Natural genomic amplification of cholinesterase genes in animals

Arnaud Chatonnet¹, Nicolas Lenfant¹,², Pascale Marchot² and Murray E. Selkirk³

¹Dynamique Musculaire et Métabolisme, INRA, Université Montpellier, Place Viala, Montpellier France.

²Architecture et Fonction des Macromolécules Biologiques, CNRS/Aix-Marseille Université, Faculté des Sciences, Campus Luminy, Marseille, France.

³Department of Life Sciences, Imperial College London, London SW7 2AZ, United Kingdom

Corresponding author: A. Chatonnet, Phone (33) 499612814, Fax (33) 467545694, E-mail arnaud.chatonnet@inra.fr

Abstract

Tight control of the concentration of acetylcholine (ACh) at cholinergic synapses requires precise regulation of the number and state of the ACh receptors, and of the synthesis and degradation of the neurotransmitter. In particular, cholinesterase activity has to be controlled exquisitely. In the genome of the first experimental models used (man, mouse, zebrafish, drosophila) there are only one or two genes coding for cholinesterases, whereas there are relatively more genes for their closest relatives 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 cases, 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.

Index Entries: acetylcholinesterase; α/β-hydrolase fold proteins; amplification; butyrylcholinesterase; carboxylesterase; nematode; tick

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

Introduction

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 acetylcholine. Every partner of this pathway (choline acetyltransferase, muscarinic or nicotinic receptors, cholinesterases) can be found together or separately in non-neuronal tissues. Reduction of cholinesterase activity by inhibitors or by genetic mutation affects both neuronal transmission and the non-neuronal cholinergic systems. Likewise, experimental over-expression of AChE also alters cholinergic neurotransmission (Beeri et al. 1997).

Acetylcholinesterase belongs to a subset of the alpha/beta 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. neuroligin, thyroglobulin) that all have many representatives in fungi and animals (Lenfant et al. 2013). Divergence of the carboxylesterases/cholinesterases and the non-catalytic families occurred early in the evolution of metazoa. In 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 acetylcholinesterase-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 over-expression is not fully understood.

**Number of cholinesterase genes in animals**

Most vertebrates have one to two genes encoding AChE or butyrylcholinesterase (BChE) (Pezzementi et al. 2015, Pezzementi & Chatonnet 2010). 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, Cha & Lee 2015) Kim & Lee 2013). 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 defence against xenobiotics (Kang et al. 2011b, Kang et al. 2011a). Natural amplification of genes encoding 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 human modification of the environment. 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 (Figure 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 (Pezzementi & Chatonnet 2013). 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 evolutionary rate between the two genes, the smallest value is not always associated with the gene that retained the main neuronal function (Cha & 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 the 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 an AT-rich region whereas that for AChE is in a CG-rich region, which are not equally prone to mutations (Chatonnet & 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 (Prody et al. 1989, Lapidot-Lifson et al. 1989). Massive changes in karyotype of cancer cells is very frequent, however specific amplification of cholinesterase genes have been described (Zakut et al. 1990, Lapidot-Lifson et al. 1989, Boberg et al. 2013, Bernardi et al. 2010). These amplifications modify the cholinergic system locally and thus may not interfere with the neuronal cholinergic system. Yet the 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). Over-expression of cholinesterases in cancer cells may also disturb normal gene regulation provided by miRNAs (Soreq 2015).

Amplification of carboxylesterase genes

Amplification of carboxylesterase genes is not uncommon, and analysis of these occurrences can help understand similar 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 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 esters in the environment, either as elements of their food or poisons produced as defense strategies in plants (Rane et al. 2016)
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 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 has also been found in aphids (Field & 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 due to 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 upregulation 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 (Milesi et al 2016). These amplified genes can co-exist 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 acetylcholine. Sequence comparison indicates that they are more closely related to cholinesterases than to carboxylesterases. Three butyrylcholinesterase genes are found in Takifugu rubripes and Tetraodon nigroviridis. These puffer fish are known to accumulate tetrodotoxin in the liver and ovaries by dietary uptake or symbiosis of bacteria producing this compound. Tetrodotoxin serves as an antipredator defence, 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 & Venkatesh 2006), but other adaptations of cholinergic proteins such as over-expression of butyrylcholinesterase might also play a role in resistance.

**Amplification of acetylcholinesterase 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, over-expression 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 be due to the deleterious effect of genes co-amplified 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 (Temeyer et al. 2012, Bellgard 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). The sequenced genome of the two-spotted spider mite, Tetranychus urticae, also revealed expansions and radiations in the carboxylesterase/cholinesterase family (Van Leeuwen & Dermauw 2016, Wu & 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 acetylcholinesterase 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, Rothwell et al. 1973, Rapson et al. 1986, Ogilvie et al. 1973). 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, Hussein et al. 2002, Lazari et al. 2003, Hussein et al. 2000, 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 colonise mucosal surfaces (Selkirk et al., 2005). Whilst 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 neutralising acetylcholine 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. 2005). Cholinergic signaling plays a role in a number of immune cell functions, including acting as a co-stimulatory 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 minimise the possibility of host antibodies neutralising parasite secretory AChE activity.

**Concluding Remarks**

It is clear that natural amplification of cholinesterase genes has been a common occurrence 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 role in neurotransmission, and we still have a lot to learn about cholinergic signaling outside the neuromuscular system.
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 can be retrieved in the ESTHER database (http://bioweb.supagro.inra.fr/esther) (Lenfant et al. 2013). Individual references and links to genbank and uniprot can be retrieved from ESTHER. 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 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 & Nei 1987). Bootstrap test (1000 replicates) was used (Felsenstein 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling 1965) and are 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 dataset. Phylogenetic analyses were conducted in MEGA6 (Tamura et al. 2013). Maximum likelihood analysis was performed using PhyML (http://www.atgcmtpellier.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: Dicyoecaulus 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))).
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). Grey 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 Acetylcholinesterases.

Fig. 2. Network representation of the metazoan CO-esterase family

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