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REVIEW

Natural genomic amplification of cholinesterase genes in animals

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Tight control of the concentration of acetylcholine at cholinergic synapses requires precise regulation of the number and state of the acetylcholine receptors, and of the synthesis and degradation of the neurotransmitter. In particular, the cholinesterase activity has to be controlled exquisitely. In the genome of the first experimental models used (man, mouse, zebrafish and drosophila), there are only one or two genes coding for cholinesterases, whereas there are more genes for their closest relatives the carboxylesterases. Natural amplification of cholinesterase genes was first found to occur in some cancer cells and in insect species subjected to evolutionary pressure by insecticides. Analysis of the complete genome sequences of numerous representatives of the various metazoan phyla show

that moderate amplification of cholinesterase genes is not uncommon in molluscs, echinoderms, hemichordates, prochordates or lepidosauria. Amplification of acetylcholinesterase genes is also a feature of parasitic nematodes or ticks. In these parasites, over-production of cholinesterase-like proteins in secreted products and the saliva are presumed to have effector roles related to host infection. These amplification events raise questions about the role of the amplified gene products, and the adaptation processes necessary to preserve efficient cholinergic transmission.

Keywords: acetylcholinesterase, amplification, butyrylcholinesterase, carboxylesterase, nematode, tick, α/β -hydrolase fold proteins.

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Cholinergic transmission involves the synthesis and release of the neurotransmitter, acetylcholine (ACh), and its binding to the postsynaptic receptor, triggering their activation and channel opening. Acetylcholinesterase (AChE) is involved in the termination of impulse transmission by rapid hydrolysis of ACh. Every partner of this pathway (choline acetyltransferase, muscarinic or nicotinic receptors and cholinesterases) can be found together or separately in non-neuronal tissues. Reduction in cholinesterase activity by inhibitors or by genetic mutation affects both neuronal transmission and the non-neuronal cholinergic systems. Likewise, experimental overexpression of AChE also alters cholinergic neurotransmission (Beeri *et al.* 1997).

Acetylcholinesterase belongs to a subset of the α/β -hydrolase fold superfamily, the carboxylesterase B (CO-esterase) family, which is defined by a prosite motif containing a cysteine involved in a first N-terminal disulfide

bond (Prilusky *et al.* 2011; Lenfant *et al.* 2013). This family comprises both enzymes (e.g. carboxylesterase, cholinesterase, bile salt-activated lipase) and non-enzymatic proteins (e.g. the cholinesterase-like domains of the neuroligins or of thyroglobulin) that all have many representatives in fungi and animals (Lenfant *et al.* 2013). Divergence of the carboxylesterases/cholinesterases and their non-catalytic relatives occurred early in the evolution of metazoa. In

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Abbreviations used: AChE, acetylcholinesterase; amp, amplified; CXE, carboxylesterase; JHE, juvenile hormone esterase; non-catal, non-catalytic.

particular, divergence of the cholinesterases and neuroligins occurred at the same time as emergence of the first neural cells (Lenfant *et al.* 2014). More recent duplications of non-specific carboxylesterase and cholinesterase genes occurred in representatives of most animal classes. The possible role of the extra AChE-like genes is only recently being unveiled in a few animal species. The mechanism by which efficient cholinergic neurotransmission is preserved in situations of cholinesterase overexpression is not fully understood.

Number of cholinesterase genes in animals

Most vertebrates have one to two genes encoding AChE or butyrylcholinesterase (BChE) (Pezzemanti and Chatonnet 2010; Pezzemanti *et al.* 2015). In insects, there are one or two AChE genes (*ace1*, *ace2*), of which at least one plays a major role in the central cholinergic nervous system (Huchard *et al.* 2006; Kim and Lee 2013; Cha and Lee 2015). In Arachnida (e.g. spiders and ticks), three to four genes have been described (Temeyer *et al.* 2013; Meng *et al.* 2016). Although the cholinesterase sequences in ticks can be quite divergent from the canonical sequence of cholinesterases from other phyla, they exhibit true cholinesterase activity (Temeyer *et al.* 2010). Four AChE genes have been described in *Caenorhabditis elegans* (Grauso *et al.* 1998; Combes *et al.* 2000). At least three genes are conserved in rhabditididae (Grauso *et al.* 1998; Combes *et al.* 2000), and in many nematodes including vertebrate, insect or plant parasites (Selkirk *et al.* 2005), for example, in pinewood nematodes where the soluble AChE provides chemical defense against xenobiotics (Kang *et al.* 2011a,b). Natural amplification of genes coding for cholinesterases has been found in arthropods which gained insecticide resistance. However, genome sequences available for representatives of many animal phyla contain multiple copies of cholinesterase-like genes independently of modification of the environment by man. Figure 1 represents a phylogenetic tree of the true orthologs of cholinesterase genes in animals together with some representatives of the amplified genes. For sake of clarity, only representative sequences of complete amplified genes that had conserved a complete catalytic triad and the important tryptophan 84 residue involved in the choline-binding site were used. Branches of some amplified genes in hemichordates, prochordates, nematodes, ticks, reptiles are visible. To show the relationship of the full set of amplified genes in comparison with the carboxylesterase and cholinesterase genes, the whole carboxylesterase B family is presented as a group network (Fig. 2). Groups of amplified genes in parasitic nematodes and ticks are clearly separated from the non-specific carboxylesterases. The occurrence of amplification events raise the question of how neo-functionalization preserves cholinergic transmission efficiency, but this most likely requires tissue-specific expression and, in the case of

soluble enzymes, compartmentalization in order to minimize the impact on neuronal function. Measuring evolutionary distance in groups of amplified genes and comparing them with parent genes or between particular phyla could give hints about acquisition of functions of amplified genes. A full analysis of this aspect is out of the scope of this review. Previous studies showed that BChE is less conserved (i.e. accumulated more non-synonymous substitutions) than AChE in vertebrates (Pezzemanti & Chatonnet 2010). A more thorough study determined that duplicated AChE genes in insects are under purifying selection and that some new specialization or essentiality appeared in the most divergent copy. Even though there are differences in the evolutionary rates of the two genes, the smallest value is not always associated with the gene that retained the main neuronal function (Cha and Lee 2015). However, carboxylesterases and AChE are rather large proteins and the number of amino acid residues crucial for the catalytic activity is limited (Sussman *et al.* 1991). The substitution of residues in surface loops can evolve more randomly according to the position of the gene in the genome. For example, in vertebrates the gene for BChE is embedded in a AT-rich region, whereas that for AChE is in a CG-rich region, which are not equally prone to mutations (Chatonnet and Lockridge 1989). The AChE gene can accommodate a great number of mutations that do not alter the enzyme activity of the protein (Goldenzweig *et al.* 2016).

Amplification of cholinesterase genes in cancer cells

Amplification of cholinesterases was first described in some cancer cells and in blood cells of humans exposed to organophosphates (Lapidot-Lifson *et al.* 1989; Prody *et al.* 1989). Massive changes in karyotype of cancer cells is very frequent; however, specific amplification of cholinesterase genes have been described (Lapidot-Lifson *et al.* 1989; Zakut *et al.* 1990; Bernardi *et al.* 2010; Boberg *et al.* 2013). These amplifications modify the cholinergic system locally only and thus may not interfere with the neuronal cholinergic system. Yet, cholinesterase overexpression may modulate the immunological or inflammatory response toward the cancer cells. Recently, it was shown that cholinesterase genes contain sequences that can interact with microRNAs, and a subgroup of miRNAs (mirs) associated with the regulation of cholinergic elements have been termed 'cholinomiRs' (Soreq 2015). For example, mir-132 suppresses AChE expression, and transgenic mice which over-express 3'-UTR null AChE show excessive production of inflammatory cytokines (Shaked *et al.* 2009). Overexpression of cholinesterases in cancer cells may also disturb normal gene regulation provided by miRNAs (Soreq 2015).

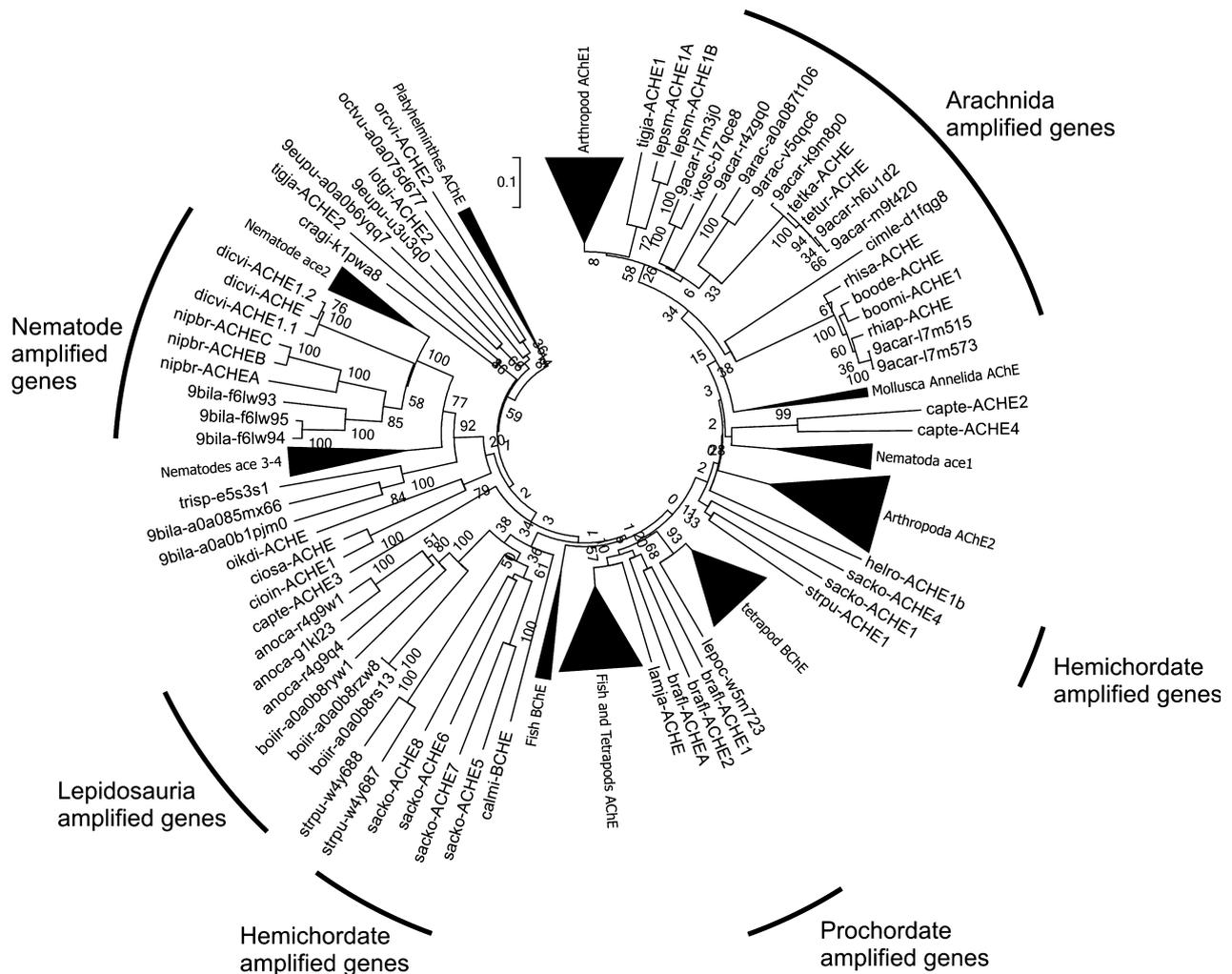


Fig. 1 Phylogenetic tree of the cholinesterase and cholinesterase-like sequences. The sequences were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>), Uniprot (<http://www.uniprot.org/>), the current assembly of the genomes using the BLAST servers dedicated to species from DOE Joint genome institute (<http://genome.jgi-psf.org/>), ENSEMBL (<http://www.ensembl.org/>), NCBI (<http://www.ncbi.nlm.nih.gov/>) or Genoscope (<http://www.genoscope.cns.fr/>). The sequences used along with Individual references and links to Genbank and Uniprot can be retrieved in the ESTHER database (<http://bioweb.su.pagro.inra.fr/esther>) (Lenfant *et al.* 2013). The cholinesterase and cholinesterase-like sequences were used. From the 781 entries in the initial set, we retained only the 404 sequences for which the catalytic triad residues and tryptophan 84 were conserved (Torpedo fish AChE numbering (Sussman *et al.* 1991)). Alignment was performed with ClustalO (Sievers *et al.* 2011). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). Bootstrap test (1000 replicates) was used (Felsenstein 1985). The evolutionary distances were computed using the Poisson correction method

Amplification of carboxylesterase genes

Amplification of carboxylesterase genes is not uncommon, and analysis of these occurrences can help understand similar

(Zuckerkl and Pauling 1965) and are displayed/expressed in the units of the number of amino acid substitutions per site. The scale on top of the figure is for 0.1 unit. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 241 positions in the final data set. Phylogenetic analyses were conducted in MEGA6 (Tamura *et al.* 2013). Maximum likelihood analysis was performed using PhyML (<http://www.atgcmontpellier.fr/phyml/>) (Guindon *et al.* 2009). Subtrees containing only orthologues (one sequence for one species) are compressed as a black triangle. The 5 character nomenclature of Uniprot is used for species (for example: nipbr: *Nippostrongylus brasiliensis* (rat hookworm), dicvi: *Dictyocaulus viviparus* (bovine lungworm), anoca: *Anolis carolinensis* (green anole) (American chameleon), boiir: *Boiga irregularis* (brown tree snake), sacko: *Saccoglossus kowalevskii* (acorn worm), ixosc: *Ixodes scapularis* (blacklegged tick or deer tick), boomi : *Boophilus microplus* (*Rhipicephalus microplus* (cattle tick))

events in the cholinesterase family. Non-specific carboxylesterases are the closest enzyme relatives of cholinesterases. These are thought to play a role in digestion, detoxification, or hydrolysis of odorant molecules (Holmes

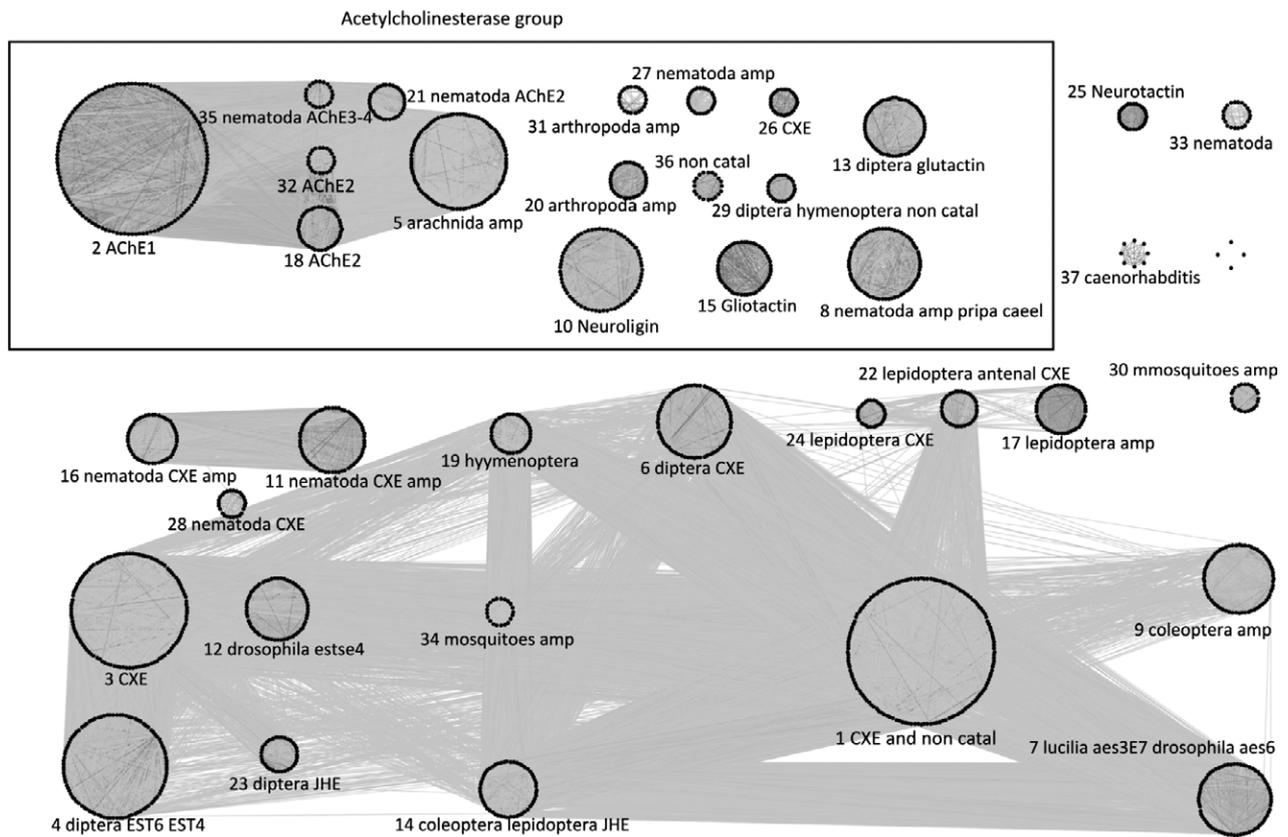


Fig. 2 Network representation of the metazoan CO-esterase family. The total number of 3777 sequences of metazoan carboxylesterase B retrieved from ESTHER was reduced to a set of 2032 sequences excluding fragments or sequences from very close species (for example, we retained only sequences from *Drosophila melanogaster* and not the other 20 *Drosophila* species). An all-versus-all blast comparison was performed (Camacho *et al.* 2009) and a blast bit-score matrix was generated. The result is presented as a network, where nodes represent sequences and edges represent Blast bit-

score between two sequences. Each sequence is represented by a dot that makes the circumference of each group. Groups are ordered by the number of sequences that they contain (size of the circle, e.g. group 1 contains the largest number of sequences). Gray lines between dots indicate that the BLAST bit-score between two sequences belonging either to the same group or to distinct groups is higher than 345. The rectangular form includes the groups closely related to AChEs.

et al. 2010; Hatfield *et al.* 2016). They can hydrolyze a great variety of xenobiotics both *in vitro* and *in vivo*, but clearly defined natural substrates have not been identified. The fact that genes organized in arrays or contigs can be found in the same order on chromosomes of distantly related species indicates that amplification occurred early in evolution in animals (Robin *et al.* 2009; Hatfield *et al.* 2016; Howe *et al.* 2016). These amplified genes are conserved in animals in contact with many environmental esters, either as elements of their food or poisons produced as defense strategies by plants (Rane *et al.* 2016). In humans, the CES1 gene is complex with a head to tail pseudogene replaced in some individuals by a copy of the normal gene. Multiple haplotypes are found in different populations and some are found more frequently in cancer patients (Sai *et al.* 2010).

One of the first molecularly described mechanisms of resistance of mosquitoes to insecticides was amplification of

a carboxylesterase gene (Mouches *et al.* 1990; Raymond *et al.* 1991). Similar amplification of esterase E4 was also found in aphids (Field and Devonshire 1997). The protein is responsible for sequestration of the insecticide before it can reach the AChE target. Since amplification of carboxylesterase did not interfere with the metabolism of the insect, the only drawback of amplification was probably a reduction in fitness because of the massive hijacking of RNA and protein synthesis. The amplified protein can account for 10% of the total protein of the insect (Fournier *et al.* 1987). Different alleles with up regulation or carboxylesterase gene amplification have been described. Each of these can coexist in populations of insects and can be differentially selected according to the distance from the treatment zone or level and duration. This led to an exceptionally long (30 years) and unique analysis of evolution of fitness toward a well defined selective pressure in a natural environment (Miles

et al. 2016). These amplified genes can coexist with the mutations of the AChE gene which result in a more specific resistance to organophosphates or carbamates.

Amplification of AChE genes in deuterostomes

Some extra cholinesterase-like genes have been found in the genome of *Strongylocentrotus purpuratus* (purple sea urchin, Echinodermata), *Saccoglossus kowalevskii* (acorn worm, Hemichordata), *Branchiostoma floridae* (Florida lancelet, amphioxus, Cephalochordata), snakes, turtles and some fish. Most of these animals are not commonly used as laboratory model organisms, and thus far no experiments have been performed to understand the role of the products of the extra genes. It is not known if these genes are expressed and if the products actually hydrolyze ACh. Sequence comparison indicates that they are more closely related to cholinesterases than to carboxylesterases. Three BChE genes are found in *Takifugu rubripes* and *Tetraodon nigroviridis*. These puffer fish are known to accumulate tetrodotoxin in their liver and ovaries by dietary uptake or symbiosis of bacteria producing this compound. Tetrodotoxin serves as an antipredator defense, functioning as a voltage-gated sodium channel blocker in nerves and muscle cells. Mutations of the sodium channel of the fish are expected to produce varying degrees of resistance toward the toxin (Soong and Venkatesh 2006), but other adaptations of cholinergic proteins such as over-expression of BChE might also play a role in resistance.

Amplification of AChE genes in arthropods

Amplification of AChEs in arthropods exposed to insecticides has been well documented. At least 35 amino acid positions (alone or in combinations) have been found mutated in cholinesterases of more than 30 arthropod populations resistant to insecticides (Hotelier *et al.* 2010) (<http://bioweb.supagro.inra.fr/ESTHER/preformed.pl?cat=insecticide%20resistance>). The most common mutations which result in cholinesterase insensitive to insecticide give rise to enzymes which are less efficient in terms of catalysis of acetylcholine. In spider mites, overexpression of the AChE gene can compensate for the reduced catalytic activity caused by resistance-conferring mutations (Kwon *et al.* 2012; Lee *et al.* 2015). In mosquitoes, similar mutations were found (Weill *et al.* 2003). Secondary amplification of the unmutated allele occurred, and gave rise to a resistant population with both resistant and sensitive enzymes. Consequently, fitness and efficient cholinergic function was preserved (Labbe *et al.* 2014; Assogba *et al.* 2015). Heterogeneous amplifications (resistant and susceptible copies) give lower resistance and fitness costs, whereas homogeneous duplications (only resistant copies of the gene) increase both resistance and fitness costs, and both phenotypes were described in different locations. The alleles selected depend

on the intensity and distribution of the selective pressure. An optimum of three mutated copies may proceed from the deleterious effect of genes coamplified in the amplicon encompassing the *ace-1* gene (Assogba *et al.* 2016). Chlorpyrifos resistance is associated with mutation and amplification of the AChE-1 gene in the tomato red spider mite, *Tetranychus evansi* (Carvalho *et al.* 2012).

The initial search for mutations in the three AChE genes of the cattle tick *Rhipicephalus microplus* which might be responsible for insecticide resistance was delayed because the three genes are present at an amplified copy number in the genome (Bellgard *et al.* 2012; Temeyer *et al.* 2012; Bendele *et al.* 2015). Subsequently, it was found that AChE genes are also amplified in the genome of *Ixodes scapularis*, the tick vector of Lyme disease (Gulia-Nuss *et al.* 2016). Sequencing of the genome of the two-spotted spider mite, *Tetranychus urticae*, also revealed expansions and radiations in the carboxylesterase/cholinesterase family (Van Leeuwen and Dermauw 2016; Wu and Hoy 2016).

In addition to conventional membrane-bound enzymes associated with neuromuscular transmission, secreted AChEs have been found in the saliva of several blood-feeding arthropods. Four variants were described in the bed bug *Cimex lectularius* (Francischetti *et al.* 2010), and two transcripts were found in salivary glands of the kissing bug *Triatoma matogrossensis* (Assumpção *et al.* 2012). More recently, AChE activity has been described in saliva of *R. microplus* (Temeyer & Tuckow, 2016). The saliva of blood-feeding arthropods contains numerous bioactive compounds which aid feeding, for example, inhibitors of coagulation, vasodilators, painkillers, anti-inflammatory agents and immunomodulators (Ribeiro & Francischetti, 2003). The latter are important as biting arthropods may stay in place on the skin for some time, and immunity can be acquired upon tick infestation (Trager, 1939). Although the function of AChE in arthropod saliva is not known, parasitic nematodes also secrete AChE (see below) and there is mounting evidence that this most likely acts to modulate host immunity.

Amplification of AChE genes in parasitic nematodes

Four genes for AChE (*ace-1-4*) have been described in *C. elegans* (Grauso *et al.* 1998) although *ace-4* is likely to be non-functional in terms of enzymatic activity (Combes *et al.* 2000). On the whole, these genes are expressed in discrete neurons and muscle cells, with a few examples of coordinated expression (Combes *et al.* 2003). This implies specific functions for each enzyme, and anatomical separation of expression ensures that neuromuscular function is precisely regulated. This hints at an evolutionary scenario which would include modification of expression of one extra copy, followed by amplification of the gene with the most relaxed constraint for cholinergic transmission (Combes *et al.* 2003).

Secretion of AChE by parasitic nematodes has been known for a long time (Bremner *et al.* 1973, Ogilvie *et al.* 1973; Rothwell *et al.* 1973; Rapson *et al.* 1986). The discovery of AChE proteins secreted by *Nippostrongylus brasiliensis* and *Dictyocaulus viviparus*, with characteristic sequence differences from the canonical neuronal AChE were the first demonstration of AChE gene amplification in nematodes beyond the 3–4 genes encoding neuronal enzymes (Hussein *et al.* 1999, 2000, 2002; Lazari *et al.* 2003; Selkirk *et al.* 2005). Not all animal-parasitic nematodes secrete AChE into the host environment, and this adaptation appears to be predominantly restricted to parasites which colonize mucosal surfaces (Selkirk *et al.* 2005). While soluble AChEs have been described in plant-parasitic nematodes, there is little evidence to suggest they are secreted into plant tissues, as they appear to be absent from secretomes defined by proteomics or from other analyses of effector proteins (Rehman *et al.* 2016). Most of these studies are focused on pre-parasitic J2 larvae, however, and may miss identification of secreted AChE proteins, as in animal-parasitic forms, expression of secreted AChEs is absent in free-living stages and is activated on transition to the host (Huang *et al.* 2010).

Various hypotheses have been proposed for the function of nematode secreted AChEs, based on neutralizing ACh in its diverse signaling roles. Cholinergic signaling promotes smooth muscle contraction and peristalsis in the gut, mucus secretion by goblet cells, and fluid secretion from enterocytes, all of which contribute to the ‘weep and sweep’ response which helps to promote parasite expulsion, so it would clearly benefit parasites to block this (Selkirk *et al.* 2005a,b). Cholinergic signaling plays a role in a number of immune cell functions, including acting as a costimulatory factor for T lymphocyte activation and cytokine secretion (Darby *et al.* 2015), and there are many ways in which AChEs could inhibit or modulate these responses. Alternatively, secreted AChEs could protect against inhibitors ingested in foodstuffs, i.e. providing a shield much like that previously discussed in insects resistant to pesticides. However, these hypotheses are not mutually exclusive. Recently, a direct role for parasitic nematode AChEs in altering the host cytokine environment to promote parasite survival was suggested via expression *in vivo* (Vaux *et al.* 2016). Irrespective of the functions of the secreted AChEs, it is important that they do not interfere with the cholinergic system of the parasite, and the restricted expression of relevant genes in specialized amphidial and secretory glands ensures that the enzymes are anatomically segregated prior to secretion to the exterior.

More recently, whole genome sequencing showed a remarkable amplification of AChE-like genes in *Strongyloides* and *Parastrongyloides* species, although this was not evident in the closely related free-living nematode *Rhabditophanes* (Hunt *et al.* 2016). Interestingly, these gene

sequences predict proteins which lack the C-terminal membrane anchors found in neuronal AChEs and are likely to be secreted. Consistent with this interpretation, proteomic analysis has identified 20 AChE-like proteins in the secretome of adult female parasitic *Strongyloides ratti* (Hunt *et al.* 2016). Nevertheless, the vast majority of these sequences lack residues critical for AChE activity, such as those which make up the catalytic triad, the choline-binding site, or those lining the active site gorge, and are almost certainly catalytically inactive. The function of these proteins is therefore unclear, but they may act as a smokescreen to minimize the possibility of host antibodies neutralizing parasite secretory AChE activity.

Concluding remarks

It is clear that natural amplification of cholinesterase genes has occurred commonly in many animals, in particular those subjected to environmental or evolutionary pressures, for example via exposure to pesticides, or in the case of endo- or ectoparasites, challenge from the immune system of their host. During this process, AChEs have acquired functions unrelated to their classical enzymatic role in neurotransmission, and we still have a lot to learn about cholinergic signaling outside the neuromuscular system.

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