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# Characterisation of acetylcholinesterase release from neuronal cells

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ABSTRACT

Although acetylcholinesterase (AChE) is primarily a hydrolytic enzyme, metabolising the neurotransmitter acetylcholine in cholinergic synapses, it also has some non-catalytic functions in the brain which are far less well characterised. AChE was shown to be secreted or shed from the neuronal cell surface like several other membrane proteins, such as the amyloid precursor protein (APP). Since AChE does not possess a transmembrane domain, its anchorage in the membrane is established via the Proline Rich Membrane Anchor (PRiMA), a transmembrane protein. Both the subunit oligomerisation and membrane anchor of AChE are shared by a related enzyme, butyrylcholinesterase (BChE), the physiological function of which in the brain is unclear. In this work, we have assayed the relative activities of AChE and BChE in membrane fractions and culture medium of three different neuronal cell lines, namely the neuroblastoma cell lines SH-SY5Y and NB7 and the mouse basal forebrain cell line SN56. In an effort to understand the shedding process of AChE, we have used several pharmacological treatments, which showed that it is likely to be mediated in part by an EDTA- and batimastat-sensitive, but GM6001-insensitive metalloprotease, with the possible additional involvement of a thiol isomerase. Cellular release of AChE by SH-SY5Y is significantly enhanced by the muscarinic acetylcholine receptor (mAChR) agonists carbachol or muscarine, with the effect of carbachol blocked by the mAChR antagonist atropine. AChE has been implicated in the pathogenesis of Alzheimer's disease and it has been shown that it accelerates formation and increases toxicity of amyloid fibrils, which have been closely linked to the pathology of AD. In light of this, greater understanding of AChE and BChE physiology may also benefit AD research.



## 1. Introduction

### 1.1. The cholinergic system and the cholinesterases

The cholinergic basal forebrain system is strongly linked to AD [1,2]. There are reports specifically and consistently linking cholinergic hypofunction and cognitive decline. For reasons unknown, cholinergic neurons of the basal forebrain are specifically targeted in AD [3]. However, this system is not limited to neurotransmission as the predominant neurotransmitter in this system, acetylcholine (ACh) and its principal degradative enzyme, acetylcholinesterase (AChE) have been suggested to have trophic functions [4,5]. Although AChE is primarily a hydrolytic enzyme, there are reports of its significant non-catalytic functions [6,7].

Several forms of AChE can be generated from the *ACHE* gene as splice variants, with these variants (hydrophobic (H), readthrough (R) and tailed (T)) differing in membrane association and also localisation on a tissue and sub-cellular level. The individual subunits of AChE can associate with each other, forming both dimers and tetramers [8,9]. In the brain, tetramers of $AChE_T$ form the functional units at cholinergic synapses, which reveals an important role for the Proline Rich Membrane Anchor (PRiMA), a 20 kDa protein responsible for both AChE tetramerisation and its anchorage to the membrane in neuronal cells [10–13]. PRiMA has a Proline Rich Attachment Domain (PRAD), like its counterpart ColQ, which serves as a membrane anchor for AChE at neuromuscular junctions. The AChE-PRiMA association occurs between the C-terminal t peptides of AChE and the PRAD of PRiMA. It has also been suggested that disulphide bonds form between four Cys residues at the N-terminus of PRiMA and the C-terminal Cys in the AChE t peptide [11]. Immunofluorescence studies have shown strong co-localisation between AChE and PRiMA in cholinergic neurons, but no

*Abbreviations:* Aβ, amyloid beta; ACh, acetylcholine; AChE, acetylcholinesterase; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; BChE, butyrylcholinesterase; Iso-OMPA, tetraisopropyl pyrophosphamide; mAChR, muscarinic acetylcholine receptor; MMP, matrix metalloprotease; nAChR, nicotinic acetylcholine receptor; PDI, protein disulphide isomerase; PRAD, proline rich attachment domain; PRiMA, proline rich membrane anchor; TSHR, thyroid stimulating hormone receptor.

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localisation of PRiMA in either dopaminergic or GABAergic neurons [12]. The topology of membrane-bound AChE is similar to the thyroid-stimulating hormone receptor (TSHR), as shown in Fig. 1. The membrane bound TSHR β- subunit is analogous to PRiMA, while the membrane anchored α- subunit is able to be shed and is thus analogous to AChE. In TSHR, the subunits are linked by both disulphide and peptide bonds. An initial cleavage step by a metalloprotease is followed by reduction of the disulphide bonds by cell surface protein disulphide isomerase (PDI) [14,15].

Another cholinesterase characterised in the brain, but which differs from AChE structurally, genetically and kinetically, is BChE. Although BChE cleaves ACh, its major substrate is butyrylcholine (BCh). BCh is not present in mammalian nervous systems, so it has been postulated that ACh is a physiological substrate for BChE [16]. However, despite these differences, oligomerisation and membrane anchorage of BChE seem very similar to AChE [17]. In the brain of AChE$^{-/-}$ mice, which are viable, but severely defective, it has been suggested that BChE has a compensatory activity, hydrolysing ACh [18–20]. Studies regarding its localisation are fairly sparse, but it has been shown that BChE can be found on both nuclear and plasma membranes in rat cerebrum and cerebellum. BChE distribution in the brain has been shown to be far more glial than neuronal, whereas the reverse is true for AChE [21].

### 1.2. Cellular release of AChE

The existence of soluble AChE, released from the cell, has been recognised for over 30 years [22]. However, the mechanism by which AChE is released is still unclear. It is likely that two or more pathways for cellular AChE release exist. One is a shedding-type process [13,23], while the other is suggested to be an exocytosis-like process, regulated by $Ca^{2+}$ [24,25]. Furthermore, within the latter, it is probable there is more than one pathway, as suggested by Schweitzer [26]. It could also be the case that these different pathways are responsible for cellular release of different cholinesterase species, shedding to release PRiMA-bound tetramers and exocytosis to release less tightly associated cholinesterase species, not associated with PRiMA.

For AChE shedding, there have been suggestions of involvement of proteolytic enzymes, specifically those of the ADAM family [23]. This is a family of proteases responsible for shedding of many cell surface proteins, including enzymes, e.g. angiotensin converting enzyme (ACE) [27] and ACE-2 [28] and cleavage of the amyloid precursor protein (APP) in the non-amyloidogenic pathway [29,30]. Many members of this family of enzymes and also the matrix metalloprotease (MMP) family are inhibited by the hydroxamate inhibitors batimastat and GM6001 [31]. In the case of some cell surface proteins which are membrane-anchored by disulphide bonds, protein disulphide isomerase (PDI), has been shown to be involved in their shedding, such as TSHR [15].

### 1.3. AChE in Alzheimer's disease (AD)

The hallmarks of AD are pathological accumulation of amyloid β peptide (Aβ), forming senile plaques, and of neurofibrillary tangles comprised by hyperphosphorylated tau protein [32]. Loss of AChE and its non-classical actions could have a pronounced effect on brain function, as in AD, brain $G_4$ AChE levels are seen to fall as the disease progresses, while $G_1$ and $G_2$ levels rise, as compared to normal brains [33,34]. However, AChE and BChE were shown to be localised in Aβ plaques, leading to the suggestion that they can promote Aβ aggregation and increase amyloid fibril neurotoxicity [35,36]. This is to the extent that, in some brain regions, virtually all of the AChE is localised in these complexes [37]. Furthermore, AChE has been shown to regulate other key cholinergic proteins, such as α7 nAChR [38].

### 1.4. Aims and outcomes

The aims of this study were to compare several mammalian neuronal cell lines as to their applicability for investigating the biology of the cholinesterases. We also investigated the extent to which shedding contributes to the cellular release of acetylcholinesterase and which enzymes may be responsible.

## 2. Methods and materials

### 2.1. Cell culture

Cholinergic SN56 cells (kindly provided by Prof. A. Szutowicz, Medical University of Gdańsk, Poland) were cultured in DMEM (Lonza, Basel, Switzerland), supplemented with 10% foetal bovine serum and 1% penicillin, streptomycin and L-glutamine at 37 °C and 5% $CO_2$ in a humidified atmosphere. SH-SY5Y and NB7 cells were cultured as described previously [39]. Cells transfected with $APP_{695}$ were also treated with 0.3% (w/v) Hygromycin B (Sigma Aldrich, Gillingham, Dorset, UK).

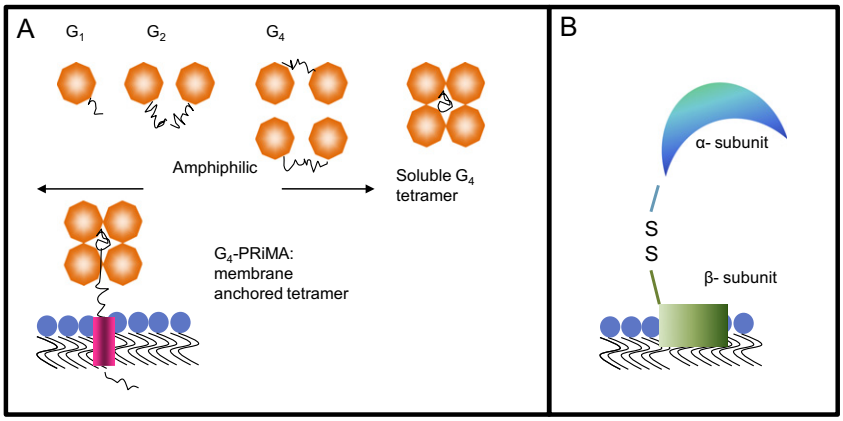


**Fig. 1.** Topologies of membrane-bound AChE and TSH receptor. (A) AChE and BChE are very similar with regard to subunit assembly, this figure being representative of the process in the nervous system. The $G_4$ tetramer is assembled starting with the $G_1$ monomer, which dimerises to form $G_2$. The $G_2$ dimer then itself homo-dimerises to form the $G_4$ tetramer, which can be either soluble or membrane bound via PRiMA. (B) The thyroid stimulating hormone receptor (TSHR) has a similar topology. The membrane bound TSHR β-subunit is analogous to PRiMA, while the membrane anchored α-subunit, able to be shed, is equivalent to AChE. Furthermore, the disulphide bonds between the two components are similar in both systems.