



HHS Public Access

Author manuscript

Biochem Pharmacol. Author manuscript; available in PMC 2019 July 01.

Published in final edited form as:

Biochem Pharmacol. 2018 July ; 153: 205–216. doi:10.1016/j.bcp.2018.01.044.

CHOLINESTERASES AND THE FINE LINE BETWEEN POISON AND REMEDY

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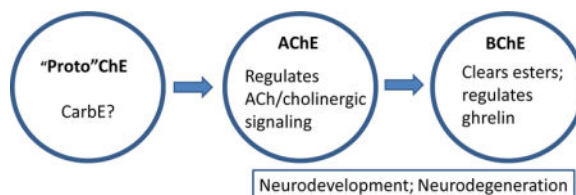
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Abstract

Acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) are related enzymes found across the animal kingdom. The critical role of acetylcholinesterase in neurotransmission has been known for almost a century, but a physiological role for butyrylcholinesterase is just now emerging. The cholinesterases have been deliberately targeted for both therapy and toxicity, with cholinesterase inhibitors being used in the clinic for a variety of disorders and conversely for their toxic potential as pesticides and chemical weapons. Non-catalytic functions of the cholinesterases (ChEs) participate in both neurodevelopment and disease. Manipulating either the catalytic activities or the structure of these enzymes can potentially shift the balance between beneficial and adverse effect in a wide number of physiological processes.

Graphical abstract



Keywords

acetylcholinesterase; cholinergic signaling; butyrylcholinesterase; cholinesterase inhibitors; ghrelin metabolism; Alzheimer's; organophosphates

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II. INTRODUCTION

An often paraphrased statement by the 16th century Swiss physician and philosopher Paracelsus is that “dose separates poison from remedy”. Students in basic pharmacology and toxicology learn early on of the ‘therapeutic index’, a quantitative relationship between efficacy and toxicity and a direct conceptual descendent of Paracelsus’ edict. The importance of dose-response relationships in pharmacology and toxicology is difficult to overstate, as they provide chemical-specific views of drug and toxicant potency, efficacy, and selectivity. The ChEs, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.18) are related enzymes expressed throughout much of the animal kingdom. AChE plays a well-defined role in regulating cholinergic signaling while the physiological impact of BChE has remained unclear until very recently. Here we provide a brief overview on the biology of ChEs and consider how drug- or toxicant-induced changes in their esterase activity, or in the proteins themselves, can shift the balance between benefit and harm.

III. TWO CHOLINESTERASES

Acetylcholinesterase (AChE) and its “sister” enzyme butyrylcholinesterase (BChE) are widely expressed throughout the animal kingdom [1]. AChE and BChE share roughly 50% sequence homology and have relatively similar tertiary and quaternary structures. They both possess a catalytic triad of three amino acids (serine, glutamate and histidine) located deep inside a “gorge” in the tertiary structure [2–4]. Evidence suggests that these enzymes emerged from a carboxylesterase superfamily, with “true” AChE first emerging hundreds of millions of years ago in Platyhelminthes [5]. Higher vertebrates have one AChE gene and one BChE gene, while some lower species express multiple genes of one or both [1]. The cyclostomes, jawless fish including the lamprey and hagfish, only express AChE, suggesting that BChE arose later in evolution by gene duplication and divergence from AChE [1, 2, 6].

IV. ACETYLCHOLINESTERASE AND CHOLINERGIC SIGNALING

The concept of a synapse between a neuron and an innervated cell, and the receptors that mediated their interaction, was developed by Bernard, Ehrlich, Sherrington, Langley and others (see [7–9]). It was long debated whether transmission of nerve impulses to muscle cells occurs by electrical or by chemical signals until the work of Otto Loewi and Henry Dale, later recognized by their Nobel Prize in 1936 [10]. The gains in understanding of various physiological processes by these early investigators and others were aided by using natural toxins. In fact, Loewi [11] considered the primary objective of pharmacology as “revealing physiological functions by the reactions of living matter to chemical agents”. While this narrow description does not encompass the multifold aspects of modern pharmacology, the experimental use of xenobiotics has played an essential role in gaining an understanding of neurotransmission and cholinergic signaling.

Over a century ago, Dale [12] compared the effects of selected choline esters with the mushroom toxin, muscarine, and was the first to describe “muscarine-like” and “nicotine-like” actions. The relative potency of choline esters in isolated organ systems vs intact

animals led him to posit that the “evanescence” of their effects could be due to rapid hydrolysis by an esterase. In Loewi’s classic studies [13], stimulating the vagus in a nerve-heart preparation in physiological solution triggered release of a substance called *vagusstoff* (i.e., vagus substance) that mimicked the effect of nerve stimulation when the fluid medium was transferred to a second heart with no vagal connection. Importantly, Loewi also showed that the effect of the vagal substance was (like that of acetylcholine) enhanced by eserine, a known inhibitor of ChEs [14,15]. Soon thereafter, Dale and Dudley [16] reported the isolation of acetylcholine from tissue (horse spleen), confirming its endogenous presence. These studies and others laid groundwork for an enormous amount of research on the role of acetylcholine in synaptic signaling and its regulation by AChE.

There is now a widespread consensus that AChE is the paramount or sole enzyme regulating neurotransmission in vertebrate cholinergic pathways that include brain, skeletal muscle and the autonomic nervous system. AChE serves this role in all mammals by selectively inactivating acetylcholine, within seconds or milliseconds after it is released from a presynaptic cholinergic neuron. AChE is one of the most efficient enzymes in the body, with a catalytic rate that approaches the limit of diffusion [17,18]. AChE’s function appears equally important in brain and the periphery. This view is supported by the intensely concentrated localization of this enzyme at cholinergic synapses throughout the body, and by the diversity of effects elicited by inhibiting AChE either in the brain or in the peripheral compartment.

V. Physiological role of BChE

In contrast to the long-established and well-defined role of AChE in regulating cholinergic signaling, a true physiological function for BChE remained elusive over many decades. BChE exhibits much broader substrate specificity than AChE. For example it hydrolyzes butyrylcholine *and* acetylcholine while AChE only hydrolyzes the latter. Also, while BChE expression in many tissues exceeds that of AChE, it exists at much lower concentrations in the brain, skeletal muscle, and peripheral nerves [19]. Although exogenous butyrylcholine has been shown to modulate intrinsic cardiac neuron activity in canines [20,21], to our knowledge no synapses in higher vertebrates use butyrylcholine as a neurotransmitter. In fact, a longstanding consensus holds that such synapses do not exist. Evidence to support that view is that, in our unpublished studies, selective inhibitors such as *iso*-OMPA (tetra isopropyl pyrophosphoramidate) can completely inhibit BChE catalysis without eliciting obvious physiological disturbance. Not surprisingly, BChE knockout mice with no BChE expression appear perfectly healthy [22]. In particular, they show no apparent change in motor, autonomic or cognitive function. Under casual observation they are indistinguishable from wild-type mice. Moreover, there are isolated human populations who have been identified as completely lacking a functional BChE gene, but again, by all accounts, they exhibit a normal phenotype. Their only physiologic difference from “wild-type” is an elevated risk when exposed to bioactive esters in food or ester-type muscle relaxants in the clinic [23,24].

Thus, until quite recently, BChE was considered to lack an important function apart from serving as a “backup” for AChE in regard to neurotransmission, and as a modestly protective

bioscavenger of bioactive esters in the food supply. The latter could be regarded as a feature that enables humans and other species to obtain nutrients from plants, many of which could be toxic if their endogenous esters were not efficiently hydrolyzed. The enzyme's ability to hydrolyze esters has been harnessed in surgical procedures using ester-based pharmaceuticals such as the muscle relaxant succinylcholine, which it rapidly inactivates. However, it became apparent early on that certain patients required exceptionally long recovery times after treatment with succinylcholine. The basis for this difference in clinical response is pharmacogenomic in nature, i.e., these individuals were found to express an "atypical BChE" with active site mutations leading to catalytic efficiency far below that of the native enzyme. Because of this BChE variant, they cleared succinylcholine slowly and needed assisted respiration and clinical surveillance for extended periods.

More recently, it was discovered that BChE hydrolyzes the neuropeptide gut hormone, ghrelin [25–28]. Nonetheless, because the enzyme reaction is very slow, those who first reported this finding were initially reluctant to attribute a real physiological role for that phenomenon. Our own views changed when we accidentally linked high level gene transfer of BChE in group-housed male mice to reduced stress, reduced aggression and reduced levels of plasma ghrelin [29]. We are now confident that ghrelin modulation represents an important physiological role for this enzyme and, in light of that role, there is real potential for using BChE to modulate ghrelin's impact in many types of emotional disorders.

Ghrelin is a 28-amino acid peptide with a serine residue acylated by octanoic acid. This feature is essential for binding and activating its primary target, the G-protein-coupled growth hormone secretagogue receptor (GSHR)[30,31]. The major source of plasma ghrelin derives from endocrine cells in the stomach, which release it into the general circulation. Circulating ghrelin then feeds back on the stomach to stimulate gastric muscles that produce "hunger pangs." It triggers afferent vagal neurons in the stomach to activate CNS regions involved in food seeking. At the same time plasma ghrelin also penetrates the blood brain barrier to stimulate GHSR and drive food cravings. In addition, brain neurons in or near the pituitary gland and hypothalamus produce and release ghrelin locally. Activating GHSR in the pituitary leads to growth hormone secretion, while activation elsewhere plays a role in many other processes including glucose homeostasis and fat storage [32,33]. Because BChE is present in both the bloodstream and the brain, its hydrolytic activity plays a role in regulating ghrelin signaling by cleaving its acyl group to form desacyl-ghrelin, which is the dominant form of the peptide in plasma and CSF [34]. Therefore, changes in blood BChE activity, e.g., in response to an anticholinesterase (anti-ChE), can shift the balance of GHSR signaling in favor of active acyl-ghrelin. However, desacyl-ghrelin also has multiple effects in a GHSR-independent manner. These relationships further highlight the physiological implications of altering ghrelin metabolism by reducing BChE activity with enzyme inhibitors or raising it with BChE gene transfer 29, 35–37].

Ghrelin serves as a stimulant for hedonic feeding, promoting food intake and fat storage [38–40]. Healthy lean individuals experience a drive for food in response to ghrelin pulses [38]. Circulating ghrelin levels are influenced by food intake, being high before a meal and low afterwards [41]. The most obvious role for BChE's modulation of ghrelin is to regulate feeding behavior/food intake. Under ordinary conditions, circulating BChE is stable, with

little change from hour to hour, day to day, or week to week. In contrast, levels of ghrelin released by the stomach or within the brain can change sharply across time. If BChE levels are too low, the drive to eat could be heightened. It has been reported that obese humans and dogs have modestly higher plasma BChE and lower plasma ghrelin than their lean counterparts [42,43]. Similar findings have been reported in mouse models of obesity [44]. By the same token, when obese humans and mice succeed in recovering their original healthy weight, plasma BChE falls and plasma ghrelin rises, often markedly. Evidence to date thus suggests that the levels of BChE activity and ghrelin activity are inversely coupled. This implies a potential for changes in plasma or tissue BChE to influence ghrelin metabolism and thereby impact the respective signaling pathways of acyl- and desacyl-ghrelin [45]. Following up these insights it seems feasible to manipulate BChE levels to impact ghrelin-driven overeating and obesity [46].

VI. Cholinesterases and pharmacological effects of inhibitors in the periphery

It is now known that cholinergic signaling plays a vital role in controlling many peripheral functions including skeletal and smooth muscle contraction, autonomic postganglionic neuron activation, parasympathetic end-organ activation (e.g., salivary, lacrimal and bronchial glands), regulation of cardiac activity, and others. Figure 1 diagrams peripheral motor and autonomic functions regulated by cholinergic signaling and illustrates relationships among cholinergic receptor activation, acetylcholinesterase activity, and functional responses.

As noted previously, early research on cholinergic signaling was focused on the neuromuscular junction. Claude Bernard's studies of neuromuscular blockade by the arrow poison, curare, were instrumental in developing the concept of nerve-muscle synapses [47]. Adequate control of muscle tone is an essential goal in many surgical and emergency settings. Neuromuscular blocking agents have been extensively used to facilitate tracheal intubations, perform endoscopic evaluations and maintain immobility during surgery. Curare acts as a competitive, non-depolarizing blocker of nicotinic receptors at the neuromuscular junction. A variety of non-depolarizing neuromuscular blockers such as atracurium, vecuronium and rocuronium have been developed and implemented clinically to block neuromuscular transmission. When neuromuscular blockade is no longer needed, it has to be reversed for rapid recovery of muscle tone. Cholinesterase inhibitors (e.g., neostigmine) have been used to reverse the effects of these non-depolarizing neuromuscular blocking agents. While cholinesterase inhibitors are still used for this purpose, a reversal agent that does not act by inhibiting acetylcholinesterase (sugammadex, a cyclodextrin-based scavenger) is being increasingly used to reverse the effects of rocuronium and vecuronium [48,49].

In contrast to the non-depolarizing receptor blockers, succinylcholine, a structural analog of acetylcholine, is a depolarizing blocker that remains the gold standard for neuromuscular blockade in rapid procedures such as emergency intubations [50]. Succinylcholine's neuromuscular blockade is ordinarily reversed very quickly by the catalytic activity of

plasma BChE [51]. However, as noted above, problems did arise in some individuals, owing to genetic differences in the catalytic activity of their BChE [52,53].

Obviously, a cholinesterase inhibitor would be of no use in reversing neuromuscular blockade induced by succinylcholine, and could in fact be counterproductive. But inhibiting acetylcholine breakdown by blocking AChE activity may be beneficial in disorders wherein acetylcholine signaling is impaired [54]. One clinical condition that can be improved by blocking acetylcholine hydrolysis is myasthenia gravis, a group of neuromuscular disorders involving autoimmune-mediated destruction of neuromuscular nicotinic receptors or associated proteins at the neuromuscular junction [55]. By virtue of their ability to block degradation of acetylcholine at the neuromuscular junction and enhance the activation of nicotinic receptors, cholinesterase inhibitors (e.g., pyridostigmine) have been used for decades to enhance muscular performance in human and veterinary patients [56]. However, one form of myasthenia gravis, associated with antibodies to muscle-specific kinase, does not benefit from blocking AChE and symptoms may in fact be exacerbated by cholinesterase inhibitors. Another cholinesterase inhibitor, the rapid-acting and reversible inhibitor edrophonium (Tensilon®), is often used in the Tensilon test to diagnose myasthenia gravis. Intravenous administration of edrophonium leads to a rapid (seconds) and dramatic, short-term improvement in muscle tone. Edrophonium may also be helpful in diagnosing other neuromuscular disorders, such as cervical dystonia and blepharospasm [57,58].

Cholinesterase inhibitors can be helpful in treating glaucoma, a leading cause of blindness worldwide [59]. Generally, increased intraocular pressure from aqueous humor accumulation leads to degeneration of the optic nerve and retinal ganglion cells. Strategies that reduce intraocular pressure are the only approaches proven to treat most forms of glaucoma [60]. Ocular administration of an anti-ChE can facilitate contraction of the ciliary muscle and increase the flow of aqueous humor through the trabecular meshwork to reduce intraocular pressure. Other drugs, e.g., sulfonamide carbonic anhydrase inhibitors such as acetazolamide and dorzolamide that decrease the formation of aqueous humor and prostaglandin F analogs (e.g., latanoprost, bimatoprost) that enhance aqueous humor outflow have largely supplanted cholinesterase inhibitors for these conditions [61]. Interestingly, the reversible cholinesterase inhibitor galantamine was recently shown to protect retinal ganglion cells and improve local blood flow in experimental models of glaucoma, but in a manner independent of intraocular pressure [62,63].

Gastrointestinal tone can be effectively modulated by cholinesterase inhibitors. As parasympathetic fibers release acetylcholine to contract the circular and longitudinal intestinal muscles, AChE inhibition can facilitate those actions. Postoperative ileus (delayed gastric emptying after surgery) can arise from the surgical procedure itself, anesthetic agents, opioid medications and other factors. Neostigmine can accelerate gastric emptying, but some studies suggest it contributes to adverse bowel effects, e.g., serious leaks from intestinal anastomoses [64,65]. Similarly, conditions such as functional dyspepsia, gastroparesis and colonic pseudo-obstruction may be treated with a cholinesterase inhibitor such as acotiamide [66–68]. Other prokinetic agents, e.g., metoclopramide, are used to facilitate gastric emptying and activate GI motoneurons as do cholinesterase inhibitors, but they act by increasing acetylcholine release rather than by blocking its degradation [69,70].

As noted earlier, individuals expressing BChE variants with low or absent catalytic activity are generally symptomless, and only a limited amount of research has been conducted on the pharmacological/toxicological consequences of BChE inhibition. However, the enzyme's putative role in ghrelin signaling is likely to be a therapeutic (or toxic) target in the future. Li and colleagues [26] reported that BChE knockout mice maintained on a high-fat diet gained considerably more weight than BChE^{+/+} littermates. Interestingly, in that study, the mice on a path to obesity did not seem to show increased food consumption. In fact, our later studies along the same lines suggest that this apparently negative outcome may have been an artifact of the means for measuring food intake, which is easily distorted by losses hidden in bedding material. In any case, the plasma concentration of acyl-ghrelin in BChE knockouts was almost twice as high as in wildtype littermates. Other hydrolases besides BChE, including, carboxylesterases and proteases, may cleave the acyl group from acyl-ghrelin or degrade the peptide itself [26,71]. As rodents have much higher carboxylesterase blood levels than humans, such differences may influence the relative impact of BChE activity on ghrelin signaling in man compared to some test species. Nonetheless, recent findings by our research teams showed that a marked rise in plasma BChE activity after gene transfer was associated with drastically reduced acyl-ghrelin in plasma [72]. Interestingly, high-BChE mice were seen to exhibit lower levels of aggressive behavior (both spontaneous and intruder-provoked). A number of studies suggest a link between ghrelin and emotional/affective behaviors [73–78]. The role of BChE in ghrelin signaling is both intriguing and important but it remains incompletely understood [79]. Therefore, studies to evaluate the effects of BChE and its inhibitors in the complex signaling associated with ghrelin's numerous physiological impacts appear well worthwhile. Figure 2 illustrates the widespread potential influence of BChE on ghrelin signaling and associated functions in the brain.

VII. Cholinesterases and Pharmacological Effects of Inhibition in the CNS

A number of elegant morphological and immunochemical studies led to the description of six cholinergic cell nuclei in the brain, termed Ch1-Ch6 [80–83]. Figure 3 is taken from [80], showing the cholinergic nuclei and their general projections in the mammalian brain. The diffuse innervation by these different cholinergic fibers affects many physiological functions including cognition, respiration, locomotion, and others. Moreover, some brain regions (notably striatum) contain multiple networks of cholinergic interneurons [84,85]. Thus, AChE inhibition in the CNS has the potential to influence an extremely wide array of functions, and this has been targeted in treating early-stage Alzheimer's disease (AD).

AD is a devastating neurodegenerative disorder affecting an estimated 35 million people worldwide [86]. Findings of extensive loss of cells in Ch4 (nucleus basalis) and widespread reduction of cholinergic markers in Alzheimer forebrain [87–92] led to the cholinergic hypothesis of dementia (reviewed in [93,94]). This hypothesis opened the door to examine potential therapeutic benefits of cholinesterase inhibitors [95–97]. Since that time, many natural and synthetic cholinesterase inhibitors that bind selectively or show multifunctional characteristics have been evaluated to treat Alzheimer's disease (see recent reviews [98–102]).

Currently, according to Mielke and colleagues [103], drugs with US Food and Drug Administration (FDA) approval for Alzheimer therapy include the following: galantamine (Razadyne®, 4aS,6R,8aS-5,6,9,10,11,12- hexahydro- 3-methoxy- 11-methyl- 4aH [1], benzofuro[3a,3,2-ef] [2] benzazepin- 6-ol), rivastigmine (Exelon®, [3-[(1S)-1-(dimethylamino)ethyl]phenyl] N-ethyl-N-methylcarbamate) and donepezil (Aricept®, 2-((1-Benzylpiperidin-4-yl)methyl)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one). In 1993, the cholinesterase inhibitor tacrine (Cognex®, 1,2,3,4-tetrahydroacridin-9-amine) was the first drug with FDA approval to treat Alzheimer's disease, but its use was limited by short half-life (requiring 4× daily dosing) and evidence of hepatotoxicity. A recent meta-analysis of the efficacy of cholinesterase inhibitors in Alzheimer's disease reported that donepezil, rivastigmine and galantamine all produced relatively minimal but significant benefits including enhanced cognition and improved behavioral outcomes, while none increased the risk of serious toxicity [104]. Donepezil and galantamine are selective for CNS ChEs, but all three can elicit nausea, diarrhea and other unwanted effects [104–108]. Unfortunately, they have little if any impact on disease progression. Interestingly, Tricco and coworkers [104] reported that galantamine was associated with a *decreased* odds ratio for death, while pharmacovigilance databases in both the US and Canada detected an *increased* odds ratio for death with rivastigmine [105]. Thus, while current cholinesterase inhibitors do have minor therapeutic benefits, the search continues for more effective or multi-purpose inhibitors acting on ChEs and other macromolecular targets [109–111].

Non-catalytic Roles of CNS Cholinesterases

In addition to serving as drug targets for inhibitors that increase synaptic acetylcholine levels, both AChE and BChE have been proposed to play *non-catalytic* roles in neurobiology. A number of studies suggest that both cholinesterases have non-catalytic roles in neurodevelopment, possibly playing a “morphogenic” role in vertebrate systems [112–116]. Some of the most striking findings in support of a morphogenic role for the cholinesterases includes the spatiotemporal expression patterns of AChE in the thalamocortical and geniculocortical projections during neonatal rat brain development [113,117]. These neurons are not cholinergic nor do they receive cholinergic innervation in adulthood, but during a discrete window of development (peaking during week 2 of postnatal development correlating with the timing of respective fiber ingrowth into the cortex) they exhibit intense AChE staining. Interestingly, inhibition of the transiently expressed AChE activity had no effect on subsequent patterning of innervation [118,119]. On the other hand, Yang and colleagues [120] reported that chlorpyrifos and chlorpyrifos oxon could disrupt axonal growth in cultured sensory neurons expressing AChE at low concentrations not inhibiting the enzyme, but similar exposures had no such effect in AChE $-/-$ neurons. Transfection of the neurons with cDNA coding for AChE restored sensitivity. Using chicken neurodevelopmental models, Layer and coworkers [121] provided evidence suggesting both BChE and AChE play morphogenic roles in vertebrate neurodevelopment. Of particular interest was the “foreshadowing” of AChE-positive areas cholinergic fibers by BChE expression during neural tube development [112,121,122]. It was proposed that these enzymes both regulate neurite extension by inactivating acetylcholine, at least partially independent from cholinergic synaptogenesis. The ChEs may play non-catalytic roles in the adult CNS as well. For example, Graybiel and Ragsdale [123] reported marked, differential

staining of AChE and BChE in the primate visual pathway. BChE showed distinct labeling of parvocellular layers of the lateral geniculate and area 17 of the visual cortex, independent of AChE labeling. The authors suggested that the differential distribution of both cholinesterases could be an indicator that BChE played some role in signaling within this pathway.

Substantial evidence points to non-catalytic roles for AChE and BChE in the development and progression of Alzheimer's disease. Alzheimer's disease greatly enhances the rate of irreversible neuronal cell death in the aging brain. Its characteristic lesions are accumulations of amyloid protein and neurofibrillary tangles that replace functional synaptic structures. These lesions typically incorporate deposits of excess AChE and BChE, in patterns that have no clear relation to functional neuronal circuitry. This feature has raised a key question: Are the enzyme deposits compensatory reactions to amyloid toxicity, or are they actually damaging stressed neurons? Adding to complexity, in addition to modulating synaptic acetylcholine levels, AChE appears to have non-cholinergic functions that involve protein–protein interactions rather than enzymatic catalysis [124–128]. Thus, studies on neuronal development suggest that AChE promotes neurite outgrowth, possibly through adhesive interactions with the extracellular matrix [128].

In AD research, consistent local accumulations of AChE in senile plaques led early on to the hypothesis that this enzyme binds beta-amyloid (A-beta) and promotes its deposition. Indeed, *in vitro* models have shown that AChE accelerates the formation of insoluble fibrils of A-beta and AChE proteins, which are more cytotoxic than A-beta alone. Crystal structure data suggest a potential locus of interaction between amyloid and the AChE protein [129]. This locus is near the enzyme's peripheral site, located on the external surface near the entrance to the catalytic gorge. Consistent with this structural inference, peripheral site-directed AChE ligands like propidium iodide and certain monoclonal antibodies have been found to prevent AChE from enhancing the formation of amyloid fibrils *in vitro* [130]. These observations are intriguing, but their relevance to the pathogenesis of Alzheimer's disease has been largely speculative. Now we need a realistic appraisal of AChE's influence on amyloid deposition in the intact brain.

In vitro studies with neuronal tissue culture have shown that AChE interacts with A-beta to promote its deposition and aggregation, which lead to neuronal death [130]. To test that the hypothesis *in vivo*, Tg2576 mice were engineered in one of our labs to express human AChE, and then crossed with a line of mice that express human amyloid precursor protein [126]. These subjects reliably developed plaques at 9 months. The resulting F1 hybrids expressed both of these human transgenes in brain, and by 6 months of age their cerebral cortex showed authentic plaques that stained readily by immunochemistry for A-beta 1–40 and 1–42. Those early onset plaques also stained positively for other components found in the brain lesions of human patients as hallmarks of AD pathology. These included Cd11b, GFAP, and again, AChE. Overall, plaque onset in the hybrid mice began 30%–50% sooner than in either of the two parental lines. Their plaque burdens increased with age, growing markedly larger and more numerous in these doubly transgenic animals at 9 and 12 months. Quantitative immunoassay *via* ELISA also confirmed an increase of total amyloid content in brain at 9–12 months. These histological and biochemical results bear a striking resemblance

to human AD pathology, and they strongly support a conclusion that AChE plays a role in initiating and promoting the pathogenesis of Alzheimer's disease.

Taken together, several findings show increased plaque formation when A-beta is expressed against a background of high AChE levels. The mechanism for this interaction remains unclear, but a plausible sequence of pathological events can be inferred from prior *in vitro* work. Direct measurements of protein aggregation in culture show that AChE can act as a nucleating factor to promote conversion of soluble amyloid peptide into insoluble amyloid fibrils [130]. Structural analysis by X-ray crystallography, competition assays, and computational docking with AChE ligands all point to the enzyme's peripheral site as a probable locus of protein interaction with A-beta [131,132]. Therefore, the most effective compound to block this interaction would not necessarily be a "classical inhibitor" that binds in AChE's active site within the gorge. In fact it seems likely that ligands directed to more superficial regions may suppress the aggregation of beta amyloid monomers and, in turn, reduce subsequent neuronal toxicity.

Among the anti-ChEs currently approved for Alzheimer therapy, tacrine, galantamine, and rivastigmine all bind primarily at the central site of AChE [133]. Interestingly, donepezil can orient from the catalytic triad region to the peripheral anionic site via aromatic stacking with aromatic acid residues [134]. This unique feature adds interest to the debate as to whether donepezil slows disease progression, as it has appeared to do in recent clinical trials. With advanced molecular modeling technology it should be possible to discover drugs that more effectively disrupt interactions between AChE and A-beta, ideally with minimal inhibition of AChE in the periphery, especially skeletal muscle. Hybrid transgenic mice with early plaque deposition in a background of high AChE and APP may represent an especially good model for testing such agents to determine whether they can help in slowing the progression of AD.

BChE's role in AD is much more controversial. This enzyme is primarily generated in the liver for release into the systemic circulation, where, as noted earlier, it serves to inactivate various bio-active ester compounds from plant-based foods that otherwise might be toxic. But a second locus of BChE production is the brain, where it contributes to inactivation of acetylcholine and thereby participates in regulating key neural pathways. Like AChE, BChE also concentrates in amyloid plaques associated with dying neurons in AD. These features have convinced many neurologists and neurobiologists that BChE is a significant contributor to AD pathogenesis, or even a major driver. That view led to clinical trials of selective BChE inhibitors like cymserine, with the expectation of enhancing acetylcholine levels at brain synapses and restoring the neurochemistry for cholinergic transmission and cognition. In fact, this concept did lead to improved cognitive function and better short term memory, but only temporarily. Unfortunately, none of the tested BChE-selective inhibitors were able to slow, much less reverse, the inexorable process of neuronal death and irreversible cognitive loss. In other words, these drugs can indeed be useful as palliative treatments, primarily in the early stages of AD, but they are not disease modifiers. As the brain pathology inevitably progresses to severe neuronal loss, their efficacy declines accordingly.

As for the issue of BChE's mechanistic role in AD, if any, full clarity remains elusive despite intensive and ongoing research. Some investigators have recently concluded that

BChE is an actual *driver* of AD pathology [135–137] but other equally well-known scientists have published data suggesting the direct opposite. The former view is largely based on the ubiquitous presence of BChE protein in plaque lesions, and the growing abundance of this protein as lesions progress. However, one can make an argument that BChE's abundance in plaque lesions is instead a “bystander effect”. Specifically, BChE may be attracted to plaques as amyloid increases and its impact may be entirely neutral or even one that mitigates amyloid-induced damage to neuronal circuitry. Support for that concept came from Diamant and coworkers [130] who showed that native BChE *attenuates* spontaneous formation of amyloid fibrils *in vitro*, while C-terminal mutated “K-variant BChE” (strongly associated with increased risk of early onset AD) is deficient in that respect.

In one of our labs, studies have begun with so-called “5×FAD mice” prone to early-onset AD, hoping to shed fresh light on BChE's role in AD plaque lesions, neuronal death, and cognitive impairment. It is far too early to draw any conclusions from the preliminary data. Nonetheless we have been able to demonstrate an essentially permanent (i.e., lifetime) elevation of brain BChE levels after direct intracerebral injection of AAV8 viral vector encoding mRNA for this enzyme. In addition, using a novel “PHP gene transfer vector” that readily crosses the blood-brain barrier [138], we found it possible to generate very high and essentially permanent BChE expression across virtually all regions of the cerebral cortex, cerebellum, and basal ganglia [139]. This capability should facilitate studies aimed at clarifying BChE's roles in the central nervous system.

VIII. Toxicological Uses of Cholinesterase Inhibitors

While ChE inhibitors can and have been used to treat a variety of illnesses, the other side of therapy is toxicity. A remarkable number of microorganisms, plants, fungi and animals have developed anti-ChEs [140–142]. As the ChEs are widespread across the animal kingdom and play an important role in neuronal signaling in all higher species of animals, widespread expression of ChE inhibitors by these many organisms has likely evolved as a defense against predation.

The toxic potential of ChE inhibitors has been recognized for over 170 years. Robert Christison, a leading toxicologist of the time reported the first results of toxicological testing of the “trial by ordeal” bean [143]. Indigenous people in the Calabar region of West Africa were known to use an “ordeal poison” to determine whether a person was innocent or guilty of a crime, usually “witchcraft.” As we noted earlier herein, extracts of the calabar bean (*Physostigma venenosum*) contain eserine (physostigmine), subsequently found to be a potent inhibitor of ChEs. The deadly potency of the extract was noted by European missionaries as early as 1840. It has been speculated that this toxicant actually had inherent properties that led to its effective “judicial” use (see review [144]).

Since discovery of eserine's toxicity, carbamate structure and anti-ChE action, many other carbamate cholinesterase inhibitors have been synthesized and characterized [145]. A substantial number were introduced as pesticides in the 1960s and some of these continue extensive use throughout the world [146]. Before widespread introduction of the carbamates, starting in the 1940s, many organophosphorus cholinesterase inhibitors were developed as

insecticides [147]. Carbamate and organophosphorus cholinesterase inhibitors both covalently modify the active site serine residue in AChE and BChE, thereby inhibiting all choline ester hydrolysis. Cleavage of carbamoyl moieties by an esterase, while far slower than hydrolysis of the acyl group in a choline ester, is still rapid compared to the painfully prolonged reactivation of esterase inhibited by an organophosphorus agent [148].

Organophosphorus cholinesterase inhibitors did not emerge from a natural source: unlike the carbamates, they are all synthetic entities. Tetraethylpyrophosphate was the first organophosphorus inhibitor, synthesized and reported in the 1850s. Further development and characterization of organophosphorus anti-ChEs was not initiated until the mid-1930s in pre-WWII Germany, under the direction of Dr. Gerhard Schrader [149]. Schrader was searching for synthetic insecticides to address the high cost and low availability of common natural insecticides in use at that time. Tabun (N-dimethyl phosphoramidocyanidate), one of Schrader's most potent inhibitors, subsequently came under German military control to use as a chemical warfare agent [150]. Other organophosphorus compounds to come from that laboratory included parathion (O,O'-diethyl-O-*p*-nitrophenyl phosphorothioate) and its oxygen analog (O,O'-diethyl-O-*p*-nitrophenyl phosphate), paraoxon [147]. Since that time, many organophosphorus anti-ChEs have been synthesized. Most were developed for commercial use as pesticides and, in some cases, as drugs in veterinary and human therapeutics (e.g., diisopropylfluorophosphate for glaucoma, trichlorfon as an anthelmintic).

Carbamate and OP insecticides are still among the most widely used pesticides in the world [151]. In 2012, the OP insecticides chlorpyrifos and acephate were ranked 14th and 22nd overall in estimated amounts of agricultural pesticides used in the US. The carbamate insecticide carbaryl and the OP insecticides acephate and malathion were until recently among the most common pesticides for home use. Acephate was also widely used in the industrial sector. Over the last two decades, a number of OP insecticides have been withdrawn from the market, banned, or use-restricted in the US, but others remain a large part of total pesticide use. In 2012, OP insecticides made up roughly 38% of all insecticides used in the US [151]. As of now, multiple regulatory actions in the US have curtailed the use of such pesticides, but certain populations may still be exposed to cholinesterase-inhibiting levels [152].

Worldwide concern for anti-ChE pesticide exposure remains: more sinister is the threat of OP nerve agent intoxication. While virtually all developed countries have joined agreements to eliminate stockpiles of these extremely toxic compounds, their relative ease of synthesis makes them a continuing concern. As recently as April of 2017, the Organization for Prohibition of Chemical Weapons confirmed that the "nerve gas" sarin had been used on residents of Khan Sheikoun, Syria. Thus the potential use of OP nerve agents by rogue nations and/or terrorist groups is a remaining global problem.

A seemingly paradoxical pharmacological use of more readily reversible cholinesterase inhibitors is to block the long-term inactivation of AChE by "irreversible" inhibitors, the OP nerve agents [153]. In essence, individuals at risk of exposure to an OP nerve agent can be protected by pre-exposure to pyridostigmine or another carbamate anti-ChE, transiently blocking OP access to the active site of the enzyme. If the agent with short-lived inhibition is

given before exposure to the irreversible inhibitor, the latter cannot bind to the enzyme, which will then spontaneously reactivate. In fact this scenario has shown protection against nerve agent intoxication in several animal models [153–158]. The logic behind this prophylactic approach is based on the concept that cholinergic synapses have “spare” enzyme, so a certain level of AChE inhibition can be tolerated without disrupting the dynamics of cholinergic signaling. This clever prophylactic strategy was implemented in the first Persian Gulf War. Soldiers were given varying doses of pyridostigmine with concern for possible exposure to chemical weapons. For some time, a cloud of suspicion has hovered over this application however because of the potential role of pyridostigmine in the unexplained “Gulf War Illnesses” following the first Persian Gulf War [159]. Those disorders may well have had other contributing causes however, e.g., post-traumatic stress. Even if pyridostigmine had no role in these illnesses, delivering a dose of drug to shield enough enzyme molecules without excessive inhibition, in a diverse group of people with high variability and exposure to environmental stressors, was a risky approach. But one thing is certain: if one is to use cholinesterase inhibitors for therapy or prophylaxis, there will always be a delicate balance between beneficial and toxic inhibition of AChE. Too little drug will be ineffective, too much will be dangerous.

IX. Increasing ChEs in vivo

Elevating cholinesterase levels in the body may be beneficial to protect against chemicals that elicit toxicity by inhibiting AChE, or that are inactivated primarily by ChEs. The main incentives for enhancing ChE activity are to overcome or prevent the toxicity of anti-ChEs caused by 1) accidental or intentional exposure to anti-ChE pesticides in agricultural settings, 2) ingestion of foods carrying residual pesticides, or 3) intentional exposure to nerve agents in chemical warfare or terrorism. All these scenarios are possible in present day conditions. Some are especially likely in developing countries and regions that lack active oversight and strict environmental regulations. Increasing the levels of BChE in the circulation has been evaluated as a protective strategy in two very different settings.

First, administering large amounts of purified human BChE has been shown to protect experimental animals against lethal exposures to various OP nerve agents. BChE can sequester or scavenge OP molecules in a stoichiometric manner, inactivating the toxicant molecules before they can inhibit AChE in brain and peripheral tissues to disrupt cholinergic signaling [160]. In fact, both BChE and AChE have been evaluated as bioscavengers for OP nerve agents. In sufficient quantities, administration of these stoichiometric binding proteins can minimize AChE inhibition in target tissues and protect against lethality from OP toxicants in multiple experimental models, including primates.

There are obstacles in the prophylactic use of ChEs as OP bioscavengers including a need for large amounts of purified human protein, due in part to their rapid clearance from the circulation. For example, intramuscular (im) administration of human BChE in mice (13 mg/kg, about 0.3 mg) leads to peak blood BChE activities at about 10–12 h, but only ~ 25% of peak remains at 70 h [161]. Both im and intraperitoneal (ip) administration of 0.1–3 mg of human BChE in mice caused marked elevation of circulating BChE enzyme activity, peaking 12–24 h later, but returning to near baseline by 120 h [162]. Doctor and Saxena

[163] and Saxena and coworkers [164] reported on the pharmacokinetics of human BChE in mice and guinea pigs given im and ip administration as well as long-term stability of the lyophilized enzyme (at least 2 years), and complete, sign-free survival in guinea pigs subsequently given an LD₅₀ dosage of the nerve agents VX and soman. Higher im dosages of human BChE (up to 60 mg/kg), led to peak levels of enzyme around 24 h that remained substantial for at least four days [165]. The same investigators reported no overt physiological or behavioral signs after high-dose BChE administration, and no changes in serum chemistry or tissue histopathology. Intravenous administration of 30 mg/kg human BChE in rhesus monkeys led to marked elevation of blood BChE activity that returned to within about 25% of peak levels by 100 h [166]. These and other recent studies illustrate the potential for exogenously administered human BChE to elevate circulating enzyme activity and protect against toxicity from potent OP nerve agents, with little evidence of adverse reaction to high doses of the enzyme.

As noted earlier, while exogenously administered BChE can protect against OP toxicity, its efficacy is limited by clearance of the protein from the circulation within a few days. A number of investigations have attempted to increase the circulation time of ChEs by chemical modification, gene transfer, and “nanoformulation.” Studies using AChE as a bioscavenger [167–171] led to the conclusion that the degree of AChE sialylation has a direct influence on the enzyme’s circulatory residence time. Chemical modification of recombinant BChE by polysialylation led to a 6-fold increase in mean residence time after iv dosing in mice, and such enzyme proved to be a bioscavenger offering protection against several OP nerve agents [172,173]. Modifying proteins with polyethylene glycol (PEG, i.e., PEGylation) has been used for decades to reduce their clearance *in vivo* [174–177]. Cohen and coworkers [171] reported that PEGylation of rhesus and human AChE led to large increases in mean residence times of recombinant rhesus and human AChE given to rhesus monkeys. PEGylation also doubled the mean circulatory residence time of recombinant tetrameric BChE from 18.3 to 36.2 h [178]. An almost 10-fold increase in circulatory time was gained by PEGylating recombinant monomeric BChE [179]. Thus, modifying ChEs by PEG generally leads to increased circulation times, but there are reports of antibody development and more rapid clearance of enzyme activity with subsequent doses of PEGylated proteins [179,180].

Some studies have evaluated gene transfer as an approach to increase and prolong BChE activity for protection against OP toxicants. Chilukuri and coworkers [179,181] showed that BChE^{-/-} mice treated (iv but not ip) with recombinant adenoviruses encoding rHu-BChE showed elevated blood BChE levels (about 200-fold higher than wild-type controls) peaking about 5 days after treatment, but returning to baseline by 10 days post-inoculation. Antibodies to the native protein were detected in the serum. In a similar approach, Parikh and coworkers [182] reported up to 3,400- fold increase in plasma BChE activity and transient protection against high (5-30-fold LD₅₀) doses of the OP anti-ChEs echothiophate and VX (O-ethyl-S-2-N,N-diisopropylaminoethyl methylphosphonothiolate). Again BChE activity returned to baseline by day 10, however.

Nanoformulations of BChE that might be useful as bioscavengers have been reported. Gaydoss and coworkers [183] described a polyionic complex made of BChE with a poly-L-

lysine and poly(ethylene glycol) copolymer, with an estimated diameter of about 15 nm. Fluorescence-labeled BChE-copolymer complexes injected into mice showed a small amount of BChE entered the brain. In 2015, Pope and colleagues [184] reported on a series of BChE-copolymer complexes synthesized following the general approach of Gaydess et al., [183]. A subset of these complexes was spherical, with a median diameter of about 35 nm [185]. *In vitro* sensitivity to the OP anti-ChE paraoxon, resistance to proteases and heat-inactivation, and *in vitro* “bioscavenging” activity against paraoxon were all equivalent to native BChE [184,185]. Recombinant dimeric BChE has also been conjugated with CdSe/CdZnS quantum dots [186]. These conjugates retained partial enzyme activity and showed similar sensitivity to paraoxon. Sokolov and colleagues [187] reported that human BChE conjugated with gold nanoparticles had a diameter about 15 nm and showed an interesting increase of BChE activity. In another recent study, equine serum BChE was coated with a zwitterionic polymer gel [188], leading to nanoparticles of 15–30 nm diameter that resisted inactivation by heat and trypsin. They also showed about a 3-fold increase in circulating time versus the free enzyme, and importantly no immune sensitization with repeated dosing. Rahhal and colleagues [189] reported an interesting formulation of equine BChE, laminating a film of protein onto a mold to produce 1 μ m BChE microparticles. After purification, the preparation was administered by insufflation, with BChE retention times similar to free enzyme following oro-tracheal administration (48–72 h). Optimization of such formulations holds promise for further development of bioscavengers for OP and possibly other toxic chemicals.

It has long been known that native BChE is a major factor in the inactivation of cocaine, an ester-type drug of abuse [190]. But recently some researchers conceived the idea that BChE mutations could improve that function to a point that would favorably impact cocaine overdose. Rapid progress was made in different laboratories approaching this goal, assisted by computer-based models of cocaine docking in the enzyme’s active site, which led to successful predictions to improve drug binding and hydrolysis [29]. At present, near-optimal BChE versions that enhance the rate of cocaine inactivation by more than a thousand-fold have been generated [191]. Efficacy with exogenous administration of such cocaine-hydrolyzing BChE variants can also suffer because they are more rapidly cleared from the circulation than native BChE. However, site-directed mutagenesis introducing disulfide bonds between two mutant BChE subunits led to an approximate doubling of circulatory half-life in rats [192]. In our hands, mice and rats treated with a cocaine hydrolase showed no reaction whatsoever to doses of cocaine that would ordinarily have been lethal within 1–2 minutes. In contrast, they merely continued normal cage-side activities, eating and grooming, just like vehicle-controls [72].

The theoretical importance of a highly effective cocaine hydrolase is that treatment-seeking addicts who have been “clean” for a while would be less likely to relapse after, in a weak moment, “one last hit” delivered a greatly reduced drug reward. For that effect to be maintained for periods of months to years the most promising approach would be one that doesn’t require former users to make repeated decisions to remain in treatment on a daily or weekly basis. We consider that only a durable gene transfer can meet that requirement. In fact, our recent studies with such agents indicate that very high levels of BChE can be generated by gene transfer with no overt disturbances. Furthermore, these levels were

sustained for more than a year in experimental animals ranging from mice to Rhesus monkeys (S. Brimijoin and M. Carroll, unpublished data).

Several studies on the safety of BChE as an OP bioscavenger or cocaine hydrolase have reported no overt signs of toxicity, even with massive increases in circulating BChE activity. While the severity of either nerve agent intoxication or cocaine abuse may warrant taking the risk for possible side effects of large increases in circulating BChE levels for extended periods, the emerging role of BChE in the metabolism of ghrelin could be of concern. Long-term studies should be conducted to evaluate more subtle physiological changes that may accompany prolonged BChE elevation. Of special note, altered affective behaviors in mice with high levels of genetically-modified BChE were disconcerting at first, but they led to highly positive effects such as reductions in aggression and stress-induced fear [72,193,194].

Brain AChE and BChE both concentrate in senile plaques associated with amyloid protein. While a systemic administration of BChE would not be expected to lead to increased activity in the CNS, gene transfer approaches may in fact lead to increased expression in the brain. If BChE has some role in the aggregation of amyloid and plaque generation, the potential for its enhancement by increasing BChE expression would be of great concern. However, as noted earlier, at Mayo, extensive but unpublished studies on mice carrying genes for premature formation of brain plaques of amyloid and tau proteins with neurofibrillary tangles showed no increase in lesions when brain BChE levels were raised many fold.

X. Conclusion

In summary, AChE and BChE are related enzymes that evolved throughout the animal kingdom. Complex organisms have developed chemical signaling pathways relying primarily on AChE as a key regulator. On the other hand, a physiological role for BChE has previously been obscure. In fact, there was no real consensus that this enzyme had any role beyond the detoxification of bioactive esters in plant foods and ester-type medications, but its status is now rapidly changing. A number of clinical disorders have been therapeutically addressed by use of cholinesterase inhibitors. In general, any condition that involves reduced activation of acetylcholine receptors in cholinergic synapses may be partially or fully relieved by application of a cholinesterase inhibitor. Such conditions include neuromuscular disorders, autonomic dysfunction, and Alzheimer's disease. All share a deficiency of cholinergic receptor activation that can be restored by inhibiting AChE. Of course, excessive AChE inhibition in these patients can lead from therapy to non-selective toxicity. Abnormally low levels of BChE can lead to inefficient clearance of esters in the food supply and slowed inactivation of ester-based drugs, potentially leading to toxicity. Moreover, recent studies point to a likely role for BChE in regulating levels and activity of the peptide hormone ghrelin, with altered ghrelin signaling a potential consequence of xenobiotics that affect BChE activity.

It seems fair to say, the two cholinesterase enzymes, AChE and BChE, have catalytic properties with important impacts on neuromuscular, cognitive and emotional functions. In addition they show adhesive and other properties that contribute to protein-protein interactions, with potential roles in disorders like Alzheimer's disease. For this reason,

manipulating either the protein levels of AChE or BChE or their enzymatic activities may influence the toxic aggregation of proteins in this and other neurodegenerative diseases, with positive (therapeutic) or negative (toxic) outcomes. While each of these two ChEs has been studied for decades, remarkable new information continues to emerge regarding their pleiotropic relationships with key signaling pathways and functions in the nervous system. It has become clear that changes in either catalytic or structural properties of these ChEs are likely to shift the delicate balance between poison and remedy in regard to numerous crucially important physiological functions. And, with the rising tide of discovery regarding the range and power of these roles we can look forward to harnessing this knowledge to avoid toxicity while reaping a harvest of advances in treatments for a wide range of medical conditions.

Acknowledgments

This work was primarily supported by grants from the Defense Threat Reduction Agency (HDTRA1-13-1-0042, CP), National Institute for Environmental Health Sciences (ES008739, CP) and a Translational Avant-Garde Award from the National Institute on Drug Abuse (DA42492, SB).

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Cholinergic Receptor Activation

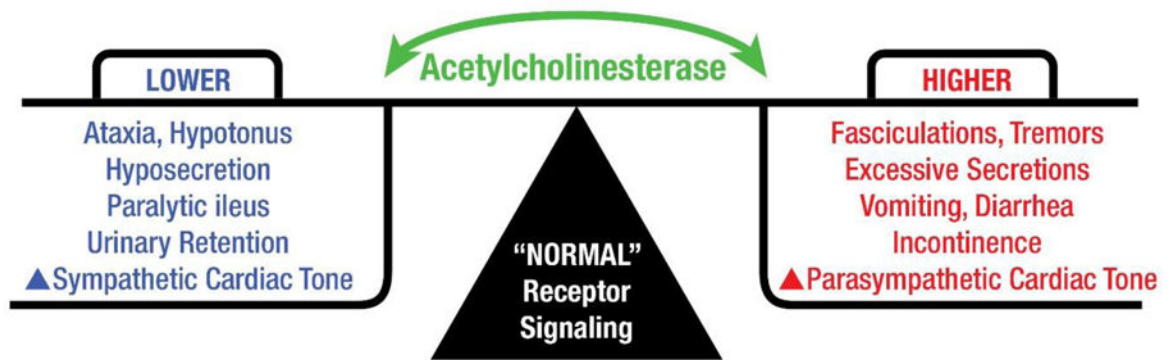


Figure 1.

Acetylcholinesterase and cholinergic receptor activation. Under normal physiological conditions, AChE plays a dynamic role in regulating cholinergic signaling, rapidly degrading acetylcholine to terminate neurotransmission. Cholinergic motoneurons release acetylcholine to activate skeletal muscles for ambulation and fine motor control. Similarly, parasympathetic neurons stimulate smooth muscles to drive gut peristalsis, contraction of the bladder, secretion from salivary glands, slow heartbeats at the sinoatrial node. When AChE activity is suppressed, cholinergic receptor activation causes increased muscle contractions, exocrine secretions, GI disturbances, incontinence and increased parasympathetic tone leading to decreased rate and force of heartbeats. When AChE activity is high, reduction of cholinergic receptor activation, lowers force and tone in muscle and heart, and reduced exocrine secretions.

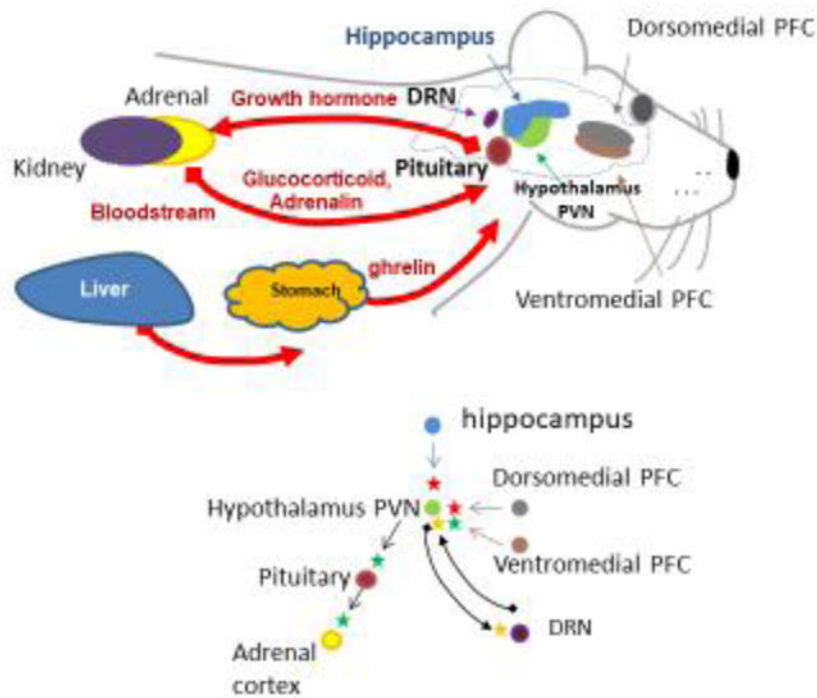


Figure 2. Role of BChE in ghrelin metabolism and signaling in the brain. Top panel: Main organs, brain regions, and pathways involved in fear response. Red arrows indicate blood-borne delivery. Bottom panel: Key interacting centers: parvo-ventricular nucleus of the hypothalamus (PVN); dorsomedial and ventromedial prefrontal cortex (PFC); dorsal raphé nucleus (DRN); adrenal cortex; and pituitary gland. Arrows indicate direction of interactions. Green = activation; red = inhibition; and yellow = modulation.

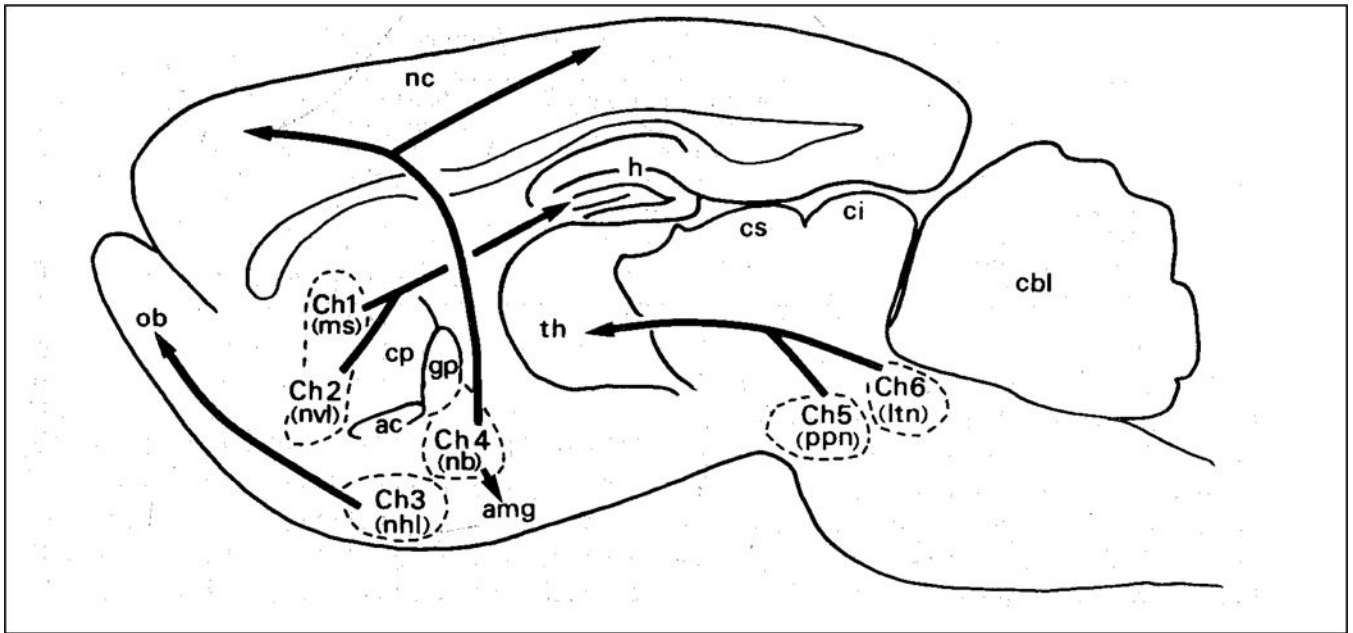


Figure 3.

Cholinergic nuclei and projections in the rat brain. Classic schematic diagram from Mesulam *et al.*, 1983. Ch1, medial septal nucleus; Ch2, vertical limb nucleus of the diagonal band; Ch3 Lateral part of horizontal limb nucleus of the diagonal band; Ch4, Nucleus basalis of Meynert, globus pallidus, Substantia innominate; nucleus of the ansa lenticularis; Ch5, Nucleus pedunculopontinus; Ch6 Laterodorsal tegmental nucleus. Other structures: nc, neocortex; ob, olfactory bulb; th, thalamus; h, hippocampus; cbl, cerebellum.