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REVIEW

Neuronal AChE splice variants and their non-hydrolytic functions: redefining a target of AChE inhibitors?

M Zimmermann

Department of Pharmacology, School of Pharmacy, Goethe University Frankfurt, Frankfurt am Main, Germany

Correspondence
M Zimmermann, Department of Pharmacology, School of Pharmacy, Biocentre N260, Max-von-Laue Str. 9, Goethe University Frankfurt, 60438 Frankfurt am Main, Germany.
E-mail: Martina.Zimmermann@em.uni-frankfurt.de

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AChE enzymatic inhibition is a core focus of pharmacological intervention in Alzheimer's disease (AD). Yet, AChE has also been ascribed non-hydrolytic functions, which seem related to its appearance in various isoforms. Neuronal AChE presents as a tailed form (AChE-T) predominantly found on the neuronal synapse, and a facultatively expressed readthough form (AChE-R), which exerts short to medium-term protective effects. Notably, this latter form is also found in the periphery. While these non-hydrolytic functions of AChE are most controversially discussed, there is evidence for them being additional targets of AChE inhibitors. This review aims to provide clarification as to the role of these AChE splice variants and their interplay with other cholinergic parameters and their being targets of AChE inhibition: AChE-R is particularly involved in the mediation of (anti-)apoptotic events in cholinergic cells, involving adaptation of various cholinergic parameters and a time-dependent link to the expression of neuroprotective factors. The AChE-T C-terminus is central to AChE activity regulation, while isolated AChE-T C-terminal fragments mediate toxic effects via the α7 nicotinic acetylcholine receptor. There is direct evidence for roles of AChE-T and AChE-R in neurodegeneration and neuroprotection, with these roles involving AChE as a key modulator of the cholinergic system: *in vivo* data further encourages the use of AChE inhibitors in the treatment of neurodegenerative conditions such as AD since effects on both enzymatic activity and the enzyme's non-hydrolytic functions can be postulated. It also suggests that novel AChE inhibitors should enhance protective AChE-R, while avoiding the concomitant up-regulation of AChE-T.

Abbreviations

aa, amino acid; Aβ, amyloid β; AChE-R, readthrough AChE; AChE-T, tailed AChE; AChE-Tt, hydrolytically cleaved AChE-T; AD, Alzheimer's disease; APP, amyloid precursor protein; ARP, C-terminal fragment of AChE-R; DLB, dementia with Lewy bodies; HACU, high-affinity choline uptake; mAChR, muscarinic ACh receptor; nAChR, nicotinic ACh receptor; PAS, peripheral anionic site; PDD, Parkinson's disease dementia; PRiMA, proline-rich membrane anchor; T14 and T30, C-terminal fragments of AChE-T of 14 and 30 aa length respectively

AChE: from gene to structure and neuronal splice variants

AChE is responsible for the termination of cholinergic transmission, that is, the enzymatic breakdown of ACh (Radic *et al.*, 1997). It is known as one of the fastest enzymes of our body (Nair *et al.*, 1994), degrading, as a tetramer, about 25 000 molecules of ACh per second. The AChE gene is located on chromosome 7 and spans seven kilobases. It contains six exons, three of which (E2, E3 and E4) encode for the core peptide that is common to all enzyme variants and

harbours the information for the enzyme's activity. The AChE pre-mRNA is susceptible to alternative splicing (Taylor and Radic, 1994), which leads to three post-transcriptional species that, however, all derive from the same gene (Marsh *et al.*, 1984; Aziz-Aloya *et al.*, 1993; Grisaru *et al.*, 1999). The C-terminal domains of these different splice variants determine the post-translational processing and, thus, location (both within the organism and the single cell) as well as role of the enzyme (Massoulie *et al.*, 1998; Massoulie, 2002; Camp *et al.*, 2010; Hicks *et al.*, 2011). Further AChE variants originate from different promoter use and, thus, N-terminal alterations (Meshorer and Soreq, 2006; Toiber *et al.*, 2008; 2009), but these forms are less extensively characterized.

Two splice variants are particularly relevant to neuronal tissue. Specifically, the synaptic form of AChE is the one predominantly expressed in the CNS and muscle tissue (Massoulie and Millard, 2009). It is formed by splicing of exons 4 to 6, yielding a E1-E2-E3-E4-E6 transcript. The translation of this mRNA leads to the C-terminal extension of the common core by a peptide containing a cysteine, which favours dimerization; in view of this 'tailed' extension, this variant is also denominated tailed AChE (AChE-T; Liang *et al.*, 2009). This C-terminal extension leads to AChE-T post-translational modifications involving, in nervous tissue, the link between a proline-rich membrane anchor (PRiMA) and a WAT domain on the very AChE-T C-terminus (Inestrosa and Perelman, 1989; Xie *et al.*, 2010; Chen *et al.*, 2011). This membrane-tethered form of AChE presents the bulk of AChE activity in neuronal tissue.

The other AChE splice variant relevant to neuronal events is the readthrough form of AChE (AChE-R), which obtains its name from the continuous transcription through intron I4, which yields the E1-E2-E3-E4-I4-E5 transcript. The extension of its C-terminus over the common core is shorter and lacks cysteine (Li *et al.*, 1991; Camp *et al.*, 2010). Hence, AChE-R is destined to remain monomeric, with its consequential solubility and swift distribution in tissue being closely linked to its apparent, though highly debated role in stress-related conditions (Soreq and Seidman, 2001; Massoulie *et al.*, 2008): as both *in vitro* and *in vivo* work shows, powerful inductors for AChE-R are stress, for example, forced swim stress, continuous use as well as toxic concentrations of AChE inhibitors or inflammation (Kaufer *et al.*, 1998; Nijholt *et al.*, 2004; Dori *et al.*, 2007; Evron *et al.*, 2007), with stress-inducible changes in AChE gene expression being mediated by histone deacetylase (Sailaja *et al.*, 2012). Figure 1 offers a scheme of these different AChE splice variants and their C-terminal peptides.

Among the splicing factors involved in the alteration of the AChE expression pattern are SC35 (Meshorer *et al.*, 2005) and hnRNPA1 (Berson *et al.*, 2012), with the latter having recently been demonstrated to assemble into self-seeding fibrils, revealing prion-like activities in an array of neurodegenerative conditions (Kim *et al.*, 2013). This observation adds to deliberations on the striking mechanistic commonalities among neurodegenerative disorders, including, for example, alternative splicing and ubiquitination (Fotuhi *et al.*, 2009; Ferraiuolo *et al.*, 2011; Robberecht and Philips, 2013).

Given that cholinergic neurotransmitter levels drastically decrease in conditions like Alzheimer's disease (AD; Arendt *et al.*, 1992; Mufson *et al.*, 2008), AChE is usually discussed in relation to enzyme inhibition since this strategy can temporarily increase ACh levels. In fact, since the FDA approval of tacrine (1993) and donepezil (1997) as the first AChE inhibitors for the treatment of AD, the efficacy and suitability of these drugs have been considered in countless reviews (a PubMed research for 'acetylcholinesterase inhibitors' yields nearly 3000 hits in the 'review' category), and several more agents have reached the market since, among them rivastigmine and galantamine.

The discussion regarding cholinergic deficits as being centrally involved in the pathogenesis of neurodegenerative conditions such as AD (Francis *et al.*, 1999; Mufson *et al.*, 2008; Schliebs and Arendt, 2011) particularly focuses on the loss of cholinergic neurons and the ensuing decrease of neurotransmitter levels and the enzymatic machinery responsible for its synthesis, that is, ChAT and high-affinity choline uptake (HACU), taking also into account alterations of glutamatergic signalling (Francis *et al.*, 2012). In the light of the more recently discussed non-hydrolytic functions of AChE, this review specifically focusses on the role of AChE inhibitors in the context of AChE alternative splicing. Moreover, in discussing the role of AChE splice variant expression in conditions of acute and chronic neurodegeneration, the review broaches the issue of the impact of AChE inhibitors on AChE alternative splicing, deliberates on the potential limited usefulness of AChE inhibitors and hopes to instigate a discussion on concepts for future drug development.

Non-hydrolytic functions of AChE: first clues for the use of AChE inhibitors

The enzyme's complex structural polymorphism supports the theory that the different AChE molecular forms play distinct physiological roles not only in the cholinergic system: AChE has been detected in adult non-cholinergic neurons, for

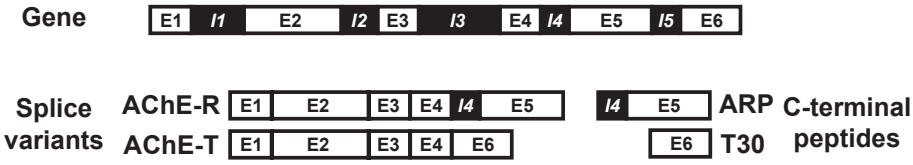

Figure 1

AChE splice variants and their C-terminal peptides. The figure shows the AChE gene (top; omitting the promoter region), AChE splice variant transcripts (bottom left) and the transcripts from which the translated C-terminal moieties that contain the AChE peptides are derived. E, exon; I, intron.

example, substantia nigra and cerebellar cells (Greenfield *et al.*, 1983), as well as hematopoietic, osteogenic and even various neoplastic cells (Zakut *et al.*, 1992; Karpel *et al.*, 1994; Small *et al.*, 1996; Grisaru *et al.*, 2001; Soreq and Seidman, 2001; Deutsch *et al.*, 2002). In addition, AChE is known for its widespread occurence during early development (Vogel-Hopker *et al.*, 2012). Further roles may be inferred from the non-neuronal functions of ACh itself in sustaining barrier functions (Yoshida *et al.*, 2006; Kurzen *et al.*, 2007; Kummer *et al.*, 2008). In addition, non-neuronal roles of ACh in conditions like inflammation (Fujii and Kawashima, 2001; Pavlov and Tracey, 2005; del Rey and Besedovsky, 2008) may give further clues as to non-enzymatic roles of AChE. This is particularly true since neuronal, stress-induced increase in interleukin-1 seems to mediate stress-related increased AChE expression (Li *et al.*, 2000b).

In fact, various AChE inhibitors are successfully used to support the recovery in elderly, cognitively impaired stroke patients (Oldenbeuving *et al.*, 2008; Whyte *et al.*, 2008; Hong *et al.*, 2012). For example, donepezil was tested in a phase II trial as an adjuvant therapy to standard medical care of stroke patients (Barrett *et al.*, 2011) and supported cognitive improvement in a pilot study (Chang *et al.*, 2011). In this context, the degree of recovery from stroke has been correlated to stroke-induced effects on the immune system that can lead to infections (Dirnagl, 2012). Such events involve a mechanism described as the inflammatory reflex (Tracey, 2002; Huston and Tracey, 2011). Since vagally released ACh is able to suppress the release of inflammatory cytokines, the positive effect of AChE inhibitor treatment in post-stroke patients may well extend to the effects of non-neuronal ACh.

In addition, non-hydrolytic functions of AChE are suggested by the spatio-temporally regulated expression of AChE in early embryogenesis, embryonic neurite extension and synaptogenesis (Fitzpatrick-McElligott and Stent, 1981; Layer, 1990): the mutually exclusive expression patterns of AChE and its enzymatically less specific sister molecule butyrylcholinesterase, BuChE, (and the pre-synaptogenetic role of AChE) have been described as early as 1983 (Layer, 1983). AChE is transiently expressed in the developing nervous system, in particular, during periods of neuronal proliferation, migration and axonal outgrowth (Layer and Sporns, 1987; Layer, 1990, 1991). Exogenous, purified AChE promotes and regulates axonal and neurite growth from chick nerve cells in culture. This function is not the result of the enzymatic activity *per se* since this function was not attenuated by treating the cell culture with various active site inhibitors, which depressed enzymatic AChE activity (Layer *et al.*, 1993). In line with these findings, others showed that neurite outgrowth is retarded following AChE inhibitor treatment (Dupree and Bigbee, 1994) and that a secondary, peripheral anionic site (PAS) on the AChE molecule achieves this adhesive function (Small *et al.*, 1995).

A breadth of studies carried out in various cellular systems continued to corroborate these findings throughout the 1990s (Koenigsberger *et al.*, 1997; Grifman *et al.*, 1998; Bigbee *et al.*, 2000). Yet, observations for an AChE knockout mouse seemingly casted major doubt on these insights: transgenic mice not carrying any AChE allele were alive, even though they required a liquid diet in view of muscle weakness (Xie *et al.*, 1999). This mouse model establishes central cholinergic pathways and uses BuChE to hydrolyse the enormous quantities of ACh present in the cerebral extracellular space (Li *et al.*, 2000a; Mesulam *et al.*, 2002; Hartmann *et al.*, 2007). At the same time, however, this mouse model revealed severely disturbed retinal development and neuritogenesis (Bytyqi *et al.*, 2004), thus, further suggesting a mandatory role of AChE in development. What is more, AChE shares – as reviewed previously (Soreq and Seidman, 2001) – high homology with the extracellular domain sequence of, firstly, gliotactin, a transmembrane protein transiently expressed on peripheral glia that is required for the formation of the peripheral blood-nerve barrier (Auld *et al.*, 1995); secondly, neurotactin, which is known for its interneuronal interactions (de la Escalera *et al.*, 1990); and, thirdly, neuroligin, a further non-catalytic transmembrane protein with its extracellular sequence being composed of a catalytically inactive esterase domain homologous to AChE. Such evidence further underlines a likely role of AChE in development. It even suggests – as put forward earlier (Layer, 1995) – that the function of AChE in neurodegeneration may equally be attributed to 'non-classical' functions of the enzyme. These very functions are likely affected by AChE inhibition, with this issue being explored in the following sections.

AChE and amyloid: enzymatic activity in a new light

Nibaldo Inestrosa's work contributed significantly to our understanding of AChE as being involved in amyloid fibre assembly (Inestrosa *et al.*, 1996), which continues to be considered – and discussed as – one of the core hallmarks of the condition (Hardy *et al.*, 1986; Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Armstrong, 2011; de la Torre, 2011; Benilova *et al.*, 2012; Reitz, 2012). In particular, amyloid beta (Aβ) aggregation is promoted by AChE forming a complex with growing fibrils (Alvarez *et al.*, 1997); furthermore, these stable complexes change the biochemical properties of the enzyme and increase the neurotoxicity of the Aβ fibrils (Alvarez *et al.*, 1998); thirdly, AChE promotes peptide aggregation by a mechanism not involving the hydrolysis of the amyloid precursor protein (APP; Campos *et al.*, 1998); and finally, the neurotoxicity of AChE Aβ peptide aggregates is dependent on the type of Aβ and the AChE concentration present in the complexes (Munoz and Inestrosa, 1999). These authors further showed, most notably and in reference to the above discussion on the role of the enzyme's PAS, that propidium, an AChE PAS ligand, could (in contrast to edrophonium, an active site inhibitor) prevent amyloid fibre assembly.

As such, one should assume that specific AChE inhibitors may prevent further aggregation of Aβ fibrils. In fact, researchers encourage future work in the area of AChE inhibitor drug synthesis to move towards inhibitors that reduce the deposition of amyloid (Pohanka, 2012). Such suggestions receive further support from the fact that a co-localization of AChE and Aβ was also reported in autopsy studies of AD patients' brains: these findings suggest that AChE activity is intimately associated with the process of amyloid formation and accumulation in senile plaques *in vivo* (Ulrich *et al.*, 1990;

Moran *et al.*, 1994). The source of esterase activity of senile plaques has been ascribed to glia cells by Mesulam and his co-workers (Wright *et al.*, 1993). Yet, they also demonstrated that BuChE was as much involved in these processes as AChE itself: firstly, AChE and BuChE activities with pH preferences and inhibitor selectivities identical to those of plaque-bound cholinesterases are found in the astrocytes and oligodendrocytes of control and AD brains; secondly, these glial-type cholinesterases are selectively inhibited by indolamines and protease inhibitors; and, thirdly, in control and AD brains, AChE-positive glia are distributed throughout the cortical layers and subcortical white matter. What is more, Sultan Darvesh and colleagues strongly emphasize the expression of BuChE in brain structures involved in cognition (Darvesh *et al.*, 2001), and claim a central role of BuChE, alongside AChE, in the occurrence, symptoms and progression of dementia (Ballard *et al.*, 2005), also in the light of the association of BuChE with Aβ plaques (Darvesh, 2013). These findings are not easily reconciled with Inestrosa's insights that BuChE, which lacks the PAS, does not affect amyloid formation (Inestrosa *et al.*, 1996). Indeed, they support work advocating the use of BuChE inhibitors in the treatment of AD (Giacobini, 2001; Giacobini, 2003), especially since these compounds seem to impact on the enzyme's splice variant expression and consequential attenuation of amyloidogenesis (Greig *et al.*, 2005; Podoly *et al.*, 2009). Effects of AChE inhibitors on gene expression are discussed in detail further below.

Nevertheless, these discrepancies may well be owed to the fact that a good part of the above work was carried out in isolated test tube conditions, which can hardly account for the individual patient's complex physiology or cerebral pathomorphology. As recently reviewed by Inestrosa himself, BuChE is able to extend the nucleation phase of Aβ polymerization, reducing the rate of fibril formation, instead stabilizing soluble Aβ assemblies (Inestrosa *et al.*, 2008). Whether BuChE affects Aβ oligomerization remains unclear. Still, the hypothesis persists that senile plaques may be formed from the terminals of AChE containing neurons. As such, AChE PAS inhibitors may be preferable to purely competitive enzyme inhibitors. Novel AChE inhibitors are targeted against this site (Silman and Sussman, 2005), and their use is, additionally, supported by evidence suggesting that they are involved in modulating the APP metabolism (Racchi *et al.*, 2004; Zimmermann *et al.*, 2005a; 2005b; Garcia-Palomero *et al.*, 2008).

AChE-T, its C-terminal peptides and neurodegeneration: AChE inhibitors with new functions

With respect to the non-hydrolytic roles of AChE in the context of AD-related amyloid burden, further hypotheses have been put forward for the role of AChE. In view of the non-hydrolytic actions of AChE in development, its known involvement in AD pathology and its functional parallels to APP – both are secreted from neurons, have trophic action and their levels decrease in AD (Greenfield and Vaux, 2002) – molecular similarities between the two proteins were investigated. At the same time, a role for two AChE C-terminal peptides (14 and 30 amino acids (aa) in length and, thus, denominated T14 and T30, respectively) in neurodegeneration were hypothesized (Greenfield *et al.*, 2008). AChE-T as well as T14/T30 exhibit, depending on dose and exposure time, trophic or toxic actions. These were seen in hippocampal neurons as well as organotypic slices, and could be related to opening, specifically and selectively, the L-type Ca^{2+} channel (Day and Greenfield, 2002; 2003; Bon and Greenfield, 2003; Emmett and Greenfield, 2004; Greenfield *et al.*, 2004; Zbarsky *et al.*, 2004).

In order to further corroborate their hypothesis, Greenfield and colleagues explored the possibility of there existing a form (and role) of AChE-T, in development and degeneration, having undergone cleavage as suggested by the metabolism of the homologous APP sequence. Such an approach was encouraged by the identification of a truncated, 543 to 547 aa long form of AChE-T in development (Saxena *et al.*, 2003). This form is supposedly the result of proteolytic cleavage (Camp *et al.*, 2010) – a likely assumption, given that a similar mechanism has been detected for BuChE (Blong *et al.*, 1997). Cleaving the T30 peptide – which by itself is, as referenced above, implicated in both developmental and neurodegenerative processes *in vitro* – off the enzyme's C-terminus would yield a truncated enzyme (AChE-Tt) devoid of a dimerization favouring cysteine in its remaining C-terminus. Hence, this AChE-Tt would remain monomeric. Such an AChE-T C-terminal shedding seemed then further likely, when considering that the form of AChE relevant in neurodegeneration and development has been identified as being monomeric (Arendt *et al.*, 1992; Inestrosa *et al.*, 1994), and that it has lost its characteristic of substrate inhibition (Arendt *et al.*, 1992; Moreno *et al.*, 1996). In this context, work on a genetically engineered form of truncated AChE-T (Bourne *et al.*, 1999) aimed at uncovering a mechanistic link between the supposedly cleaved, residual AChE-Tt enzyme and the free floating peptides in neurodegeneration (Zimmermann *et al.*, 2008; 2009).

While criticism regarding this work continues to focus on the still lacking *in vivo* evidence of the AChE-T C-terminal peptide(s) (Massoulie *et al.*, 2008), progress has been made regarding evidence for their potential site of action: experiments using a range of nicotinic ACh receptor (nAChR) blockers in various model systems provided indirect proof that T14 binds selectively to an allosteric site on the α7-nAChR [abbreviations for receptors follow the *British Journal of Phamacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2011)], thus, modulating Ca^{2+} influx, which is involved in short-term plasticity and chronic, long-term trophic and toxic effects (Day and Greenfield, 2002; 2003; Emmett and Greenfield, 2004; Greenfield *et al.*, 2004). These actions were sensitive to α7-nAChR blockade in the nanomolar range. Notably, the α7-nAChR is co-expressed along with AChE in the same transient period and within the same brain regions during developmental (Taylor *et al.*, 1994; Broide *et al.*, 1996; Torrao *et al.*, 2000) and degenerative processes (Dineley *et al.*, 2001; Fodero *et al.*, 2004). Moreover, it can bind Aβ (Wang *et al.*, 2000; Dineley *et al.*, 2002; Lain *et al.*, 2005; Dineley, 2007), with this finding, however, having been challenged in cell culture work (Small *et al.*, 2007). All the same, the receptor has been shown to be involved in neuro-

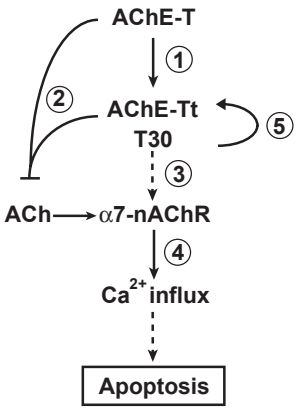

Figure 2

The interrelation between AChE-T and the α7-nAChR, mediated by T30. For numbers, see text; ⟶, induction of or leading to; ⟞, blocking or inhibiting; ⇢, either inducing or inhibiting, depending on concentration.

nal development (Liu *et al.*, 2007) as well as neurodegenerative pathogenesis (Dineley *et al.*, 2001; Nagele *et al.*, 2002; Duda, 2004) and oncogenesis (Schuller, 2009). As regards degenerative processes, the role of both AChE – specifically AChE-T – and the α7-nAChR have been widely discussed (Sternfeld *et al.*, 2000; O'Neill *et al.*, 2002; Jin *et al.*, 2004; Rees *et al.*, 2005; Holzgrabe *et al.*, 2007; Schliebs and Arendt, 2011). Recent work now conclusively reveals that the two AChE C-terminal peptides T14 and, particularly, T30 are involved in binding interactions with the α7-nAChR, modulating its mRNA as well as protein expression (Bond *et al.*, 2009).

Always considering that the above results have been obtained in different *in vitro*, even test tube model systems, they may, taken together, offer a possible scenario for mechanisms active under developmental/apoptotic conditions (Figure 2): AChE-T is cleaved through proteolysis, as has been suggested previously (Saxena *et al.*, 2003), and the C-terminal peptide, T30, is freed (①). The resulting, AChE-Tt remains enzymatically active (②, Zimmermann *et al.*, 2008). T30, in turn, manipulates α7-nAChR expression (③, Bond *et al.*, 2009), and trophic-toxic actions are triggered by means of Ca^{2+} influx (④), depending on the T30 dose (Greenfield *et al.*, 2004). AChE-Tt activity is further enhanced by T30 (⑤; Zimmermann *et al.*, 2008), on which breakdown of α7-nAChR-stimulating ACh is contingent (②). Such a possible scenario would be in agreement with the selective sensitivity of cholinergic neurons to a dysregulation of Ca^{2+} levels (Toiber and Soreq, 2005; Zundorf and Reiser, 2011). Additionally, it would fit to the mandatory role of the α7-nAChR in learning and memory (Thomsen *et al.*, 2010), with neuroprotective effects being mediated via the downstream PI3K/pAkt pathway (Akaike *et al.*, 2010).

In view of the seminal role of AChE-T and α7-nAChR in degenerative processes, the *in vivo* identification of T30 and, to a lesser extent, T14 is eagerly awaited. Still, AChE-T itself has been described to exert toxic effects. For example, specific AChE-T overexpression is linked to programmed cell death (Greenberg *et al.*, 2010), and mice transgenic for human AChE-T carry progressive accumulation of clustered, heat shock protein 70 immunopositive neuronal fragments as well as a high incidence of reactive astrocytes (Sternfeld *et al.*, 2000), and show accelerated stress-related neuropathology including loss of dendritic arborization and spines (Cohen *et al.*, 2002; Meshorer and Soreq, 2006). Likewise, N-extended forms of AChE-T lead to apoptosis (Toiber *et al.*, 2008; 2009), possibly as it is involved in apoptosome formation (Park *et al.*, 2004). While these findings already suggest that AChE inhibitor treatment should avoid leading to an increase in the expression of AChE-T, I will address this question further below, in conjunction with deliberations on changes in AChE-R expression, with these being explored in the following paragraph.

AChE-R, stress-related conditions and neuroprotection: AChE and cholinergic plasticity

In comparison to AChE-T, AChE-R was shown to exert short- to medium-term beneficial effects under acute stress (Kaufer *et al.*, 1998): mice overexpressing AChE-R display normal neuromuscular function and their brains are relatively protected from stress associated pathological hallmarks that would otherwise cause age-dependent neurodeterioration (Berson *et al.*, 2008). Similarly, the C-terminal peptide pertaining to AChE-R, denominated C-terminal fragment of AChE-R (ARP), has been shown to promote neuronal development and plasticity (Dori and Soreq, 2006a). In addition, extensive experimental work could demonstrate a role for AChE-R in peripheral tissue as a marker of stress-related conditions. For example, modified testicular expression of AChE-R predicts male infertility (Mor *et al.*, 2001), while sera of mothers, who just had given birth, reveal increased AChE-R levels *post partum* (Pick *et al.*, 2004). Moreover, AChE-R is expressed in hematopoietic progenitors, and ARP promotes hematopoietic progenitor proliferation (Grisaru *et al.*, 2001), while stress-induced cholinergic signalling promotes inflammation-associated thrombopoiesis (Pick *et al.*, 2006).

However, Jean Massoulié and colleagues could not identify this form of AChE following heat shock, organophosphate AChE inhibition or stress – neither in cell culture nor *in vivo* (Perrier *et al.*, 2005); in addition, immobilization stress of rodents did not lead to detectable levels of AChE-R in their hands (Perrier *et al.*, 2006). However, their *in vivo* work focused on 24 and 48 h, then 7 and 14 days post-stress, while their *in vitro* test was ended immediately after stress. In comparison, Soreq and co-workers reported peaks of AChE activity changes 1 and 2 h post-stress in hippocampal and cortical tissue respectively (Kaufer *et al.*, 1998). Their findings are supported by recent work exploring the role of alternative splicing of AChE under conditions of oxidative stress (Härtl *et al.*, 2011) and other apoptotic stimuli, namely, staurosporine (Li *et al.*, 2012a) and Aβ (Li *et al.*, 2012b). These studies were carried out in a model of cholinergic cells (Pahlman *et al.*, 1984; 1995), which enabled an analysis of conditions that closely matched those in the cell type particularly lost in neurodegeneration of the Alzheimer's type.

The most important findings of this cell culture-focused work were that, firstly, AChE-R levels rise significantly following exposure to mild to moderate oxidative stress (Härtl *et al.*, 2011) or pathophysiological amounts of amyloid (Li *et al.*, 2012b), and that, secondly, AChE-R is released into the cell media in large amounts (Härtl *et al.*, 2011), which is in agreement with its solubility as a monomer.

In these models, controlled apoptotic events are observed, which seem connected to the expression of AChE-R and a time-dependently linked, sharp increase in Bcl-2 (Härtl *et al.*, 2011; Li *et al.*, 2012b). Moreover, AChE-R has been observed in the context of mitochondrial hyperactivity (Mor *et al.*, 2008) and described as interacting with the receptor for activated C-kinase 1, RACK1 and PKC (Dori and Soreq, 2006a). Increased mitochondrial activity provides additional ACh precursor acetyl-coenzyme A. As a consequence, ACh levels rise, as also suggested by the increased choline turnover itself (Kuhar and Murrin, 1978; Jope, 1999) as well as the observed increase in ChAT activity (Li *et al.*, 2012b). Likewise, the known interaction between RACK1 and PKC, promoted by AChE-R, may well drive choline transporter as well as ChAT activity, as they are dependent on their phosphorylation status (Dobransky *et al.*, 2004; Gates *et al.*, 2004; Dobransky and Rylett, 2005; Kim *et al.*, 2006). ACh, in turn, likely exerts protective effects via muscarinic ACh receptors (mAChRs) and PKC activation (Fisher, 2007; Tiong *et al.*, 2010).

Notably, high concentrations of Aβ destroy the balance of the cholinergic players up-regulated following the exposure to low Aβ concentrations, with AChE-R being up-regulated only transiently (Li *et al.*, 2012b). As such, slowly increasing Aβ levels, as is the case in AD, may cause cholinergic adaptation, likely associated with AChE-R expression. Long-term accumulation of Aβ peptides, in contrast, will overwhelm the adaptive capacity of cholinergic neurons, leading to their death. This hypothesis is in agreement with the analysis of human hippocampal tissue samples that revealed a drastic decrease in functional AChE-R (Berson *et al.*, 2008), suggesting that a high dose of Aβ, applied over an extended period of time, impairs this neuroprotective AChE-R. This biphasic phenomenon is further confirmed by work using the phosphorylation inhibitor staurosporine (Li *et al.*, 2012a), which shows that reduced AChE activity may result only under conditions when regulatory effects involving cholinergic adaptation and AChE-R up-regulation, are exhausted, that is, once the system has tipped towards cellular necrosis.

These findings suggest that the selective increase of AChE-R levels may well be desirable in neurodegenerative conditions (Meshorer and Soreq, 2006), especially since chronic AChE-R overexpression has been shown to enhance cognitive performance *in vitro* (Sklan *et al.*, 2006) and *in vivo* (Farchi *et al.*, 2007). Interestingly, treatment with AChE inhibitors presented with an eightfold increase over control levels in AChE mRNA and a concomitant decrease in the mRNAs encoding for ChAT and the vesicular ACh transporter (Kaufer *et al.*, 1999). Stress-related changes in brain microRNA expression, and microRNA-183 in particular, were shown to regulate AChE splicing and cholinergic neurotransmission (Meerson *et al.*, 2010). Therefore, future work should address as to whether and how AChE inhibitors do impact on brain microRNA expression, and whether likely effects differ among the various drugs.

There is evidence of a shift towards AChE-R splicing following muscarinic modulation (Salmon *et al.*, 2005), and recent studies could detect long-lasting AChE splice variant variations in AD patients treated with AChE inhibitors (Darreh-Shori *et al.*, 2004). In particular, donepezil has been associated with decreased levels of AChE-R as compared with AChE-T, whereas rivastigmine, a pseudo-irreversible cholinesterase inhibitor, increases the AChE-R/AChE-T ratio. Even though the clinical effects of these observations still need to be established fully, several authors suggest – in the light of the beneficial effects of AChE-R outlined above – that the synthesis and design of new drugs should aim for the specific up-regulation of AChE-R, but not AChE-T (Greenberg *et al.*, 2010; Pohanka, 2012). In addition, circadian changes in the expression of AChE-T (Erb *et al.*, 2001) and AChE-R (Shaltiel *et al.*, 2013) should be taken into account for therapeutic considerations.

More generally, AChE inhibitors also find application in other types of dementia. For example, rivastigmine showed modest but significant benefits in the treatment of cognitive and neuropsychiatric symptoms in Parkinson's disease dementia (PDD) and dementia with Lewy bodies (DLB; Bullock and Cameron, 2002), and also donepezil showed positive effects (Mori *et al.*, 2012). Indeed, it is noteworthy that AChE alternative splicing has been implicated in PD and Parkinsonism (Benmoyal-Segal *et al.*, 2012), with AChE-R confering resistance to dopaminergic cell death in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model (Ben Shaul *et al.*, 2006). Likewise, the efficacy of targeting specific AChE splice variants as suitable for the therapy of chronic cholinergic malfunctioning has been highlighted for the case of myasthenia gravis: Monarsen, a 20-base antisense oligodeoxynucleotide directed against the human AChE gene is effective in improving muscle action potential in a myasthenia gravis rodent model and showed, in part, dramatic effects in patients afflicted by myasthenia gravis (Dori and Soreq, 2006b; Sussman *et al.*, 2008), where AChE-R serum accumulation is noted (Brenner *et al.*, 2003).

Animal models of modulated AChE activity: evidence for non-enzymatic AChE inhibitors?

Cholinergic activity increases following stress – in various cellular systems (Melo *et al.*, 2003; Tyagi *et al.*, 2010), *in vivo* (Dumont *et al.*, 2006; Saxena *et al.*, 2008) and in clinical studies (Correa *et al.*, 2008) alike. At the same time, ACh itself has been shown to exert neuroprotective effects via mAChRs (Liu *et al.*, 2011) and consequential PKC activation (Fisher, 2007; Tiong *et al.*, 2010). In this paragraph, I would like to turn to questions as to the enzymatic impact of AChE inhibitors. In particular, I want to address whether the limited success obtained with pro-cholinergic therapeutics in conditions such as AD may be linked to their not sufficiently explored non-enzymatic effects.

In view of the drastic loss of cholinergic neurons and the consequential depression of ACh levels in conditions of neurodegeneration (Schliebs and Arendt, 2011), the therapeutic approach of choice remains the inhibition of AChE activity

so as to keep neurotransmitter levels high for as long as possible (Mufson *et al.*, 2008). Yet, both *in vivo* and clinical studies reveal that AChE inhibitor achieved enzyme inhibition rates are far from complete and probably remain below 50% (Giovannini *et al.*, 1998). At the same time, advanced stages of AD present with significantly reduced levels of AChE (Perry *et al.*, 1978; Shinotoh *et al.*, 2000). As such, it is of note that ACh neurotransmitter levels in a genetically engineered mouse model displaying single AChE allele deletion (Xie *et al.*, 1999) are scarcely altered when comparing with levels measured in the wild-type counterpart (Mohr *et al.*, 2012). This data sheds further doubt on an enzyme-related effect of clinically used AChE inhibitor concentrations, especially since it was recently demonstrated that 5 to 10 $mg \cdot day^{-1}$ donepezil administered to AD patients led to only 20–25% of AChE inhibition (Bohnen *et al.*, 2005). These findings may well explain the low efficacy of these drugs, even though individual patients report positive effects (Zimmermann, 2011). Similarly, *in vivo* studies detected no more than 27 to 39% reduction of enzyme activity in rats after treatment with donepezil (1 or 1.5 $mg \cdot kg^{-1}$) (Scali *et al.*, 2002; Cerbai *et al.*, 2007).

Notably, these models cope with reduced enzymatic function by means of cholinergic compensation. In particular, HACU and, thus, choline turnover are significantly increased (Mohr *et al.*, 2012), suggesting an increased ACh firing rate (Jope, 1999). This finding, notably, parallels data from AD brain rapid autopsy (Bissette *et al.*, 1996) and AChE nullizygous mice (Hartmann *et al.*, 2007). It is then noteworthy that AChE inhibitors are more effective in enhancing ACh levels in these heterozygous mice, on a background of already low basal AChE activity (Mohr *et al.*, 2012), which encourages the use of these drugs for the treatment of advanced cholinergic dysfunction of the AD type (Passmore *et al.*, 2005; Roman *et al.*, 2005). What is more, there is evidence that donepezil alleviates disturbances in energy metabolism (Zhou *et al.*, 2001), which is significantly affected in AD (Hirono *et al.*, 2004). In confirmation of such evidence, donepezil increased glucose levels in the AChE heterozygous mice more strongly, and also transiently pushed up choline levels (Mohr *et al.*, 2012), hence, up-regulating the availability of both precursors of ACh, an effect that may contribute to the increase of ACh levels.

Therefore, it is not far fetched to assume that donepezil can influence cholinergic transmission independently of AChE catalytic inhibition. Yet, as an impact on AChE gene expression has to be taken into account as well as aspects of enzyme maturation and turnover, that is, protein levels as compared with actual enzyme activity (Rotundo and Fambrough, 1980; Shaked *et al.*, 2009), enzymatic and non-enzymatic effects cannot easily be distinguished. All the same, such non-catalytic effects may well be mediated by an AChE-R increase, as suggested by the discussed *in vitro* work (Li *et al.*, 2012b). In this context, it should be emphasized that a significant relative increase in AChE-R will have little impact on ACh breakdown in absolute terms, since AChE-R baseline levels are close to zero. As such, the *in vivo* study on AChE heterozygous mice suggests that treatment of AD-related cognitive impairment involves AChE inhibition in early to moderate stages, likely involving non-cholinergic in addition to directly enzyme activity-related effects.

Consequentially, AChE activity *per se* might not be the only target of the AChE inhibitor-related treatment of cholinergic degenerative processes in AD, nor an exclusive indicator of cholinergic degeneration and cell death. Yet, how can we reconcile these findings with the evidence of the role of AChE-T C-terminal fragments in triggering neurodegenerative processes? Again, genetically engineered mouse models might give clues. AChE-T is the predominant form of AChE in the CNS, and it is tethered to the neuronal plasma membrane by the small transmembrane protein PRiMA (Perrier *et al.*, 2002; 2003), which interacts with the WAT domain of the AChE-T C-terminus. Earlier work in a PRiMA knockout mouse had shown that the membrane anchor is necessary for targeting and stabilizing nascent AChE in neurons (Dobbertin *et al.*, 2009). But at the same time, these mice had a phenotype similar to their wild-type counterpart in terms of weight, body temperature and ventilation (Boudinot *et al.*, 2009), thus, further challenging the role of AChE activity in neurodegenerative processes.

Recent work on this mutant reveals a thorough adaptation of the PRiMA knockout mouse to the genetically induced excess of cholinergic neurotransmitter (Farar *et al.*, 2012). These high levels significantly surpass the EC_{50} of mAChRs, but not that of most nAChRs, which may explain the cholinergic adaptation of mAChRs – both in terms of their density and functionality – as was also observed in the case of AChE knockout mice (Li *et al.*, 2003; Volpicelli-Daley *et al.*, 2003a,b). In comparison, a corresponding adaptation of nAChRs is scarcely seen (Volpicelli-Daley *et al.*, 2003a). As such, the question arises as to whether pharmacological intervention targeting the disposition of AChE at the neuronal surface could support cholinergic neurotransmission under circumstances where AChE activity needs to be hampered.

This question becomes even more focussed when studying a genetically engineered mouse model presenting deletion of the AChE-T exon 6, which carries the WAT domain: it similarly lacks functional AChE in the synaptic membrane (Dobbertin *et al.*, 2009; Camp *et al.*, 2010), but reveals a significant phenotype (Camp *et al.*, 2010), even though the originally intended effect of preventing AChE from anchoring to the synaptic membrane by means of interaction with PRiMA corresponds to that achieved in the PRiMA knockout mouse. These findings further suggest the mandatory role of the AChE-T C-terminus in supporting the enzyme's function and the organism's physiological development. That these mice show a significantly reduced number of nAChRs (Girard *et al.*, 2006) only further highlights the intricate relationship between AChE-T and the nAChR.

Summary, conclusion and outlook: AChE in neurodegeneration, AChE inhibitors in neuroprotection

Various hypotheses have been put forward and controversially discussed in relation to the pathogenesis of AD (Hardy *et al.*, 1986; Hardy and Higgins, 1992; Francis *et al.*, 1999; Hardy and Selkoe, 2002; Armstrong, 2011; Reitz, 2012; Takata and Kitamura, 2012). At the same time, recent deliberations suggest that atrophy in the cortex and hippocampus, which

continue to be considered the best determinant of cognitive decline with aging (deToledo-Morrell *et al.*, 2007; Pihlajamaki *et al.*, 2009; Schliebs and Arendt, 2011; Li *et al.*, 2012c), results from a combination of AD pathology, inflammation, Lewy bodies and vascular lesions (Fotuhi *et al.*, 2009). Likewise, altered alternative splicing seems intricately involved in pathogenetic mechanisms of neurodegenerative conditions (Ferraiuolo *et al.*, 2011; Gagliardi *et al.*, 2012; Mills and Janitz, 2012). Against this background – and in the light of the insights collected here, there seems to be an urgent need to understand, firstly, whether and how strongly cholinergic cell death features in a broad range of neurodegenerative conditions, not least since cholinergic impairment is most convincingly related to cognitive decline (Pinto *et al.*, 2011), and, secondly, whether and to what extent we can see a converging cholinergic picture in neurodegeneration. Conditions have to be determined, under which AChE variants trigger apoptotic processes or exert protective functions, with the pharmacologist's final aim being to further the activation of the protective variant.

Figure 3 summarises the findings discussed here and suggests both enzymatic and non-enzymatic roles for AChE inhibition: while stress-induced AChE activity (②) depresses ACh levels (⑤), neurotransmitter stores are replenished by stress-mediated induction of HACU and ChAT (③), as well as via AChE-R (① and ④) and, linked, mitochondrial hyperactivity. Neuroprotective ACh effects are mediated by both the α7-nAChR (⑥) and mAChRs (⑦). In the event of prolonged or enhanced stress, AChE-R-relayed synthesis of ACh via PKC and RACK1 (④) is exhausted (its half-life is significantly shorter than that of AChE-T) so that protective ACh signalling (⑥ and ⑦) is not achieved any more. Apoptosis (or, in the case of high concentrations of toxic agents or prolonged exposure times, necrosis) sets in (⑧). Regardless of the ongoing debate about the actual *in vivo* existence of AChE-T C-terminal peptides as well as a form of AChE-Tt, the scenario set out could be complemented by data on AChE-T C-terminal fragments (see also Figure 1): a stress-related rise in AChE-T leads, following proteolysis, to increased levels of T30. The peptide induces and interacts with the α7-nAChR, which, in turn, mediates protective effects. In the event of excessively rising AChE-T levels and, hence, activity, blocking of the α7-nAChR is brought about.

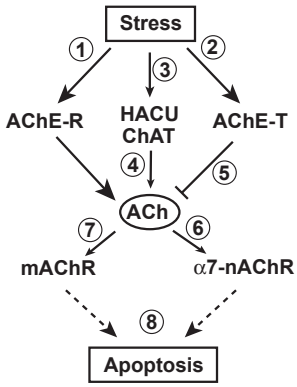

Figure 3

A possible scenario for how cholinergic dynamics involving AChE-R and AChE-T might stave off cell death. For numbers and the role of AChE inhibition, see text; ⟶, induction of or leading to; ⟞, blocking or inhibiting; ⇢, either inducing or inhibiting, depending on concentration. The figure omits that a stress-related increase in AChE-T leads, following proteolysis, to increased levels of T30, which, in turn, impacts on the α7-nAChR as well.

This schematic presentation is an attempt to put the individual findings collected in this review article into a coherent picture of cholinergic mechanisms. Yet, it does not want to suggest a representation of a general action network of AChE-R and AChE-T in all stress-related conditions. Nevertheless, several targets of AChE inhibitors should be considered in the light of the insights assembled here: AChE inhibition will reduce, in the early stages of the cellular demise, AChE activity, consequentially leading to ACh levels remaining high. Likewise, inhibition of AChE activity (AChE-T and AChE-Tt) reduces choline levels, which are also discussed as being implicated in α7-nAChR activation (Alkondon *et al.*, 1997). Further effects may derive from altered AChE-R gene expression following the exposure to AChE inhibitors.

In view of the non-enzymatic mechanisms put forward for the mode of action of AChE inhibitors, it is necessary to understand whether and how they impact on the cholinergic and AChE variant scenario active in conditions such as PDD and DLB. This understanding will support the development of directly related therapeutic strategies that take into account AChE as a key modulator of the cholinergic system, both on an enzymatic and a non-hydrolytic level.

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Conflict of interest

The author has no conflict of interest to declare.

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