INVITED REVIEW

NON-CLASSICAL ACTIONS OF CHOLINESTERASES:
ROLE IN CELLULAR DIFFERENTIATION, TUMORIGENESIS
AND ALZHEIMER'S DISEASE

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Abstract—The cholinesterases are members of the serine hydrolase family, which utilize a serine residue at the active site. Acetylcholinesterase (ACHE) is distinguished from butyrylcholinesterase (BChE) by its greater specificity for hydrolysing acetylcholine. The function of ACHE at cholinergic synapses is to terminate cholinergic neurotransmission. However, ACHE is expressed in tissues that are not directly innervated by cholinergic nerves. ACHE and BChE are found in several types of haematopoietic cells. Transient expression of ACHE in the brain during embryogenesis suggests that ACHE may function in the regulation of neurite outgrowth. Overexpression of cholinesterases has also been correlated with tumorigenesis and abnormal megakaryocytopenisis. Acetylcholine has been shown to influence cell proliferation and neurite outgrowth through nicotinic and muscarinic receptor-mediated mechanisms and thus, that the expression of ACHE and BChE at non-synaptic sites may be associated with a cholinergic function. However, structural homologies between cholinesterases and adhesion proteins indicate that cholinesterases could also function as cell-cell or cell-substrate adhesion molecules. Abnormal expression of ACHE and BChE has been detected around the amyloid plaques and neurofibrillary tangles in the brains of patients with Alzheimer's disease. The function of the cholinesterases in these regions of the Alzheimer brain is unknown, but this function is probably unrelated to cholinergic neurotransmission. The presence of abnormal cholinesterase expression in the Alzheimer brain has implications for the pathogenesis of Alzheimer's disease and for therapeutic strategies using cholinesterase inhibitors. Copyright © 1996 Elsevier Science Ltd

The cholinesterases have enthralled researchers for more than 80 yr. It is probably correct to state that more is known about the structure, activity and expression of cholinesterases than any other group of enzymes. The study of cholinesterases has played a pivotal role in neurobiology and biochemistry. As they are prototypes of hydrolytic enzymes that utilize a serine residue at the catalytic site (serine hydrolases), there has been an enormous amount of research on their structure. The ease and sensitivity with which cholinesterases can be detected has made cholinesterase histochemistry a popular method for staining structures in the brain and peripheral nervous system. For this reason, there are many studies describing the distribution of cholinesterases in tissues.

However, despite the wealth of information on cholinesterase structure and expression, the biological functions of cholinesterases in many tissues are unknown. The principal function of acetylcholinesterase (ACHE) at the neuromuscular junction is to hydrolyse acetylcholine (ACh) and to inactivate cholinergic neurotransmission. However, the function of ACHE in tissues that are not innervated directly by cholinergic nerves is unclear.

The aim of this article is to review the literature on functions of cholinesterases that are unrelated to neurotransmission. The article also reviews the relevance of these functions to the therapy of neuro-
The role of cholinesterases in neurotransmission evolved from the studies of early pioneers such as Sir Henry Dale and Otto Loewi. Dale helped to establish the role of ACh as a neurotransmitter at synapses in autonomic ganglia and skeletal muscle (Dale, 1914). Using a perfused frog heart, Loewi (1921) identified an active factor that slowed the heartbeat, and which was later shown to be ACh. Studies by Dale (1914) were the first to demonstrate that ACh was rapidly hydrolysed by an endogenous enzyme. Later studies showed that physostigmine prolonged the physiological effects of ACh. Studies by Dale and Loewi (1914) helped to establish the concept of chemical neurotransmission.

2. ENZYME ACTIVITY

The cholinesterases are a family of enzymes that hydrolyse choline esters. This family is subdivided into the acetylcholinesterases (AChE) and the butyrylcholinesterases or pseudocholinesterases (BChE). AChE and BChE can be distinguished by their relative abilities to hydrolyse different choline esters (Alles and Hawes, 1940). AChE cleaves ACh most actively and is much less potent in hydrolysing other alkyl esters such as butyrylcholine. In contrast, BChE exhibits much less substrate specificity as it is able to hydrolyse ACh (almost as well as AChE) as well as many other alkyl choline esters. AChE and BChE are classified as serine hydrolases because they use a serine residue at the active site to initiate binding to the substrate (Quinn, 1987). Cleavage of ACh at the ester bond of ACh results in the production of acetate and choline. The mechanism of catalysis for AChE and BChE is different from that observed for serine proteases such as chymotrypsin and subtilisin, which have a different three-dimensional structure at their catalytic sites (Matthews et al., 1967; Wright et al., 1969). However, all of these enzymes rely on a catalytic triad or charge relay system involving a serine, a histidine and an acidic residue at the catalytic site (Carter and Wells, 1988; Schrag et al., 1991;
Shafferman et al., 1992). In AChE, the catalytic triad consists of serine-200 (using the Torpedo AChE residue numbers), histidine-440 and glutamate-327 (Shafferman et al., 1992).

Catalysis by cholinesterases is an acid–base reaction that involves a two-step reaction with the generation of a single proton (Rosenberry, 1975; Quinn, 1987). The first step involves the formation of an acyl intermediate between the serine residue and the substrate. The second step involves deacylation with nucleophilic attack upon the acyl intermediate by a molecule of water. The turnover number of AChE is approx. 10^6 M⁻¹ s⁻¹, which is the fastest of all the serine hydrolases and is one of the fastest of all enzymes (Quinn et al., 1991). Because of the high specific activity of AChE, it is relatively easy to assay the enzyme. Indeed, simple radiometric assays can easily measure as little as a few attomoles of AChE (Johnson and Russell, 1975).

3. GENE STRUCTURE

The first AChE sequence to be cloned was from Torpedo californica electric organ (Schumacher et al., 1986b). cDNAs encoding Torpedo marmorata (Sikorrav et al., 1987), Drosophila (Hall and Spierer, 1986), Anopheles stephensi (Hall and Malcolm, 1991), C. elegans (Arpagaus et al., 1994), mouse (Rachinsky et al., 1990), rat (Legay et al., 1993), human (Soreq et al., 1990) and chicken AChE (Randall et al., 1994) have been reported. The amino acid sequence of bovine AChE was determined by direct protein sequencing (Doctor et al., 1990). In vertebrates, AChE and BChE are each encoded by a single gene. The AChE gene is located on the long arm of chromosome 7 (7q22) in man (Getman et al., 1992) and on chromosome 5 in the mouse (Rachinsky et al., 1992). The mammalian AChE gene contains six exons. Exon 1 contains the 5′ untranslated region and the open reading frame begins in exon 2. Splicing of exons 5 and 6 regulates the assembly of certain molecular isoforms of AChE (Maul et al., 1990). In the AChE gene of lower vertebrates (e.g. Torpedo) and the BChE gene the entire open reading frame is encoded by three exons (compared with four exons in the mammalian AChE gene). The sequences of human (Arpagaus et al., 1990), mouse (Arpagaus et al., 1991) and rabbit BChE (Jbilo and Chatonnet, 1990) have been deduced from their cDNAs.

A comparison of the deduced amino acid sequences of AChE and BChE genes reveals extensive homologies, suggesting that the AChE and BChE genes arose by evolution from a common precursor. Indeed, invertebrates like Drosophila possess only a single cholinesterase gene, which produces a protein that is intermediate between AChE and BChE in its substrate specificity and inhibitor sensitivity (Toutant, 1989). The cholinesterases belong to a family of homologous proteins which includes the serine carboxyestrases (Korza and Ozols, 1988; Long et al., 1988; Jones et al., 1994), pancreatic lysophospholipase (Han et al., 1987), hormone-sensitive lipase (Hemila et al., 1994), two Drosophila adhesion proteins known as glutactin (Olson et al., 1990) and neurotactin (Barthalay et al., 1990; De La Escalara et al., 1990) and thyroglobulin (Takagi et al., 1991). The most highly conserved regions of AChE and BChE contain residues which form part of the active site. For example, the active serine residue (at position 203 in the human sequence) is found within the amino acid sequence GESAG, that is conserved in all cholinesterases. The disulphide bonding pattern (MacPhee-Quigley et al., 1986) is also highly conserved.*

The promoter regions of human and rabbit BChE (Jbilo et al., 1994b) and mouse and human AChE (Ben Aziz-Aloya et al., 1993; Mutero et al., 1995) have been sequenced. The BChE promoter contains an AP-1 site, and the AChE promoter an SP-1 site, suggesting that both BChE and AChE may have important developmental functions in the regulation of cellular growth and differentiation. This role will be discussed later in this review.

4. SECONDARY AND TERTIARY FOLDING

In 1991, Sussman et al. (1991) crystallized the catalytic subunit of the GPI-linked (dimeric amphipathic) AChE from T. californica and examined its structure using x-ray crystallography at 2.8 Å resolution (Fig. 1). The crystal structure of AChE and other homologous proteins reveals a common secondary structural motif at the active site, known as an αβ hydrolase fold (Ollis et al., 1992). Surprisingly, the active serine residue was found within a deep gorge lined with hydrophobic residues [Fig. 1(A)]. The presence of these hydrophobic residues may help to direct Ach to the active site, thus accounting for the rapid turnover rate of the enzyme. It remains unclear whether diffusion per se is sufficient to account for the removal of the positively charged choline moiety from

* Recently, two more proteins with structural similarity to cholinesterases have been reported. These proteins are neuropilin (Ichtchenko et al., 1995) and gliotactin (Auld et al., 1995).
Fig. 1. Three-dimensional structure of *Torpedo californica* AChE as determined by x-ray crystallography (Sussman et al., 1991). The structure was displayed using RasMol Version 2.5 molecular modelling software (Roger Sayle, U.K.). Two views of the structure are shown. In the first view (A), residues lining the active site gorge are shown in a space-filling model. The three residues of the catalytic triad (ser-200, glu-327 and his-440) are found at the bottom of the gorge. In the second view (B), the backbone of the polypeptide chain is represented by a strand. This view shows that the globular structure consists of both $\alpha$ helix and $\beta$ sheet.

The concept that cholinesterases have functions unrelated to cholinergic neurotransmission is now firmly supported by experimental evidence. There are essentially five separate lines of evidence that indicate that cholinesterases have non-classical functions.

1. The topography of cholinesterase expression does not always correlate with the topography cholinergic synapses. Furthermore, the developmental pattern of cholinesterase expression does not always occur coordinately with synaptogenesis.

2. Cholinesterases exist in multiple molecular forms (discussed later). It is unclear why there should be multiple isoforms of AChE if the enzyme has only a single function.

3. Cholinesterases have structural similarity to cell adhesion molecules. This suggests that they may have functions in the regulation of cell–cell or cell–substrate interaction.

4. Cholinesterases have been shown to have direct actions on several different types of cells. These actions are apparently unrelated to a neurotransmission role.

5. There is increasing evidence that that ACh has functions unrelated to neurotransmission. Thus
cholinesterases may be expressed at non-synaptic sites to regulate non-classical actions of ACh.

Each of these points will be discussed in more detail in the following sections.

Distribution of cholinesterases

For a thorough analysis of the distribution of cholinesterases in vertebrate and invertebrate tissues, there is an excellent monograph by Silver (1974). More recent exhaustive reviews are also available (Toutant, 1989; Massoulié et al., 1993).

The presence of cholinesterases in tissues that are not cholinergically innervated provides the most compelling evidence for the non-classical actions of AChE and BChE. The most striking example of this is the presence of cholinesterases in unicellular organisms (Sastry and Sadavongvivad, 1979). ACh-hydrolysing activity has been found in *Paramecium* (Bayer and Wense, 1936), *Tetrahymena geleit* (Seaman and Houlihan, 1951), *Tetrahymena pyriformis* (Schuster and Herschenov, 1969) and in *Physarum polycephalum* (Nakajima and Hatano, 1962). To explain the presence of cholinergic enzymes in unicellular organisms Augustinsson and Gustafson (1949) have proposed that ACh and AChE may regulate ciliary movement. However, the presence of cholinesterase activities in unicellular organisms must be viewed with caution. Because the proteins that hydrolyse ACh in these studies have not been characterized, it is possible that they may be unrelated to the cholinesterase family. Nonetheless, it seems likely that some sort of ACh-hydrolysing enzyme is present in these organisms. Also surprising is the detection of an AChE-like activity in lichens (Raineri and Modenesi, 1986), pea chloroplasts (Roshchina, 1988) and in *Vicia faba* L., *Nicotiana glauca* Graham and *Kalanchoe diagremontiana* (Madhavan and Sarath, 1995).

The presence of cholinesterases in primitive organisms lacking a nervous system suggests that the cholinesterases (and perhaps ACh) may predate the development of the nervous system. According to Pantin (1956), neuronal cells may have adapted AChE and ACh to serve in neurotransmission. Indeed, this concept is very logical. It seems highly unlikely that primitive organisms would have evolved both the neuronal architecture and a neurotransmitter system at exactly the same point in evolution. It is far more likely that organisms would have adapted a pre-existing biochemical system for the purposes of neurotransmission.

Cholinesterase activity has also been reported in sponges (Lentz, 1966), which possess a very primitive nervous system that lacks synapses or nerve endings. Again, it is unlikely that the cholinesterases have any neurotransmission function in sponges. Although there have been studies that have failed to identify cholinesterase activity in sponges (Bullock and Nachmansohn, 1942), the failure to detect activity in these early studies may be due to the low sensitivity of the assays employed.

Although the presence of cholinesterases in unicellular organisms is controversial, this is not the case in some invertebrates. It is well established that AChE is expressed in *Caenorhabditis elegans* (Johnson et al., 1988), *Schistosoma mansoni* (Espinosa et al., 1991), *Steineremema carpocapsae* (Arpagaus et al., 1992), *Nectator americanus* (Pritchard et al., 1991, 1994) and *Trichosonstrongylus colubriformis* (Griffiths and Pritchard, 1994). AChE is present in very high concentrations in the excretory secretory products of nematodes (Pritchard et al., 1991; Blackburn and Selkirk, 1992), where it is unlikely to have any role in cholinergic neurotransmission.

The differential localization of AChE expression and cholinergic synapses is not limited to invertebrates, but is also evident in higher vertebrates, including mammals. For example, very high levels of AChE or BChE are found in plasma (Stedman et al., 1932; Augustinsson, 1955; Hodgson and Chubb, 1983), erythrocytes (Stedman and Stedman, 1935; Ott et al., 1975; Futerman et al., 1985; Roberts et al., 1987; Toutant et al., 1989; Richier et al., 1992), lymphocytes and thymocytes (Méflah et al., 1984; Rubinstein et al., 1984; Paldi-Haris et al., 1990; Rossi et al., 1991; Richier et al., 1992; Fitzgerald and Costa, 1993; Costa et al., 1994), megakaryocytes (Jackson, 1973; Paulus et al., 1981; Paolotti et al., 1992) and platelets (Jackson, 1973; Sanchez-Yague et al., 1990).

It is equally difficult to account for the presence of AChE in the adrenal medulla (Lewis and Shute, 1969; Chubb and Smith, 1975; Somogyi et al., 1975; Carmichael, 1984; Bon et al., 1990; Small et al., 1993; Michaelson et al., 1994). Although the adrenal gland is cholinergically innervated by the splanchnic nerve (Rahwan and Borowitz, 1973), there are several studies which indicate that the AChE secreted from adrenal chromaffin cells is not required for the inactivation of the cholinergic response (Mizobe and Livett, 1982, 1983; Michaelson et al., 1994). Chromaffin cells express two different forms of AChE on their cell surface (Michaelson et al., 1994). It is difficult to explain the need for two different types of AChE to perform a single function (i.e. inactivation of acetylcholine released by the splanchnic nerve). In
addition to the endogenous AChE, the adrenal chromaffin cells secrete a soluble tetrameric AChE (Chubb and Smith, 1975). This secretion seems unrelated to a function in the inactivation of neurotransmission. Similarly, although AChE is also found in sympathetic neurons (Ferrand et al., 1986; Schotzinger and Landers, 1988; Verdière-Sahuque et al., 1990), its subcellular localization is not consistent with a function in neurotransmission (Rotundo and Carbonetto, 1987).

Even within the central nervous system, the function of AChE is not always apparent. There are regions of the brain that express high levels of AChE but receive little cholinergic innervation (Eckenstein and Sofroniew, 1983; Levey et al., 1983). Although all cholinergic neurons contain AChE, not all AChE-positive neurons are cholinergic. For example, in the zona incerta of the rat brain, numerous AChE-containing neurons are present, but very few cholinergic nerve terminals can be identified (Eckenstein and Sofroniew, 1983). There is also disparity between AChE localization and cholinergic innervation in the lateral hypothalamus, dorsal raphe nucleus and substantia nigra (Levey et al., 1983). In the striatum of the cat, AChE is localized in “striosomes” that form mosaic patterns which register with certain neuropeptides (Graybiel et al., 1981). It is noteworthy that the distribution of choline acetyltransferase is complementary to that of AChE. In the cerebellum, the discrepancy between AChE expression and cholinergic innervation is also seen. There are high levels of AChE in many regions of the cerebellum (Friede and Fleming, 1964; Kása and Csíllik, 1965; Nicolet and Rieger, 1982). However, the presence of high levels of AChE is curious, considering that cholinergic innervation is low.

Cholinesterases are not localized solely in neurons, but are also expressed in some glial cells. BChe has been identified in glial cells and gliomas (Kása and Csíllik, 1966; Lewis and Shute, 1969; Robinson, 1969; Razon et al., 1984; Dubovy, 1991; Haninec and Dubovy, 1992). However, AChE can also be found in some astrocytes (Hebb et al., 1953; Palmer and Ellerker, 1961; Abrahams and Edery, 1964; Hess, 1972; Silver, 1972; Mesulam, 1995). The function of AChE and BChE in glia is unknown.

Diversity of molecular forms

Both AChE and BChE exist in multiple isoforms, which have different molecular weights, solubilities, and subunit associations (Massoulié and Bon, 1982) (Fig. 2). Broadly speaking, AChE exists in two major groups, the homomeric and heteromeric species. Homomeric species contain between one and four identical catalytic subunits that may be either hydrophilic (soluble) or amphiphilic (membrane-bound). Heteromeric species are similar to the homomeric forms. However, they are anchored to membranes by a non-catalytic subunit that is the product of a separate gene. For example, the asymmetric species are anchored to the cell surface or basement membranes via a collagen-like “tail” subunit. A cDNA encoding the collagen-like tail has been cloned from Torpedo (electric ray) (Krejci et al., 1991a), but no mammalian homologue of this protein has been identified. Asymmetric forms of AChE are found in all vertebrates, but are not present in invertebrate species (Massoulié and Bon, 1982). Asymmetric forms may contain 1, 2 or 3 tetramers (i.e. 4, 8 or 12 catalytic subunits) (Vigny et al., 1978). The collagen-like tail contains three polypeptide subunits (Bon and Massoulié, 1976).

The globular isoforms (G-forms) of AChE represent all other forms that do not have a collagen-like component. These forms can be monomers, dimers and tetramers and may be soluble (hydrophilic) or membrane-associated (amphiphilic). Some amphiphilic forms are anchored to membranes via a glycosylphosphatidylinositol (GPI) moiety (Silman and Futerman, 1987). GPI-linked forms predominate in Drosophila and mammalian erythrocytes (Futerman et al., 1985; Toutant, 1989) and are expressed in high levels on the surface of bovine chromaffin cells (Michaelson et al., 1994).

The expression of amphiphatic globular AChE species is regulated by RNA splicing of the last two coding exons (exons 5 and 6 in the human AChE gene). mRNA containing exon 5 encodes a form of AChE with a C-terminal amino acid sequence that directs the addition of a GPI anchor (Gibney and Taylor, 1990). The C-terminal sequence is first cleaved by a protease and then a GPI anchor is added to the C-terminal amino acid residue (Ferguson and Williams, 1988). Bovine serum contains a 115 kDa phosphatidylinositol-glycan-specific phospholipase D that can release GPI-linked AChE from membranes (Heller et al., 1994). cDNAs containing exon 6 (rather than exon 5) encode other known forms of AChE. Amphiphilic tetrameric globular AChE is anchored to membranes by a 20 kDa hydrophobic subunit (Boschetti et al., 1994), whereas the major secreted form of AChE is a hydrophilic tetrameric isoform (Hodgson and Chubb, 1983). Globular isoforms of AChE may also be linked to membranes by palmitoylation (Randall, 1994).

It would be difficult to account for the presence of multiple molecular forms of AChE if the sole function
Fig. 2. Diagrammatic representation of the major isoforms of AChE (figure adapted from Taylor, 1993). Homomeric species may be soluble (G1, G2, G4) or membrane-bound (G2 amphiphilic). Heteromeric species may also be membrane-bound, anchored by a 20 kDa non-catalytic subunit (G4) or via a collagen-like tail subunit containing three polypeptide chains each of which can bind four catalytic subunits (A12). Other isoforms which are not depicted include palmitoylated species and GPI-linked monomeric species.

of the enzyme were the termination of cholinergic neurotransmission. There is little evidence to suggest that different isoforms of AChE differ significantly in their catalytic properties. Thus the presence of different membrane-anchoring mechanisms may be a means by which cells can translocate AChE to different cell-surface and extracellular loci. Studies on the expression of AChE in bovine adrenal chromaffin cells demonstrate that membrane-bound tetrameric and dimeric (GPI-linked) isoforms differ in their routes of intracellular trafficking (Michaelson et al., 1994). Expression of a collagen tail subunit directs AChE to the cell surface or to the extracellular matrix.

Structural homologies to adhesion proteins

Both AChE and BChE share amino acid sequence homology with two Drosophila adhesion proteins known as glutactin (Olson et al., 1990) and neurotactin (Barthalay et al., 1990; De La Escalara et al., 1990) (Fig. 3) (see footnote p. 455). The C-terminal domain of neurotactin and the N-terminal domain of glutactin are homologous to serine esterases, but lack the serine residue responsible for catalytic activity. Neurotactin is expressed during embryogenesis at the blastoderm stage. Later, its distribution becomes more restricted, being present at points of cell–cell contact (Hortsch et al., 1990). Cells transfected with neurotactin do not aggregate, indicating that the protein does not mediate homophilic cell adhesion. Instead, the transfected cells bind to a subpopulation of embryonic cells, suggesting that there is a specific ligand that binds to neurotactin (Barthalay et al., 1990). The presence of three copies of the tripeptide leucyl–arginyl–glutamyl sequence (LRE), known to be associated with S-laminin binding, supports this conclusion. Interestingly, an LRE sequence also occurs between residues 266–268 in Torpedo AChE (Schumacher et al., 1986a) (Fig. 3). Although the similarity between the amino acid sequence of AChE and that of neurotactin extends throughout the AChE sequence [Fig. 3(A)], if the homologous residues are mapped in the three-dimensional structure, only one face of the enzyme is structurally similar [Fig. 3(B)]. This suggests that this region of AChE, which contains the N-terminus, may have adhesion functions.

The homology between cholinesterases and adhesion molecules also extends to their glycosylation. Certain AChE species possess an HNK-1 carbohydrate epitope (Bon et al., 1987) that is a common motif of many cell adhesion molecules. Although the precise function of the HNK-1 epitope is unknown, it has been linked to cell differentiation and migration processes (Jungalwala, 1994). Studies on serum BChE suggest that the presence of an O-linked HNK-1 carbohydrate epitope is associated with a non-catalytically active form of the enzyme (Layer et al., 1995). The presence of the HNK-1 epitope has been defined as a hallmark of a protein associated with an adhesion function (Tucker et al., 1988). The possibility that cholinesterases have adhesion functions supports a role in neuronal development (see Section 7).
**Fig. 3.** Structural similarity between *Torpedo* AChE and *Drosophila* neurotactin. (A) Alignment of the amino acid sequences of AChE (residues 1-535) and neurotactin (residues 349-835). Amino acid residues are shown using the single letter abbreviation. Gaps are introduced for maximum alignment. The position of all cysteines is conserved in both sequences. Amino acid sequence for *Torpedo* AChE is from Schumacher *et al.* (1986a) and for neurotactin, from De La Escalera *et al.* (1990). The LRE sequence known to be an adhesion motif for S-laminin binding is marked with asterisks. (B) Mapping of the homologous residues on the three-dimensional structure of AChE. Homologous residues are shown in a space-filling representation. Note that only one face of the three-dimensional structure has structural similarity to neurotactin.

6. FUNCTIONS UNRELATED TO CATALYTIC ACTIVITY

The search for alternative functions of cholinesterases unrelated to neurotransmission has led to several theories suggesting that cholinesterases have activities or functions unrelated to their ability to hydrolyse ACh. This view is most strongly held for BChE, which shows less specificity for hydrolysing ACh than AChE and which is not specifically localized at cholinergic synapses. However, the lack of localization to cholinergic synapses does not necessarily imply that the cholinesterase in this region does not hydrolyse ACh. ACh can also be released at non-synaptic sites (Lauder, 1993), where it may have important actions unrelated to neurotransmission (Sastry and Sadavongvivad, 1979). This point will be discussed later in relation to cholinesterase functions. Although a number of studies have indicated that AChE and BChE have actions which are distinct from their catalytic activities, it is not always clear whether these actions are "intrinsic" or whether they are associated with "trace contaminants". It is of vital importance in these types of studies to demonstrate that an *in vitro* action is associated with enzyme preparations of the highest purity. Even a recombinant protein can be contaminated with components from...
the host cell in which the recombinant material was expressed. Rigorous analysis of this possibility reduces the risk that trace amounts of contaminating proteins may account for an observed “non-cholinergic” function.

Protease and amidase activities

A number of studies have suggested that cholinesterases cleave substrates other than ACh. Although it is generally considered that the natural substrate for AChE is ACh, the enzyme can hydrolyse a wide variety of choline and non-choline esters (Adams, 1949). The structural similarity between ester and amide bonds and the fact that serine hydrolases are known to cleave both types of bonds has raised the possibility that AChE may act as an amidase. Amidase activity was demonstrated to be associated with AChE by Moore and Hess (1975) and an aryl acylamidase activity was reported by Fujimoto (1976) and it was later shown to be associated with AChE from several sources (George and Balasubramanian, 1980). A more recent study (Checler et al., 1994) has demonstrated that this amidase activity is intrinsic and not due to the presence of contaminating enzymes.

The presence of amidase activities associated with AChE raises the possibility that AChE may cleave peptide bonds. A peptidase activity associated with AChE was first reported by Chubb et al. (1980), who found that affinity-purified preparations of AChE were capable of cleaving substance P. The ability of highly purified BChE to cleave substance P was reported by Lockridge (1982). The peptidase associated with Electrophorus and fetal bovine serum AChE was found to be weak and fairly non-specific in its specificity (Chubb et al., 1983). Subsequent studies showed that polypeptides such as proenkephalins (Millar and Chubb, 1984; Dowton and Boelen, 1988), chromogranins (Ismael et al., 1986; Small et al., 1986) and the amyloid protein precursor of Alzheimer’s disease (Small et al., 1991) were cleaved by this “peptidase” activity. The activity was mapped to a site distinct from the esteratic site on AChE (Small and Chubb, 1988) and it was found that there were two distinct peptidase/protease activities, one a trypsin-like activity and the other a carboxypeptidase-like activity (Small et al., 1987; Small, 1988).

Doubt about whether the protease activity was intrinsic to AChE came from different studies. When
the first AChE cDNA sequence was cloned (Schumacher et al., 1986a), no significant amino acid sequence homology with proteases was identified. Furthermore, Checler and Vincent (1989) reported partial separation of the protease activity associated Electrophorus AChE from the esterase activity by immunoprecipitation. Other studies have confirmed this finding (Carroll and Emmerling, 1991; de Serres et al., 1993). However, partial separation of the protease activity from AChE did not eliminate the possibility that the protease activity which remained with the purified esterase was intrinsic. The suggestion by Small and Simpson (1988), that the esterase might be a precursor of a protease form, raised the possibility that the esterase and protease activities might be separable, but still be products of the same gene. However, Michaelson and Small (1993) demonstrated complete separation of the protease and esterase activities from fetal bovine serum AChE by chromatography, and partial separation by immunoprecipitation. Subsequently, Checler et al. (1994) demonstrated that the protease activity could be completely eliminated from Electrophorus AChE. Taken together, these studies provide convincing evidence that the protease or peptidase activities associated with AChE are due to contaminants which bind the enzyme with an indeterminate affinity.

The nature of the peptidase activity associated with BChE is still unclear. Studies by Chatonnet and Masson (1985) suggested that the peptidase activity which cleaves substance P contained an active serine, with structural similarities to the esteratic site. However, as observed for AChE, the number of peptidase active sites was lower than the number of esteratic sites, indicating that the peptidase and esterase activities could not be localized on all of the BChE monomers. However, a study by Nausch and Heymann (1985) showed that serum BChE is contaminated with dipeptidyl peptidase IV, which could account for the peptidase activity. Separation of the esterase and peptidase activities of serum BChE was demonstrated by Checler et al. (1990). Nonetheless, other reports, principally from the group of A. S. Balasubramanian, suggest that BChE’s peptidase activity is intrinsic (Boopathy and Balasubramanian, 1987; Rao and Balasubramanian, 1990, 1993; Balasubramanian, 1991).

It has been argued that BChE may be a vestigial enzyme with no function in higher vertebrates, on the basis that patients lacking active BChE have no abnormal phenotype (Cooper, 1994, 1995). This point of view has been disputed (Small, 1995). It is interesting to note that Tsim et al. (1988) reported that asymmetric forms of AChE from chick muscle also possess BChE activity. The discovery of an AChE/BChE hybrid molecule suggests that at the chick neuromuscular junction the two enzymes may have inter-related functions. Such a view is also supported by the work of Layer and colleagues who have shown that the expression of AChE is dependent upon BChE expression during the development of the chick nervous system (see Section 7).

Several early reports (Harper, 1952; Hewer and Binning, 1952; Hurley and Munro, 1952) found that a select group of patients showed increased sensitivity to the muscle relaxant drug succinylcholine (suxamethonium). These reports were followed by a study (Evans et al., 1952) that showed a relationship between succinylcholine sensitivity and the activity of serum BChE. Low serum BChE levels were found to have a familial inheritance pattern (Lehmann and Ryan, 1956; Lehmann et al., 1963), suggesting that mutations in the BChE gene were responsible. Originally, three major phenotypes were identified. The so-called “atypical” or “dibucaine-resistant” BChE (caused by a mutation of an aspartate to a glycine at residue 70 [McGuire et al., 1989]) was unable to hydrolyse succinylcholine at the normal rate, and was resistant to cholinesterase inhibitors (Kalow and Genest, 1957; Kalow and Gunn, 1957; Kalow and Staron, 1957). Another variant involved a silent gene (Liddell et al., 1962), while a third variant was described on the basis of its resistance to fluoride (Harris and Whittaker, 1961). Today, a large number of variant BChEs have been identified (Neithich, 1966; Garry et al., 1976; Rubinstein et al., 1978; Delbrück and Henkel, 1979; Simpson and Elliott, 1981; Whittaker and Britten, 1987; Krause et al., 1988). Several of the mutations responsible for these variants have been identified (McGuire et al., 1989; La Du, 1989; Lockridge, 1990). The fact that patients lacking BChE activity are phenotypically normal (except for their response to drugs like succinylcholine) raises the possibility that the sole function of the enzyme may be to detoxify foreign compounds. For example, serum BChE has also been implicated in the metabolism of cocaine (Van Dyke et al., 1976; Jatlow et al., 1979). The highest levels of BChE mRNA are found in the liver and lung, which are the principal detoxification sites in humans (Jblo et al., 1994a). Although BChE is high in the lungs of some mammalian species, low levels are found in the rabbit lung (Geretzwoff, 1959). The levels of detoxifying enzymes often show considerable species variation, possibly due to the varied diets of different species.
However, there are several reasons to doubt that the sole function of BChE is detoxification. A detoxification function does not explain the highly specific spatial and temporal patterns of expression of BChE in the brain and other tissues. For example, sparse BChE staining has been identified in certain neuronal groups (Friede, 1967; Silver and Wostencroft, 1971), which seems unrelated to a detoxification role. Nor does the detoxification hypothesis explain the existence of AChE/BChE hybrid molecules. In addition, studies by Soreq and colleagues suggest a function for BChE in haematopoiesis (discussed in Section 8), a role which is hard to relate to a simple detoxification function. Thus although BChE may have an important role in detoxifying foreign compounds, it is likely to have other functions as well.

It has often been presumed that BChE does not function to hydrolyse ACh. This assumption is based on three major observations, some of which have already been discussed in this article and elsewhere (Small, 1995). First, BChE is not specific in its ability to hydrolyse ACh. Second, BChE is not specifically localized to cholinergic systems. Third, individuals lacking a functional BChE are phenotypically normal. However, some caution must be used in presuming that ACh is not a natural substrate for BChE. It is apparent that the first two points apply almost as well to AChE as to BChE. AChE hydrolyses a wide range of choline and non-choline esters (Silver, 1974). Furthermore, the fact that AChE and BChE are found in non-synaptic sites does not imply that ACh and cholinergic receptors are not present. As will be discussed later, cholinergic receptors are widespread, and are even localized to certain blood cells. The third argument, that individuals lacking BChE are phenotypically normal, can also be explained. It is known that the knockout of many genes produces no obvious phenotype. Thus, biological systems may have inbuilt redundancy which makes BChE a non-essential enzyme.

7. CHOLINESTERASES AND NEURAL DEVELOPMENT

High levels of AChE and BChE can be detected in the embryonic nervous system well before the period of synaptogenesis (Layer, 1983; Layer and Sporns, 1987; Robertson, 1987; Layer et al., 1988a; Robertson et al., 1989; Wolfgang and Forte, 1989; Small et al., 1992; Robertson and Yu, 1993). The AChE activity expressed before synaptogenesis may later decrease or disappear during the course of development of the embryo. Thus, embryonic expression does not necessarily lead to the expression of the enzyme in the same adult tissues (Layer, 1983; Robertson, 1987; Robertson and Mostamand, 1988; Robertson et al., 1989, 1990, 1991; Hanes et al., 1992; Salceda and Martinez, 1992; Geula et al., 1993). Furthermore, AChE is expressed in regions of the embryonic brain that do not show evidence of later cholinergic neurotransmission (Pannese et al., 1971; Drews, 1975; Fitzpatrick-McElligot and Stent, 1981; Graybiel and Hickey, 1982; Falugi and Raineri, 1985).

Both the presence of AChE in the embryo prior to the establishment of cholinergic neurotransmission, and the expression of AChE in cell types which, when mature, do not express the enzyme, suggest a role for AChE in the control of neuronal differentiation [Fig. 4(A)]. Support for this view comes from experiments showing that inhibition of AChE during embryonic development causes morphological changes, retardation of growth and developmental defects (Wyttengback and Thompson, 1985; Stamper et al., 1988; Meneely and Wyttengback, 1989; Veronese and Pope, 1990; Balduini et al., 1993; Kaltner et al., 1993).

Developmentally regulated (transient) expression of cholinesterases has been observed in a wide variety of systems. Transient expression of BChE by neuroblasts (Layer and Sporns, 1987), neural crest cells (Layer and Kaulich, 1991) and retinal cells (Willbold and Layer, 1992) has also been linked to the regulation of neuronal differentiation. In the developing chick brain, the expression of AChE correlates with the cessation of mitosis and the beginning of differentiation or, in the case of neuroblasts, the stage of neurite outgrowth (Layer et al., 1988a,b; Weikert et al., 1990). Similar findings have also been reported for chick retinal pigment epithelium (Martelly and Gautron, 1988; Salceda and Martinez, 1992), rat auditory cortex (Robertson et al., 1991), rat thalamus (Schlaggar et al., 1993), and chick myoblasts (Elson et al., 1992). Transient expression of cholinesterases is not restricted to mammalian and avian systems. Similar findings have been reported in zebrafish (Hanneman et al., 1988) and in amphibians (Moody and Stein, 1988).

Increased expression of AChE is also correlated with cell migration. In the neural crest of chick and quail, transient expression of AChE occurs during migration of cells to the cranial mesenchyme (Layer and Kaulich, 1991), while in the chick brain, AChE expression occurs after proliferation and after the stage in which cells migrate to the outer surface of the brain (Layer and Sporns, 1987; Vollmer and Layer, 1986, 1987; Mizoguti and Miki, 1985). In the rat thalamus, precursors of sensory neurons express...
AChE transiently during migration and their organization into definitive nuclei (Schlaggar et al., 1993).

Cells that express AChE in the developing brain are generally those that will go on to establish connections with distant targets (Weikert et al., 1990). The expression of AChE precedes the outgrowth of processes in cultured neuroblasts (Layer et al., 1988b; Weikert et al., 1990; Wilson et al., 1990), chick major cranial nerve neurons (Layer and Kaulich, 1991) and non-cholinergic neurons in the rat thalamus (Kristt, 1989). Consistent with the view that AChE is involved in the regulation of neurite outgrowth, inhibition of AChE activity has been found to reduce neurite outgrowth from cultured dorsal root ganglion neurons (Bigbee and DeVries, 1987; Bigbee and Faster, 1989; Biagioni et al., 1989). It has been proposed that the transient expression of BChE may be required for the regulation of AChE expression in the developing brain (Layer et al., 1992).

AChE may also be important for synaptogenesis and the establishment of connections with target cells (Geula et al., 1993; Schlaggar et al., 1993). Transient AChE expression by thalamocortical axons just prior to synaptogenesis has been demonstrated in the rodent (Robertson, 1987; Robertson et al., 1989) and in the primate (Kostovic and Goldman-Rakic, 1983). Increased levels of AChE have been detected in maturing neurons in chick spinal cord and in their target myotomes prior to the arrival of axons (Layer et al., 1988a). Thus, axonal pathfinding may be regulated by AChE expression in both the developing neurons and in the corresponding target cells.
The mechanism by which cholinesterases influence cellular differentiation is not clearly understood. There may be two possibilities. First, cholinesterases may regulate the local concentration of ACh, which may influence cellular differentiation via nicotinic or muscarinic receptors. Second, a non-catalytic mechanism may be involved.

While there is a considerable body of evidence to suggest the involvement of ACh in cellular differentiation (Lauder, 1993), there is also some evidence to suggest a non-catalytic mechanism. Neurotransmitters like ACh may be involved in the regulation of differentiation (Lauder, 1993), there is also some evidence that gradients of ACh may also have a tropic effect on neuronal pathfinding. Cholinesterases may be expressed on growing neurites (Rotundo and Carbonetto, 1987) as a means of scavenging ACh, thereby allowing neurites to grow through ACh-free zones (Layer, 1990).

ACh may exert its effects on neurite outgrowth through both nicotinic and muscarinic receptors. Studies by Lipton et al. (1988) using retinal ganglion cells have shown that nicotinic antagonists enhance neurite outgrowth. Similarly, Pugh and Berg (1994) have suggested that an \( \alpha \) type nicotinic receptor that binds \( \alpha \)-bungarotoxin may be involved. In cultures of chick sympathetic neurons, inhibition of neurite outgrowth by ACh is mediated by both nicotinic and muscarinic (M\(_1\)) receptors (Small et al., 1995a,b).

The influx of intracellular calcium may be responsible for ACh-mediated inhibition of neurite outgrowth. The action of ACh at \( \alpha \)-bungarotoxin binding sites causes neurite retraction from chick ciliary ganglion neurons by stimulating the influx of calcium through \( \omega \)-conotoxin GVIA-sensitive (N-type) calcium channels. The studies of Kater and colleagues (Cohan and Kater, 1986; Cohan et al., 1987; Kater and Guthrie, 1990; Mills and Kater, 1990) also implicate voltage-sensitive calcium channels in the regulation of neurite retraction. Both an increase and a decrease in the levels of intracellular calcium may inhibit neurite outgrowth, as an optimum level of intracellular calcium is required for optimum neurite outgrowth (Lipton and Kater, 1989).

Cholinesterase inhibitors have been found to inhibit neurite outgrowth (Layer et al., 1993; Jones et al., 1995). Despite this, it has been suggested that the inhibitory effects of cholinesterase inhibitors on neurite outgrowth may be due to their interaction with a site which is close to, but distinct from the esteratic site (Layer et al., 1993). This conclusion comes from the observation that not all AChE inhibitors block neurite outgrowth. The compound BW284c51 inhibits neurite outgrowth, whereas ecotoxin does not. However, the ability of BW284c51 to inhibit neurite outgrowth might not be related to its action on AChE. In support of this possibility, Small et al. (1995a) noted that only high concentrations of BW284c51 inhibited neurite outgrowth from sympathetic neurons. Low concentrations of BW284c51, which inhibit AChE activity, did not inhibit neurite outgrowth.

The similarity between cholinesterases and adhesion molecules (Bon et al., 1987; Krejci et al., 1991b) suggests that there may be domains on cholinesterases which interact with components which regulate cell–cell or cell–substrate adhesion. In support of this hypothesis, Small et al. (1995a) showed that neurite outgrowth in cultures of chick sympathetic neurons was stimulated when cells were grown on a substrate of DFP-treated AChE complexed with heparan sulphate proteoglycans. Thus AChE may interact with heparan sulphate proteoglycans in the extracellular matrix and this interaction may be involved in a neurite outgrowth function. Indeed, the biochemical makeup of the extracellular matrix may also influence AChE expression as the underlying matrix has been shown to influence secretion of AChE from cultured dorsal root ganglion neurons (Gupta and Bigbee, 1992).

8. CHOLINESTERASES AND HAEMATOPOIESIS

Cholinesterases have also been implicated in the control of proliferation and differentiation of some haematopoietic cell types. Expression of AChE has been correlated with the differentiation of cultured murine erythroid cells (Conscience and Meier, 1980) [Fig. 4(B)]. Megakaryocyte colony-forming units express AChE when mitosis ends and the cells switch to polyploidization and megakaryocyte formation (Paulus et al., 1981). Anticholinesterase drugs increase the proportion of colony-forming units (Burstein et al., 1980; McDonald et al., 1985; Kumakawa et al., 1990). The non-hydrolysable cholinergic agonist carbamyl choline stimulates megakaryocyte precursor proliferation (Burstein et al., 1980), whereas the addition of AChE decreases the growth of megakaryocyte col-
The release of soluble AChE by non-cholinergic neurons, apparently independently of cholinergic neurotransmission, has led to the proposal that secreted AChE may have a non-cholinergic function. In the substantia nigra and cerebellum, high levels of AChE are present; however, there is little ACh or choline acetyltransferase (Silver, 1974; Levey et al., 1983; Greenfield, 1984; Cuello and Sofroniew, 1985; Henderson and Greenfield, 1987). Nigrostriatal neurons are dopaminergic (Cheramy et al., 1981), but some sparse cholinergic input to the substantia nigra has been reported (Beninato and Spencer, 1987). Cells of both the substantia nigra and cerebellum have been shown to secrete AChE (Cuello et al., 1981; Greenfield et al., 1983; De Sarno et al., 1987; Appleyard et al., 1988; Taylor and Greenfield, 1989). In the substantia nigra, AChE is secreted specifically from dopaminergic neurons (Greenfield et al., 1983; Henderson and Greenfield, 1984), whereas secretion of AChE from the cerebellar cortex is probably associated with excitatory amino acid neurotransmission (Appleyard et al., 1988). AChE secretion within the substantia nigra occurs independently of stimulation of the ACh receptor (Burgun et al., 1985).

AChE has been shown to cause changes in motor behaviour indicative of enhanced excitability in the nigrostriatal pathway (Greenfield et al., 1984; Weston and Greenfield, 1985; Taylor et al., 1990; Jones et al., 1991). The release of AChE from the substantia nigra has been correlated with drug-induced changes in the firing rate of nigrostriatal neurons (Weston and Greenfield, 1986). Microinfusion of affinity-purified AChE into the substantia nigra has been shown to depress the firing of nigrostriatal neurons (Greenfield, 1991). Application of AChE to cerebellar Purkinje cells enhanced the response of the cells to the excitatory amino acids glutamate and aspartate, and increased the ability of Purkinje cells to respond to excitatory input over a wide range of frequencies (Appleyard and Jahnsen, 1992). It has therefore been proposed that secreted AChE may act as a modulator that enhances the sensitivity of some neurons to synaptic input (reviewed by Greenfield, 1984, 1991; Appleyard, 1992).

The mechanism by which AChE modulates neuronal sensitivity is unclear. Application of AChE to substantia nigra induced reversible hyperpolarization of cells, and a corresponding decrease in input resistance (Greenfield et al., 1988, 1989). In contrast, no effect on the membrane potential or input resistance was observed when AChE was applied to Purkinje cells (Appleyard and Jahnsen, 1992). AChE may influence the opening of an ATP-sensitive potassium channel in the substantia nigra (Webb and Greenfield, 1992). It has been suggested that the main ion channel by which AChE acts is located on apical dendrites.
(Hajos and Greenfield, 1992), but that distal dendrites are probably more susceptible to the action of AChE because that part of the cell is the most likely origin of secreted AChE (Nedergaard et al., 1988).

An enzymatic action can almost certainly be ruled out in trying to explain the electrophysiological properties of AChE. AChE preparations that have been heat-denatured or inhibited with soman can still produce the observed effects (Greenfield et al., 1989; Appleyard and Jahnsen, 1992). Dajas et al. (1993) proposed that there might be two pools of AChE in the substantia nigra, distinguished by their different sensitivities to the anti-cholinesterase fasciculin. The AChE that is less sensitive to fasciculin may have a non-cholinergic action.

Despite the considerable number of studies suggesting electrophysiological actions of AChE, more evidence is needed. The possibility that the physiological effects of AChE are also due to contaminants present in affinity purified preparations of the enzyme cannot be completely ruled out. As has been demonstrated in studies on the putative peptidase activities of cholinesterases, removal of contaminants can be a difficult problem. Nonetheless, what is clear is that AChE is released from cells in a fashion that is not consistent with role in cholinergic neurotransmission, therefore secreted AChE must have a non-classical function.

10. CHOLINESTERASES AND TUMORIGENESIS

Abnormal cholinesterase expression occurs in several types of tumour cells. However, the precise relationship between cholinesterase expression and tumorigenesis remains to be established. Gallango and Arends (1969) reported phenotypic variants of BChE in the serum of patients with myeloma. High levels of cholinesterase activity have been reported in primary brain tumours (Razon et al., 1984), ovarian tumours (Drews, 1975) and in the sera of patients with various types of primary carcinoma (Zakut et al., 1988). The amplification of AChE and BChE genes has been identified in tumours of nervous, reproductive and haematopoietic tissues (Lapidot-Lifson et al., 1989; Gnatt et al., 1990; Zakut et al., 1990; Soreq et al., 1992). Although genes amplified in some tumours tend to be incomplete (Zakut et al., 1992), in ovarian carcinomas, the amplified genes yielded catalytically active products (Zakut et al., 1990). Two novel AChE mRNAs have been demonstrated in tumour cell lines that are distinct from the major form encoding the G4 isoform that predominates in the mammalian brain (Karpel et al., 1994).

Tumours can express AChE forms with abnormal structural or enzymic properties (Soreq et al., 1991). For example, cholinesterases produced by brain tumours differ in substrate specificity and in their susceptibility to inhibitors (Razon et al., 1984). Unusual serum cholinesterase activity was found in the sera of patients with carcinomas (Zakut et al., 1988, 1990). This activity was inhibited by both the AChE-specific compound BW284c51 and the BChE-specific compound iso-OMPA.

Although the role of cholinesterases in tumorigenesis is unclear, the fact that AChE and BChE may be involved in the control of cell growth and proliferation during early development suggests that the amplification of cholinesterase genes could influence the ability of tumour cells to proliferate more rapidly. This view is supported by the observation that the promoter regions of the human AChE gene contain binding sites for transcription factors associated with the control of cell proliferation and growth (Aziz-Aloya et al., 1993).

It is likely that cholinesterases influence tumorigenesis through their ability to hydrolyse ACh. Studies by Williams and Lennon (1991) have shown that small-cell lung carcinomas possess muscarinic receptors which, when stimulated, inhibit cell proliferation. Thus the expression of cholinesterases might be expected to be associated with increased cell proliferation. In a more recent study, Codignola et al. (1994) reported that small-cell lung carcinoma cell lines possess nicotinic receptors as well. Stimulation of these receptors with nicotine or cytisine was found to enhance cell proliferation, an effect that was antagonized by α-bungarotoxin.

11. CHOLINESTERASES AND ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a common progressive dementia involving loss of memory and higher cognitive function. The disease is characterized by the presence of amyloid deposits in the brains of sufferers. These deposits are found both extracellularly (amyloid plaques) and intracellularly (neurofibrillary tangles). The principal constituent of amyloid plaques is the amyloid protein (Aβ) which is produced by proteolytic cleavage from the amyloid protein precursor (APP)(Evin et al., 1994). The principal constituent of neurofibrillary tangles is the cytoskeletal protein tau (Kosik, 1992).

One of the characteristic neurochemical changes observed in AD is the loss of AChE and choline acetyltransferase activity in regions of the brain such as the cortex, hippocampus, amygdala and nucleus
basalis (Whitehouse et al., 1981, 1982; Struble et al., 1982; Mesulam and Geula, 1988). The loss of cholinergic structures and markers correlates with the number of plaque and tangle lesions present, as well as with the clinical severity of the disease (Perry et al., 1978; Wilcock et al., 1982; Neary et al., 1986; Perry, 1986). It has been suggested that the loss of AChE is not due to cell death alone, but rather represents a decrease in the activity of AChE in some neurons (Heckers et al., 1982).

Several studies have shown that there are changes in the relative proportions of different isoforms of AChE in the brains of AD patients. Attack et al. (1983) reported that tetrameric globular (G4) isoforms are selectively lost from the parietal cortex of AD and later studies showed that the isoform that is lost is membrane-bound (Younkin et al., 1986; Schegg et al., 1992). This isoform represents approx. 80% of the total brain AChE (Sorensen et al., 1982). Loss of G4 AChE has been observed in the amygdala and Brodmann areas 9, 10, 11, 21 and 40, but not in the cerebellum, the hippocampus or Brodmann areas 17 and 20 (Fishman et al., 1986). However, in the CA1 and CA4 regions of the hippocampus, and in the entorhinal cortex (areas severely affected in AD), there is a decrease in the ratio of tetrameric to monomeric AChE isoforms (Siek et al., 1990). Asymmetric collagen-tailed isoforms are increased by up to 400% in Brodmann area 21, although they represent only a small fraction of the total AChE in the human brain (Younkin et al., 1986).

The assay of levels of AChE activity in the blood and the cerebrospinal fluid (CSF) has been proposed as an ante mortem diagnostic test for AD. However, no consensus has been reached as to whether the levels of AChE are consistently affected in these tissues. The level of serum or plasma AChE has been reported to be increased (Perry et al., 1982; Attack et al., 1985), decreased (Nakano et al., 1986; Yamamoto et al., 1990) or unchanged (St. Clair et al., 1986, Sirvio et al., 1989) in AD patients. The level of erythrocyte AChE has been reported as either unaffected (Attack et al., 1985; Perry et al., 1982) or decreased (Chipperfield et al., 1981). The level of AChE activity in the CSF of AD patients has been reported to be decreased (Soininen et al., 1981, 1984; Arendt et al., 1984; Tune et al., 1985; Nakano et al., 1986; Wester et al., 1988; Elble et al., 1989; Kumar et al., 1989; Sirvio et al., 1989; Urakami et al., 1989; Kawakatsu et al., 1990; Appleyard and McDonald, 1992; Shen et al., 1993) or unchanged (Johnson and Domino, 1971; Davies et al., 1979; Wood et al., 1982; Deutsch et al., 1986; Marquis et al., 1985; Pearlson and Tune, 1986; Bisso et al., 1986; Huff et al., 1986; Appleyard et al., 1987; Ruberg et al., 1987).

An anomalous isoform of AChE (distinguished by its isoelectric point) has been detected in the CSF of AD patients (Navaratnam et al., 1991; Smith et al., 1991). This isoform may represent an abnormally glycosylated form of AChE. However, it is not clear whether the presence of this isoform in CSF is specific for AD, as an abnormal AChE has also been detected in the CSF of patients with other neurological diseases (Shen and Zhang, 1993).

The loss of cholinergic innervation in the brains of patients with AD has led to the "cholinergic hypothesis" which states that the memory dysfunction that occurs in AD is a consequence of the loss of cholinergic innervation to regions such as the cortex and hippocampus. Despite the fact that this hypothesis is controversial (Dunnett et al., 1991; Fibiger, 1991), pharmacological strategies aimed at boosting cholinergic neurotransmission have been trialled for the treatment of AD. Clinical trials with AChE inhibitors have produced limited symptomatic improvement for some patients (Summers et al., 1986; Becker and Giacobini, 1988; Becker et al., 1991). Tetrahydroaminoacridine (tacrine) was the first inhibitor reported to improve the memory of AD patients (Summers et al., 1986). However, the pharmacological effects of tacrine are complex. It is rapidly distributed to the brain (Nielsen et al., 1989), and inhibits brain and blood cholinesterase in a dose-dependent fashion (Sherman and Messamore, 1988), where it produces increased levels of extracellular ACh (Xiao et al., 1993). However, tacrine also affects ACh receptors, ACh synthesis and release, ion channel function and the levels of several neuropeptides and enzymes (Hakansson, 1993).

Other cholinesterase inhibitors being tested for therapeutic efficacy include physostigmine, heptlyphostigmine and SDZ ENA (Giacobini and Becker, 1991; Enz et al., 1993), alkylcarbamyl derivatives of physostigmine (Marta et al., 1988), 1-hydroxytacrine (Shuttske et al., 1989; Johansson and Nordberg, 1993), galanthamine (Thomsen et al., 1990), metrifonate (Giacobini and Becker, 1991), E2020 (Mihara et al., 1993), huperzine A (Geib et al., 1991), and analogues of ranitidine (Sowell et al., 1992). Ideally, a therapeutic inhibitor should produce a long-lasting inhibition of AChE in the brain with a steady state of increased cortical ACh, and with few undesirable side-effects. However, the inhibitors tested in clinical trials so far fall short of this ideal. Indeed, the therapeutic benefits of drugs like tacrine have been questioned (Holttum and Gershon, 1992).
Presence of cholinesterases in plaques and tangles

Most areas of the brain that are susceptible to the formation of plaques and tangles are strongly AChE positive (Hirano and Zimmerman, 1962; Ishii, 1966; Ishino and Otsuki, 1975; Kemper, 1984; Mesulam et al., 1984; Smith and Cuello, 1984; McDuff and Sumi, 1985). In addition, amyloid deposits (both plaques and tangles) stain strongly for AChE and BChE in the cortex (Friede, 1965; Mesulam and Morán, 1987; Mesulam et al., 1987), hippocampus (Ulrich et al., 1990) and amygdala (Nakamura et al., 1992), with little enzyme activity being localized to normal cell structures. AChE is associated with the paired helical filaments of tangles and with the core protein of plaques (Carson et al., 1991; Gomez-Ramos et al., 1992). The form of AChE present in plaques and tangles is probably asymmetric, and is probably associated with heparan sulphate proteoglycans (Perry et al., 1991) via the collagen-like tail subunit (Nakamura et al., 1990; Kalaria et al., 1992).

Non-classical actions of cholinesterases in AD

Although there is some evidence that disturbed cholinergic innervation may be of pathologic relevance to the formation of amyloid plaques (Price et al., 1982), there are a number of studies which suggest that the deposition of cholinesterases in plaques and tangles is unrelated to the degeneration of cholinergic neurons. AChE is associated with plaques very early in the process of amyloid accumulation (Ulrich et al., 1990; Morán et al., 1993). However, there is little correlation between the density of choline acetyltransferase positive fibres and AChE-positive plaques (Mesulam and Morán, 1987; Mesulam and Geula, 1990; Schatz et al., 1990; Heckers et al., 1992; Wright et al., 1993a,b). This indicates that the presence of AChE in plaques is unlikely to be a consequence of the degeneration of cholinergic neurons. This view is supported by the presence of BChE in plaques, as BChE is not a specific marker of cholinergic structures.

Mesulam and co-workers have examined the possibility that the molecular form of AChE associated with plaques and tangles may be different from that associated with cholinergic neurons. Optimal histochemical staining of AChE in amyloid plaques was observed between pH 6.8 and 7.0, as compared with a pH optimum of 8.0 for AChE from age-matched controls (Mesulam et al., 1987; Geula and Mesulam, 1989). The anticholinesterase agents BW284c51, eserine and tacrine were up to 100-fold more effective at inhibiting AChE from control tissue than plaque and tangle AChE from AD patients (Geula and Mesulam, 1989). The indoleamines serotonin and 5-hydroxytryptophan, a carboxypeptidase inhibitor and bacintracin all potently inhibited amyloid-associated AChE (Wright et al., 1993a,b).

There are several possible explanations for the difference in AChE staining between AD and control tissue. First, the deposition of AChE in association with amyloid may influence the conformation of the protein, thus altering its pH optimum and inhibitor sensitivity. Second, prolonged storage of AChE in plaques and tangles may result in its denaturation or proteolytic degradation. Finally, the AChE associated with plaques and tangles may be a different isoform with a different enzymic activity. The last hypothesis has been examined by Mesulam (1995). The results from this study suggest that AChE associated with amyloid may be of astroglial origin. As yet, it is unclear what the structural basis for a difference in enzymatic activity would be.

If AChE and BChE have a non-classical function in amyloid plaques, this could be related to a growth-promoting action. Amyloid plaques are known to contain a number of adhesion molecules and growth factors (Snow et al., 1988; Stopa et al., 1990), suggesting that there may be an underlying disturbance in trophic functions occurring around the region of the plaque (Small et al., 1994a,b). If this turns out to be the case, then inhibition of AChE and BChE in amyloid plaques could have some effects on these trophic functions, which could, in turn, have effects on any regenerative responses which are occurring in the region of the plaque. So far, non-classical actions of cholinesterases have not been considered in relation to the side-effects or the therapeutic efficacy of cholinesterase inhibitors in the treatment of AD. As cholinesterase inhibitors can enhance the action of ACh on neurite outgrowth (Small et al., 1995a), and as neurite outgrowth from neurons of the hippocampus (a major site of neurodegeneration) is known to be inhibited by ACh (Mattson, 1988), drugs such as tacrine may influence the regenerative response and the expression of molecules associated with that response. Several studies have shown that ACh can stimulate the release and non-amyloidogenic processing of the amyloid protein precursor (APP) of AD (Nitsch et al., 1992). APP is able to stimulate neurite outgrowth in cell culture (Milward et al., 1992; Jin et al., 1994; Small et al., 1994b). Thus, the weak therapeutic efficacy of drugs such as tacrine might be due as much to their effects on APP processing as on any boost in cholinergic neurotransmission.
CONCLUSIONS

There is strong evidence that AChE and BChE are involved in the regulation of physiologic processes distinct from neurotransmission. Whilst it has often been presumed that the lack of cholinergic synapses means that AChE does not function in the hydrolysis of ACh, this is not necessarily the case. Cholinergic receptors are widespread. Nicotinic and muscarinic receptors have been identified on several cells of haematopoietic lineage. As ACh is also known to have actions which are unrelated to neurotransmission, both AChE and BChE may be expressed at sites distinct from cholinergic synapses to terminate these non-classical functions of ACh. Although BChE may also function as a scavenger enzyme, there is little solid evidence in the literature to suggest that BChE may not also function to hydrolyse ACh.

The structural similarity between cholinesterases and cell adhesion molecules suggests the possibility that these enzymes have actions which are distinct from their catalytic activities. However, more evidence is needed to support this case, as the non-catalytic properties of cholinesterase preparations could be related to the presence of trace contaminants. In particular, the precise domains which mediate non-catalytic functions need to be defined.

AChE inhibitors have been tested as potential therapeutic agents for Alzheimer’s disease. However, little attention has been given to the possible consequences of long-term treatment with cholinesterase inhibitors on the non-classical physiologic functions of cholinesterases. This latter point seems important, in view of the marginal reported therapeutic benefits of cholinesterase inhibitors claimed to date. The extent to which the non-classical actions of cholinesterases are involved in the side-effects or therapeutic efficacy of AD drugs needs to be examined.

Acknowledgements The authors gratefully acknowledge the editorial assistance of Ms G. Reed. Some of the research described in this review was supported by project grants from the National Health and Medical Research Council of Australia.

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