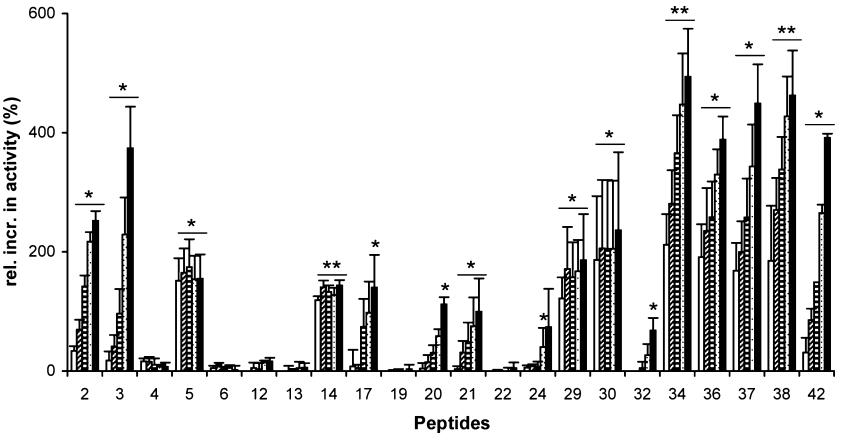


Fig. 3 Dose–response curves for the peptides enhancing T548-AChE activity. The peptides are displayed with the number codes being assigned in Table 1. T548-AChE activity enhancement is calculated as outlined in Materials and methods and displayed as relative increase in activity with the activity of non-boosted T548-AChE, therefore, being equal to zero. The final peptide concentrations (mmol/L) are as follows: 0.014, 0.028, 0.055, 0.111, 0.222 and represented by bar fillings of white, diagonally striped, horizontally striped, dotted, and black, respectively. Statistical analysis of absolute non-boosted versus absolute enhanced activity reveals a highly significant activity increase for every single data point reported in the figure (*$p < 0.05$; **$p < 0.01$). Results are displayed as the mean of three independent experiments. Error bars reflect standard error.

all 42 peptides). The amount of T548-AChE activity enhancement correlates with the amount of peptide T548-AChE is incubated with: dose–response curves can be determined for the activity enhancing peptides (Fig. 3; reported are only the 22 peptides showing enhancing qualities).

Furthermore, as can be seen in the three-dimensional diagram in Fig. 4, the increase in activity of T548-AChE following incubation with different peptides at the same, arbitrarily chosen final concentration of 0.222 mmol/L depends, in particular, on both the MW and the net charge of the relevant agent. In fact, incubation with a peptide of a net charge below +1 will, most likely, not lead to any activity enhancement (Fig. 4, black cones), while activity enhancement is generally achieved by molecules displaying a charge ≥ 1 (Fig. 4, white cones). Moreover, the MW of the agents

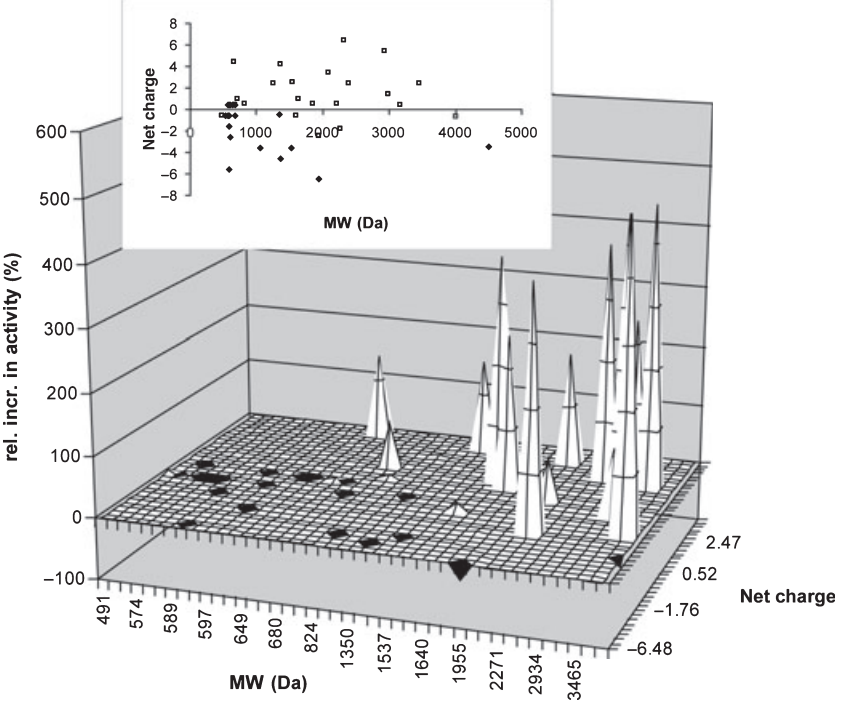


Fig. 4 T548-AChE activity enhancement in relation to net charge and molecular weight of peptides used. Peptides are used at a final concentration of 0.222 mmol/L. White cones represent the amount of activity enhancement (*z*-axis) relative to MW (*x*-axis) and net charge (*y*-axis) of the different peptides, while black cones indicate the peptides not achieving any activity enhancement. The *x*/*y* plane is also eported in the insert. T548-AChE activity enhancement is calculated as outlined in Materials and methods and displayed as relative increase in activity with the activity of non-boosted T548-AChE, therefore, being equal to zero. Results are displayed as the mean of three independent experiments. For standard errors and identification of single peaks refer to electronic Fig S1.

used plays an important role in the scenario of T548-AChE activity enhancement as, apparently, incubation with higher MW substances more likely results in more prominent T548-AChE activity enhancement (Fig. 4, white cones).

T548-AChE activity enhancement in kinetics studies

Experiments were carried out in the presence and absence of Triton X-100 (0.5% final concentration) or the AChE inhibitor procaine (1 mmol/L final concentration) as a function of substrate concentration (ATC iodide). Lineweaver-Burk plots of the reciprocal of the rate of reaction (1/velocity) versus the reciprocal of the substrate concentration (1/[ATC]) were used to determine the type of enzyme or enhancement.

Figure 5 shows the Lineweaver-Burk plot for the enzyme kinetics of the AChE-catalyzed ATC hydrolysis reaction. Curves are shown for T548-AChE alone (filled rectangle) and also in the presence of 0.5% Triton X-100 (empty triangle) or 1 mmol/L procaine (empty circle). While the inhibitor causes a decrease in enzymatic activity of T548-AChE with this being represented by a conspicuous increase in the slope of the curve (slope: 0.3232 as compared to 0.0096 for T548-AChE alone), Triton X-100 only slightly decreases the slope of the curve causing an almost parallel shift of the curve along the *y*-axis towards the *x*-axis (slope: 0.0053 as compared to 0.0096 for T548-AChE alone; *y*-intercept: 0.0243 as compared to 0.1135 for T548-AChE alone), in this way, suggesting an increase in T548-AChE enzymatic activity through an un-competitive mechanism (Fig. 5a). This effect is also reflected in a fivefold increase in the rate of reaction (V_{max} as obtained from the *y*-intercept) depicted in the close-up extrapolation of the Lineweaver-Burk plots in Fig. 5(b). Furthermore, for Triton X-100 enhanced T548-AChE, a K_M value of 0.2 as compared to a K_M of 0.08 for non-boosted T548-AChE is obtained, thus, further indicating that enhancement occurs in an un-competitive way.

The peripheral anionic site as a target for Triton X-100 in the scenario of T548-AChE activity enhancement?

Both T548-AChE alone, and T548-AChE enhanced with Triton X-100 (0.5% final concentration) were assessed for substrate inhibition by exposing them to increasing concentrations of substrate ATC. Figure 6 shows the Lineweaver-

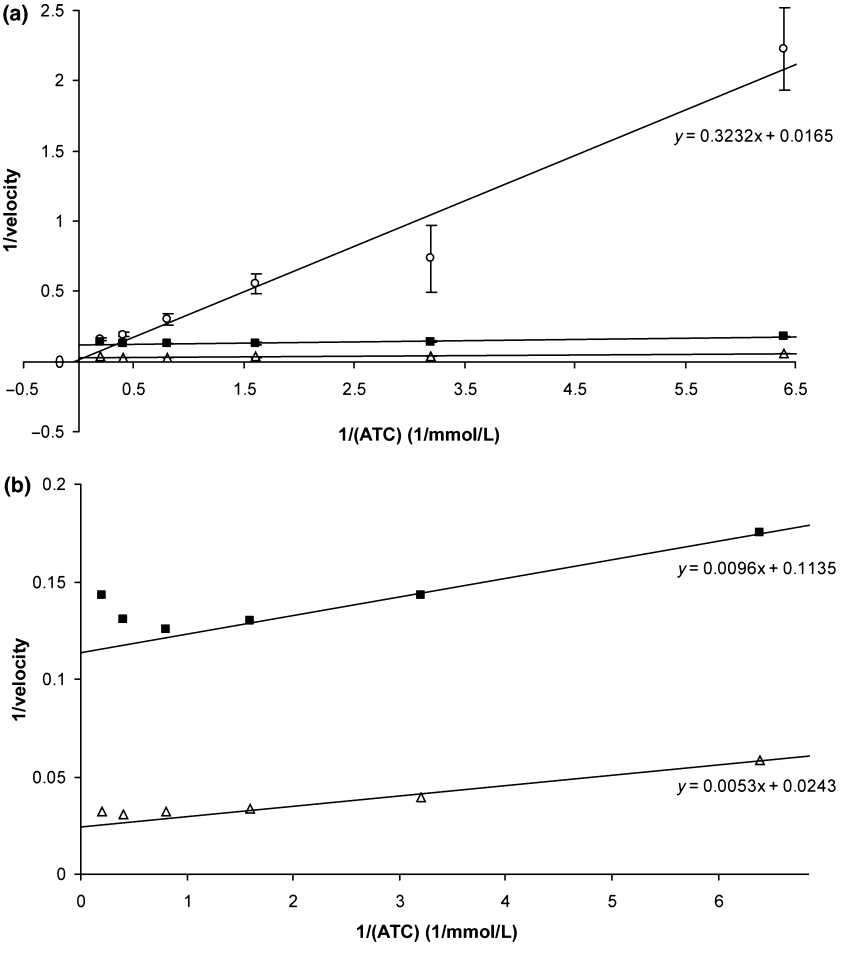
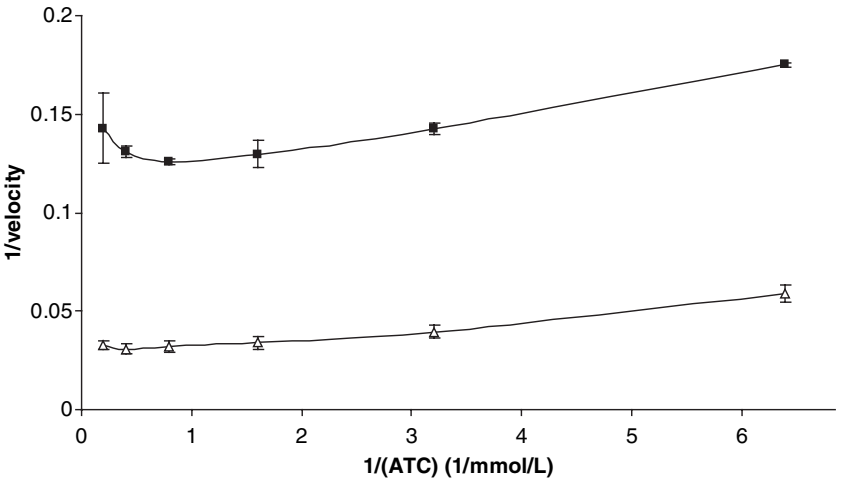


Fig. 5 Kinetic studies for procaine inhibited and Triton X-100 enhanced T548-AChE. (a) Lineweaver-Burk plots for the reciprocal of the rate of reaction (1/velocity) versus the reciprocal of substrate concentration (1/[ATC]) for T548-AChE (filled rectangle), Triton X-100 boosted T548-AChE (empty triangle) and procaine inhibited T548-AChE (empty circle). Results are displayed and extrapolation obtained for the upper end of the ATC concentrations used. Values are calculated as the mean of three independent experiments. Error bars reflect standard error. (b) Close-up of extrapolation for non-boosted versus Triton X-100 enhanced T548-AChE. K_M and V_{max} values are obtained from the *x*- and *y*-intercept, respectively.



(ATC) (mmol/L)	1/velocity T548 + buffer	T548 + triton
0.156	0.17515587	0.05868816
0.313	0.14276015	0.03939401
0.625	0.12967026	0.03391433
1.250	0.12564718	0.03198971
2.500	0.13068777	0.03090048
5	0.1428631	0.03242953

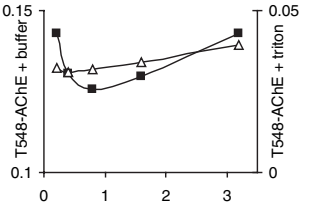


Fig. 6 Substrate inhibition delay for Triton X-100 enhanced T548-AChE. Lineweaver-Burk plots for the reciprocal of the rate of reaction (1/velocity) versus the reciprocal of substrate concentration (1/[ATC]) for T548-AChE alone (filled rectangle) and Triton X-100 boosted T548-AChE (empty triangle). By observation (see also the jolted graph), substrate inhibition is observed only at higher ATC concentrations (2.5 mmol/L as compared to 1.25 mmol/L, as listed in the table) when the activity of T548-AChE is enhanced with Triton X-100.

Burk plots for both analyzed samples. By observation, the slope changes from negative to positive at a significantly higher ATC concentration for Triton X-100 enhanced T548-AChE (0.4 1/mmol/L in the graph, i.e., 2.5 mmol/L ATC) as compared to buffer-incubated T548-AChE (0.8 1/mmol/L in the graph, i.e., 1.25 mmol/L ATC, see table and jolted close-up in Fig. 6), thus implying a change in substrate inhibition that is commonly accepted to be achieved by agents interacting with the PAS of the enzyme (Radic *et al.* 1991; Shafferman *et al.* 1992).

The lack of the C-terminal peptide in T548-AChE opens the access to the PAS

Considering that the PAS of the single AChE molecule is obstructed when oligomers are formed (Bourne *et al.* 1999), the Triton X-100 achieved enhancement should become more difficult for oligomers of AChE. To address this, T548-AChE activity enhancement was analyzed with respect to its various aggregation states. To this end, T548-AChE was subjected to sucrose density centrifugation to understand if it was purely monomeric and to analyse, if existing, the various oligomers for Triton X-100 activity enhancement. In parallel, full-length T-AChE was also analysed for Triton X-100 activity enhancement following sucrose density centrifugation that was described to clearly show the equilibrium of monomer, dimer, and tetramer species of full-length T-AChE (Bond *et al.* 2006). As can be seen from Fig. 7(a), T548-AChE is purely monomeric and every MW species of T548-AChE aggregates is significantly enhanced, i.e., irrespective of its oligomerisation state, this truncated form of AChE can be enhanced with Triton X-100 (Fig. 7a). In contrast, none of the various full-length T-AChE oligomers is significantly enhanced by Triton X-100 (Fig. 7b), thus, suggesting that full-length T-AChE activity enhancement may be hindered by the existence of the dimerization-favoring C-terminal tail of full-length T-AChE.

Discussion

The present study builds on the following background: first, the major form of AChE present in both the developing and the degenerating brain is monomeric (Arendt *et al.* 1992). Second, AChE has been shown to lose its typical characteristic of substrate inhibition in both development (Moreno *et al.* 1998) and degeneration (Arendt *et al.* 1992). Third, the sequence of the truncated form of AChE used in the study here (T548-AChE), is highly similar to that of a physiological form of AChE detected in fetal bovine serum that has lost its C-terminal tail supposedly because of proteolytic cleavage (Saxena *et al.* 2003). Furthermore, peptide sequences covered by this C-terminal tail have been shown to be crucially involved in both developmental and degenerative mechanisms *in vitro* (Bon and Greenfield 2003; Day and Greenfield 2003, 2004; Emmett and Greenfield 2004; Greenfield *et al.* 2004; Zbarsky *et al.* 2004). In this context, our work presents evidence towards a mechanistic and functional link between the form of AChE unique to both development and degeneration and a C-terminal peptide of AChE acting under these conditions.

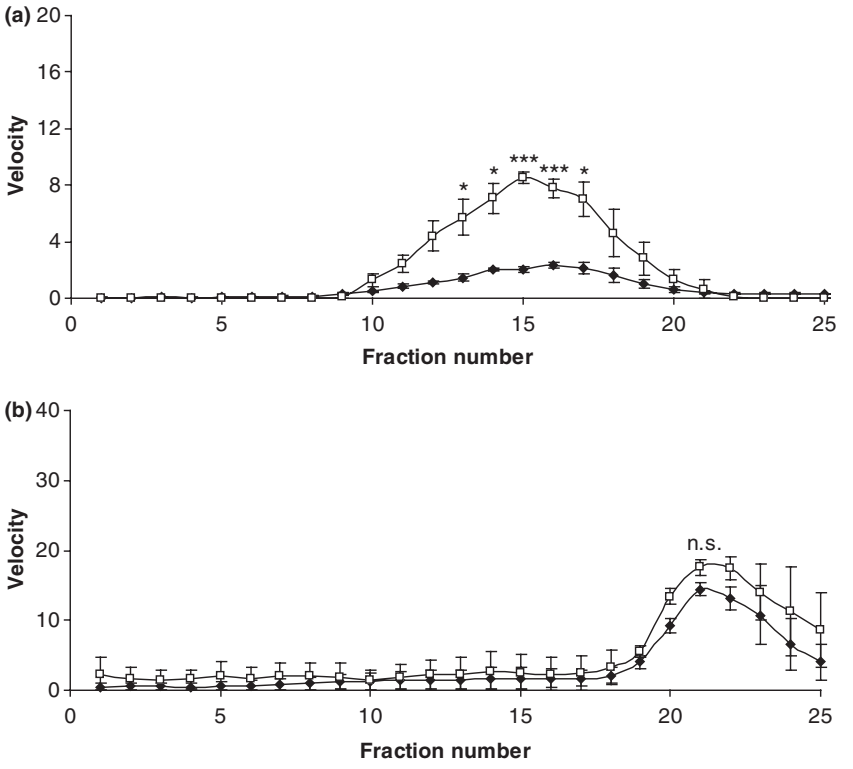


Fig. 7 Enhancement of sucrose density gradient separated T548-AChE and full-length T-AChE oligomers. (a) T548-AChE is clearly monomeric with this fact being represented by one single peak of AChE activity. The velocity displayed corresponds to the absolute velocity measured for T548-AChE alone (black rhombus) and for Triton X-100 boosted T548-AChE (white rectangle). The velocity of detergent-boosted T548-AChE is significantly higher than the velocity of not-enhanced T548-AChE. (b) The velocity of full-length T-AChE is not significantly enhanced for any of its oligomers. The velocity displayed corresponds to the absolute velocity measured for full-length T-AChE alone (black rhombus) and for Triton X-100 boosted full-length T-AChE (white rectangle). Note that gradients were pipetted off the top, i.e., the heavier components appear in the later fractions. Results are displayed as the mean of three independent experiments. Error bars reflect standard error. Statistical analysis was performed using Student's *t*-test (*$p < 0.05$; ***$p < 0.005$). Absolute activities of non-boosted enzymes as loaded onto sucrose gradients are 2.71 ± 0.03 U/mL and 2.83 ± 0.09 U/mL for full-length T-AChE and T548-AChE, respectively.

The material used is not of equal purity (Fig. 1) as we compare a commercial preparation of full-length T-AChE with a preparation of an academic laboratory (T548-AChE). However, where T548-AChE and its full-length counterpart are compared for the extent of activity enhancement following incubation, samples of same un-boosted activities are assessed to ensure the significance and relevance of our statements (Figs 2 and 7).

T548-AChE activity is boosted depending on amount, charge and size of the enhancing agent

In view of previous studies on the interactions of full-length T-AChE with Triton X-100 (Bon and Massoulie 1980; Dutta-Choudhury and Rosenberry 1984; Rosenberry and Scoggin 1984), we investigated the sensitivity of T548-AChE activity to various detergents. In contrast to full-length T-AChE activity, the activity of T548-AChE is significantly enhanced by rising concentrations of the non-ionic detergents Triton X-100 and Nonidet NP40 (Fig. 2). This finding suggests that T548-AChE, in particular, is highly sensitive to amphiphilic moieties. Considering that also cationic detergents such as benzalkonium chloride enhance T548-AChE activity, we also hypothesized that the charge of the enhancing agent may play an important role in T548-AChE activity enhancement. Importantly, the decrease in activity enhancement observed for final concentrations of benzalkonium chloride beyond 0.002% may be explained as a consequence of detergent molecules being captured in micelles rather than being available for activity enhancement.

When testing various peptides for their ability to enhance T548-AChE activity it became clear that activity enhancement correlates not only with the amount of peptide with which T548-AChE is incubated (Fig. 3) but also with characteristics intrinsic to the different agents. More specifically, both net charge and MW of the enhancing molecules are of major importance in the scenario of T548-AChE

activity enhancement (Fig. 4). In particular, a positive charge for the enhancing component is imperative for successful T548-AChE activity enhancement at low MW, with this rule becoming more relaxed with increasing MW of the enhancer.

T548-AChE activity is enhanced in an 'un-competitive' fashion

Additional experiments were undertaken to understand the mechanism of action underlying this significant enhancement of enzymatic activity. While it is a commonly acknowledged fact that a large effective negative charge on the AChE active site is a general characteristic of the enzyme's interaction with cationic ligands (Nolte *et al.* 1980), it is not clear if this specific interaction is the mechanism underlying the here discussed activity enhancement. In particular, we cannot exclude the possibility that enzymatic activation of AChE is achieved through interactions of the enhancing molecules with the PAS of the enzyme. This assumption is especially plausible for the following reason: T-AChE has been shown to be involved in amyloid fibril formation with the PAS apparently being involved in this action (Inestrosa *et al.* 1996; De Ferrari *et al.* 2001). Most importantly, this plaque-linked form of AChE that is typical for Alzheimer's Disease pathology is characterised by delayed substrate inhibition (Schatz *et al.* 1990; Alvarez *et al.* 1998), thus further supporting the hypothesis that the enhancing agents might act on the PAS of the enzyme. In this context, the enzyme kinetics studies undertaken assessed if the enhancing agents act, indeed, directly on the PAS of T548-AChE. The addition of procaine causes a decrease in enzymatic activity through a decrease of the number of enzymatically active molecules in solution (Fig. 5). This fact is reflected in a prominent increase in the slope of the Lineweaver-Burk plot. Addition of Triton X-100, in contrast, results in an almost parallel shift of the T548-AChE plot along the *y*-axis towards the *x*-axis, in this way suggesting that the detergent, apparently, behaves as an 'un-competitive enhancer' with the rate of reaction being increased fivefold (V_{max}). This finding is further supported by a striking increase of the K_M value when T548-AChE is incubated with Triton X-100. Similar observations have been made and hypotheses devised for the action of ethanol on AChE (Shin *et al.* 1991). In this context, it should be mentioned that this extraordinary feature of activity enhancement may have important implications in analytical studies where smallest amounts of enzyme ought to be detected or where Triton X-100 extracted material is assessed for AChE activity instead of protein amount (Tarrab-Hazdai *et al.* 1984; Lassiter *et al.* 2003).

T548-AChE activity enhancement is achieved through the peripheral anionic site

To further narrow down the site of action of the enhancing agents, substrate inhibition experiments were undertaken. The rationale of these experiments lies in the fact that substrate inhibition of AChE is commonly accepted to be achieved by agents interacting with the PAS of the enzyme (Radic *et al.* 1991; Shafferman *et al.* 1992). In reverse, as shown in Fig. 6, Triton X-100 acts on the PAS as an enhancer, with substrate inhibition being observed only at higher ATC concentrations for detergent-boosted as compared to non-enhanced T548-AChE. Importantly, also for butyrylcholinesterase activity enhancement has been shown to be exerted through the PAS (Stojan *et al.* 2002). The assumption that Triton X-100 interacts with the PAS is further supported by the fact that every MW species of T548-AChE aggregates is enhanced, i.e., irrespective of its aggregation state this truncated form of AChE can be enhanced with Triton X-100, while none of the different full-length T-AChE oligomers is enhanced by the detergent (Fig. 7). Considering that the PAS of the single AChE molecule is obstructed when oligomers are formed (Bourne *et al.* 1999), the Triton X-100 achieved enhancement should become more difficult for oligomers of AChE. Moreover, considering that the fundamental difference between full-length T-AChE and T548-AChE is the lack of the C-terminal tail in the synthetic AChE (Marchot *et al.* 1996), it can be assumed that the access to the enhancement supporting PAS is hindered by the existence of the C-terminal tail of full-length T-AChE, thus, hampering the activity enhancement of this form of AChE.

Even though this study describes functional characteristics of a truncated form of AChE, the results presented are of highest relevance *in vivo*. Our findings strongly support earlier work that describes the existence of a form of AChE unique to development and degeneration that has lost its characteristic of substrate inhibition (Arendt *et al.* 1992; Moreno *et al.* 1998). We present evidence towards a mechanistic link between this functional phenomenon and the strongly promoted hypothesis of the *in vivo* existence of a C-terminal peptide of T-AChE common to development and degeneration (Greenfield and Vaux 2002). While conclusive evidence for its existence is still to be provided (work ongoing in this laboratory), proteolytic cleavage of the C-terminus has already been suggested for a fetal form of T-AChE (Saxena *et al.* 2003), thus further supporting our assumption of the T-AChE C-terminus being cleaved off. In fact, a possible scenario would be that the, consequently, freed PAS of AChE is now easily receptive for specific positively charged peptides, such as the hypothesized C-terminal sequence of AChE itself (Greenfield and Vaux 2002) – that would influence the enzymatic activity of AChE and, as a consequence, delay the phenomenon of substrate inhibition. It is noteworthy that the major part of the peptides tested exists as biological entities; C-terminal peptides of full-length T-AChE are 2 and 34.

This study shows, first, how the activity of a truncated form of AChE can be enhanced through agents that contain highly positive charged moieties and, second, that the

mechanism of activity enhancement, most likely, involves the PAS of AChE. To our knowledge, this is the first time that agents acting on the PAS are shown to be enhancing rather than inhibiting for the enzymatic activity of AChE. Most importantly, the results discussed here potentially shed light onto the structure and function of a form of AChE unique to both development and degeneration.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1 The various peptides used are attributed to their relevant activity enhancement cone employing the number code displayed in Table 1 of the printed version.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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