Review TRENDS in Neurosciences Vol.29 No.4 April 2006

Virtues and woes of AChE alternative splicing in stress-related neuropathologies

Eran Meshorer[1] and Hermona Soreq[2]

[1]National Cancer Institute, National Institutes of Health, Building 41, 41 Library Drive, Bethesda, MD 20892, USA
[2]The Hebrew University of Jerusalem and the Israel Center of Neuronal Computation, Department of Biological Chemistry, Givat Ram, Jerusalem 91904, Israel

The ACh hydrolyzing enzyme acetylcholinesterase (AChE) is a combinatorial series of proteins with variant N and C termini generated from alternate promoter usage and 3′ alternative splicing. Neuronal AChE variants show indistinguishable enzymatic activity yet differ in their expression, multimeric assembly and membrane-association patterns. Differentially induced under stress, they show distinct non-hydrolytic properties and interact with different protein partners. Recent findings suggest that transcriptional and post-transcriptional regulation of AChE pre-mRNA is a neuroprotection strategy but might involve long-term damage. Specifically, variant-specific causal involvement of AChE in the progression of both neurodegenerative diseases (e.g. Alzheimer's and Parkinson's diseases) and neuromuscular syndromes (e.g. myasthenia gravis) raises the possibility that future therapeutic drugs might target specific AChE variant(s) or the corresponding RNA transcripts.

The composition and role of AChE

Much has been learned and said about both the hydrolytic and the non-catalytic roles of acetylcholinesterase (AChE) [1–3]. Its primary role undoubtedly remains that of hydrolyzing synaptic ACh. In this role, AChE is one of the most efficient enzymes in nature, capable of hydrolyzing ACh at a rate so high that it is limited only by diffusion [4]. This property makes it evolutionarily an almost perfect terminator of ACh-mediated neurotransmission. The discovery that AChE knockout mice were born alive was thus unexpected, especially because AChE is required for neuronal and muscular development in zebrafish embryos [5]. Despite their delayed physical development, the *Ache*$^{-/-}$ mice lived for up to 21 days and displayed apparently normal neuromuscular junctions (NMJs) [6]. Their survival is most probably due to partial functional redundancy with other catalytic enzymes, such as butyrylcholinesterase, that can partly compensate for some AChE functions [7].

Corresponding authors: Meshorer, E. (meshoree@mail.nih.gov), Soreq, H. (soreq@cc.huji.ac.il).

Available online 3 March 2006

The properties of AChE are modified during many CNS neuropathologies; this impedes the intricate balance between neuronal stimulation and termination, and alters synaptic properties and neurotransmission. The properties of AChE also vary in non-pathological situations, and three alternatively spliced isoforms of the enzyme have been identified. The canonical tetrameric AChE-S isoform ('S' indicates synaptic; this isoform is also known as AChE-T, with 'T' indicating tailed) is produced by joining exon 4 with exon 6 to give a cysteine-containing, and thus tetramerizing, C terminus (Figure 1a). An additional 3′ spliced form of AChE is generated by missing out the short intron 4, to join exon 4 directly with exon 5. This gives rise to the dimeric glycophosphatidylinositol (GPI)-anchored blood-expressed AChE-E isoform ('E' indicating erythrocytic; this isoform is also known as AChE-H, with 'H' indicating hydrophobic). Additional 3′ alternative splicing also generates the 'read-through' variant of AChE (AChE-R) [8,9]. Although AChE-R is found predominantly under conditions of neurodegenerative disease and stress-associated disorders, it also occurs to a minor extent in healthy tissue and myogenic cell lines [10]. AChE-R is produced by retaining intron 4 and exon 5, to yield an AChE mRNA variant that has an alternative 3′ end (Figure 1a). This transcript generates an alternative protein product that has a distinct shorter C terminus and lacks the cysteine residue present in the C terminus of AChE-S; AChE-R thus lacks multimerization potential and remains monomeric and soluble [1,11]. Therefore, AChE can be monomeric (AChE-R), dimeric (AChE-E) or tetrameric (AChE-S). Tetrameric AChE-S can further interact with collagen Q (ColQ), enabling anchorage to NMJs [11], and a proline-rich membrane anchor protein (PRiMA) is responsible for the synaptic docking of AChE-S in the brain [12]. Because AChE-E and AChE-R are incapable of anchorage to the NMJ or to synaptic membranes through ColQ or PRiMA, only the AChE-S form of the enzyme is regarded as truly 'synaptic'. The 3′ alternatively spliced transcripts described so far are common to all mammals and are ubiquitously expressed. However, an examination of the expression sequence tag (EST) database reveals that a novel 3′ alternatively spliced variant has been repeatedly observed exclusively in pooled germ-cell tumors of human origin (GenBank accession numbers BE502241,

 0166-2236/$ - see front matter doi:10.1016/j.tins.2006.02.005

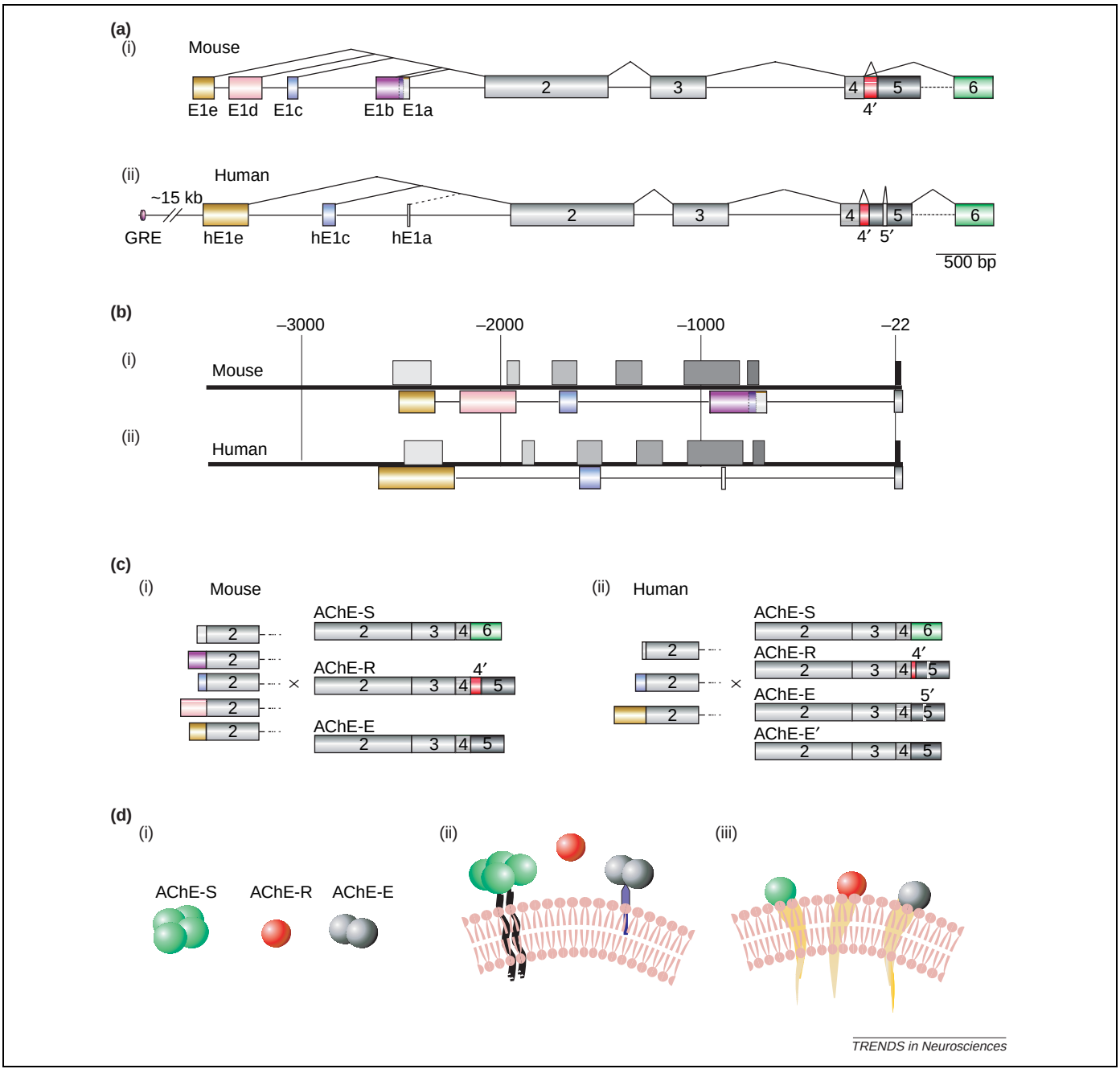

Figure 1. AChE gene structure, alternative transcripts and protein products. **(a)** Structure of the mouse *Ache* (i) and human *ACHE* (ii) genes (drawn to the same scale). Exons are depicted as cylinders, introns as horizontal lines. Splicing options are shown as lines above the genes. E1a–1e are alternative versions of mouse *Ache* exon 1, and hE1a, hE1c and hE1e are alternative versions of human *ACHE* exon 1. 4′ indicates the pseudo-intron 4 (shown in red); 5′ indicates the cryptic intron skipped in germ-cell tumors. Abbreviation: GRE, glucocorticoid response element. **(b)** Homology in the 3-kb-upstream regions of mouse *Ache* (i) and human *ACHE* (ii). Parallel homologies are shown in the same shades of gray, and alternative 5′ exons are shown underneath in the same colors as in (a). **(c)** Combinatorial complexity of mouse *Ache* (i) and human *ACHE* (ii) mRNA transcripts. Each of the five murine alternative 5′ first exons can potentially be combined with the three murine 3′ alternative transcripts, and each of the three human alternative 5′ exons can potentially be combined with the four human 3′ alternative transcripts, to yield 15 or 12 alternatively spliced isoforms, respectively. **(d)** AChE alternative protein products. (i) Globular structure of tetrameric AChE-S (green), monomeric AChE-R (red) and dimeric AChE-E (gray). (ii) Conventional wisdom regarding the membrane attachment of AChE isoforms. Synaptic docking of AChE-S is mediated by ColQ or PRiMA (black) and that of AChE-E is mediated by a GPI anchor (purple). AChE-R remains monomeric. (iii) Putative membrane docking through the extended N terminus (N-AChE, yellow) transcribed by the alternative exon E1e of AChE-S (green), AChE-R (red) or AChE-E (gray).

AW590331, AI825097, AI652210, AI632562, AI341526, AI205484, AW138046, BE504052, AI651502 and BE502360). This variant, designated AChE-E′, is generated by an exclusion of a cryptic intron within exon 5, yielding a transcript 36 bases shorter than the canonical AChE-E (Figure 1a,ii; Figure 1c,ii).

In addition to the 3′ alternative splicing of AChE that generates proteins with distinct C termini, the 5′ end is also subject to intricate regulation, with at least five alternatively spliced and differentially expressed exons in mouse and three in human [13] (Figure 1a,b). Interestingly, each of these transcripts is associated with a distinct promoter region at its 5′ end, and some are developmentally regulated and/or responsive to stress [13]. Sequence conservation between mouse *Ache* and human *ACHE* is also evident at the 5′ end, especially surrounding the

alternative 5′ exons (Figure 1b). The different 5′ versus 3′ alternative combinations can potentially give rise to 15 and 12 different AChE transcripts in mice and humans, respectively (Figure 1c).

The only hydrophobic sequence in the canonical AChE protein is its first 31 amino acids. These primarily constitute a signal peptide that docks AChE to the endoplasmic reticulum membrane and is later cleaved off during maturation. In the three AChE isoforms with distinct C termini already described, this leader sequence is removed [14]. Intriguingly, although most of the recently identified 5′-encoded exons produce alternative untranslated regions (UTRs), at least one of those exons gives rise to a protein that has an extended N-terminus in humans, designated N-AChE [13]. In principle, N-AChE-S, N-AChE-E and N-AChE-R (or various combinations thereof) might exist (Figure 1d). Because N-AChE associates with the plasma membrane in human blood cells [13], it is tempting to speculate that the extension prevents cleavage of the signal peptide, as is the case for cyclooxygenase [15]. This extension could then serve as a transmembrane domain, enabling AChE to anchor itself to the synaptic membrane (Figure 1d). The existence of N-AChE could have profound neurological implications, especially in relation to the expression of monomeric AChE-R: the N-terminal extension might provide a means by which AChE-R can dock itself – without the assistance of ColQ or PRiMA – to the synaptic membrane.

AChE in stress-related neurological disorders

Transcriptional and post-transcriptional stress-related responses interact to induce a cascade of changes in the levels and properties of brain proteins that might be involved in neuronal reactions to stimuli [16]. Proteins involved in stress-related neurotransmission pathways are particularly affected in this way. ACh levels are transiently elevated in the mammalian brain during stress responses [17], and similar increases in ACh-mediated signaling have been reported following exposure to cholinesterase inhibitors [18]. Indeed, psychological stress elicits strikingly similar neuropsychological effects to those observed after acute or chronic exposure to anti-cholinesterases [19]. In addition, acute stress and pharmacological inhibition of AChE both suppress the production of the ACh-synthesizing enzyme choline acetyltransferase (ChAT) [8]; such suppression is accompanied by rapid increases in AChE-R mRNA levels in the mouse brain in response to forced-swim stress [8,9].

Several lines of evidence suggest that AChE-S and AChE-R have distinct, and sometimes inverse, roles in the normal brain and in post-stress processes [20–22]. Transgenic TgS and TgR mice overexpressing AChE-S or AChE-R, respectively, display distinct characteristics [1]. TgS mice show accelerated stress-related neuropathology [20], including loss of dendritic arborizations and spines. This leads to progressive deterioration, neuromotor malfunctions, hypersensitivity to anti-cholinesterases and to closed-head injury, vulnerability to a switched day–night cycle, and impaired diffusion across the blood–brain barrier [1,22,23]. AChE-R-expressing TgR mice, by contrast, display normal neuromuscular function and their brains are relatively protected from the stress-associated pathology hallmarks that predict age-dependent neurodeterioration [20] (e.g. astrocytic hypertrophy). Stress-induced alternative splicing of AChE might be a cause or an outcome of this phenotype; in either case, it could predict physiological relevance for the exclusive overexpression of AChE-R following stress. Apart from this apparent protective function, AChE-R might mediate at least some of the adverse cellular changes associated with delayed stress responses. It has recently been shown that the stress-induced increase in AChE-R mRNA yields a marginal subsequent increase in the corresponding enzyme activity in many brain regions [24], suggesting that much of the newly synthesized protein is catalytically inactive. However, even a low-level increase in soluble AChE can have a profound effect, especially on non-cholinergic neurons carrying ACh receptors. Altogether, this response suggests a bimodal potential mechanism for suppressing ACh-mediated excitation after stress. Intriguingly, AChE-R mRNA was found in apical dendrites of neurons from all cortical layers following exposure to cholinesterase inhibitors [8], suggesting possible local regulation and translation of AChE in the synapses of non-cholinergic neurons. The robust and persistent post-stress AChE-R overexpression [8,9] is further accompanied by translocation of AChE-R mRNA to neurites following repeated stress in mice. There, it replaces the usually prevalent AChE-S mRNA [9]. This stress-induced shift from AChE-S to AChE-R mRNA is strongly associated with synaptic hypersensitivity to anti-cholinesterases (Figure 2). When physostigmine was applied to brain sections of mice weeks after a period of repeated stress, a dramatic increase in extracellular population field potentials (PFPs) was observed in response to stratum oriens stimulation [9]. Atropine, a selective and reversible competitive antagonist of muscarinic acetylcholine receptors (mAChRs), reduced the recorded electrophysiological activity in pre-stressed synapses to almost zero. This might suggest that a negative-feedback loop operates during stress to down-regulate the levels of postsynaptic mAChRs. Inhibition of a reduced number of receptors would reduce synaptic activity (Figure 2b). Supporting this notion, stress-induced reduction in mAChRs was reported in a rat model of Gulf War syndrome [25]. Interestingly, the evoked PFP was blocked by the NMDA receptor antagonist aminophosphonopentanoic acid (APV) and the AMPA receptor antagonist dinitroquinoxalinedione (DNQX), attesting to the glutamate-mediated (i.e. excitatory) nature of the hypersensitized synapses. This phenomenon closely resembles the hypersensitivity that has long been known to follow stressful experiences [26], suggesting involvement of AChE in stress-related symptoms and deleterious functional consequences of AChE-R overexpression.

Because stress-induced neuronal hypersensitivity occurs through intensification of ACh-induced signaling cascades [9] (Box 1, Figure 3b), it might be mediated by interaction of intracellular AChE-R with the protein kinase C (PKC)-scaffold protein RACK1 [27]. PKC mobilization to the perikaryal membrane [27] and the

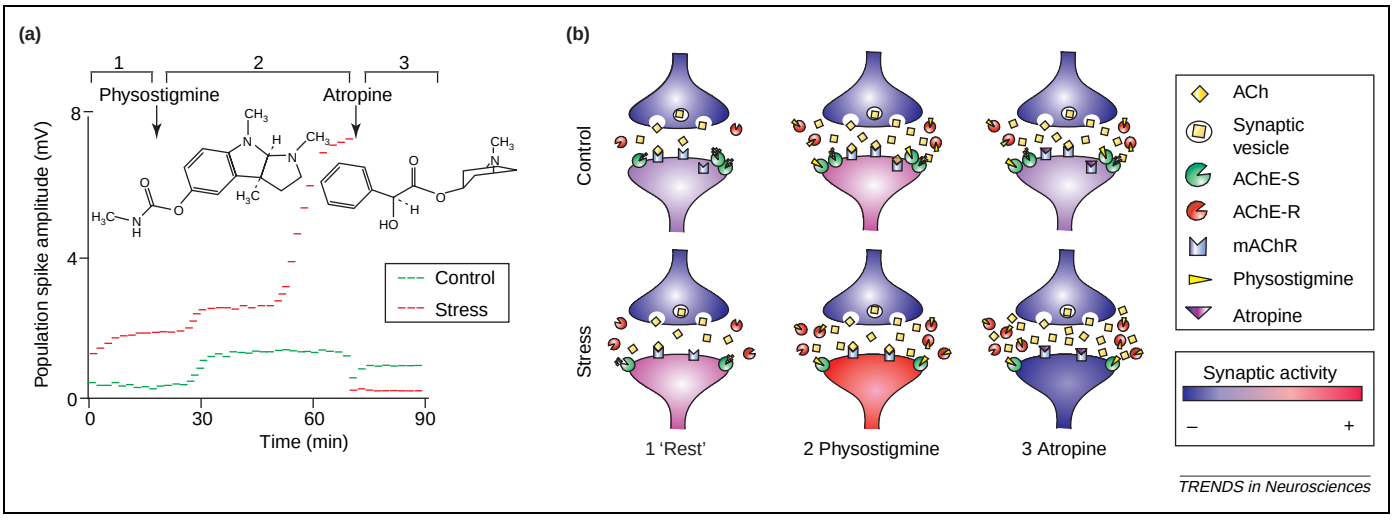

Figure 2. Prolonged stress and long-term AChE-mediated synaptic hypersensitivity. **(a)** Extracellular recordings of slices from the hippocampal CA1 area in response to stratum oriens stimulation. (1) Baseline extracellular recordings of stressed mice (red) are higher than controls (green). (2) Inhibition of AChE using physostigmine (structure as shown on the left) results in increased electrophysiological response in control mice and in severe hypersensitivity in stressed mice. (3) Atropine (structure as shown on the right) reduces the amplitude in control synapses to close to normal levels, but completely blocks electrical activity in synapses of stressed mice. **(b)** Model for the stress-induced hypersensitivity of cholinergic synapses. (1) In control mice under normal conditions (top), ACh (yellow) is released into the synaptic cleft, where it binds to mAChR (blue) on the postsynaptic membrane. Synaptic AChE-S (green) rapidly hydrolyzes ACh to convert the synapse back to resting potential. Soluble AChE-R (red) is rare in control situations. In stressed synapses (bottom), diffuse AChE-R replaces synaptic AChE-S. This reduces effective AChE levels in the synapse, increases synaptic ACh levels and increases basal electrophysiological activity (pink; see key for postsynaptic activity). Prolonged elevated ACh levels might lead to a negative-feedback response that reduces the concentration and/or signaling capacity of mAChR on the postsynaptic membrane [25]. (2) In control situations (top), inhibition of AChE by physostigmine (yellow triangles) elevates synaptic ACh levels and increased activity is recorded; levels of AChE-R are also elevated [8]. In stressed synapses (bottom), a lower starting concentration of synaptic AChE-S (and a higher concentration of AChE-R) results in hypersensitivity once the entire remaining pool of AChE-S is inhibited, and synaptic levels of ACh reach new heights. (3) In control synapses (top), blockage of postsynaptic mAChRs using atropine (purple triangles) results in decreased electrical activity. In stressed synapses (bottom), there are fewer postsynaptic ACh receptor responses. Together with effective blockage of mAChRs by atropine, this results in complete loss of electrical activity.

intensification of long-term potentiation (LTP) in AChE-R-overexpressing brain [28] could provide a new explanation for the well-known phenomenon of sensitization following stress [26,29]. That antisense suppression of AChE-R accumulation obliterates the consolidation of fear memories [28] corroborates this concept. Interactions between ACh and glutamate signaling have been demonstrated in several brain regions (Box 1) to affect CNS functions such as LTP, memory and behavior [30], all of which are subject to hyperexcitation following stress. The glutamate-mediated nature of the hypersensitized response might further be relevant to the additional functions attributed to neuritic AChE-R. In particular, based on the electrotactin properties of AChE [2], non-catalytic capacities might compete with, and mediate, cell–cell and cell–matrix interactions. Neuroligin-1, for example, is a postsynaptic cell-adhesion molecule in excitatory synapses [31] that includes an extracellular catalytically inactive AChE-homologous domain [1]. When overexpressed, AChE-R might compete with neuroligin-1 for its partner protein β-neurexin [32]. However, the distinct Ca^{2+}-binding domains in AChE and neuroligin-1 [1] predict differences in their adherence properties, suggesting that excess AChE-R might modify glutamate-mediated functions by impairing neurexin–neuroligin interactions. Non-hydrolytic roles of AChE have recently been challenged [3], suggesting that all previously reported phenotypes can also be explained by the catalytic actions of AChE on ACh. However, in a recent study, the similar effects of both active AChE-S (TgS) and inactive AChE-S (TgS_{in}) transgenes on the proliferation of neural

Box 1. Interactions of the cholinergic and glutamatergic systems

The functional synergistic interaction between glutamate and ACh neurotransmission was initially demonstrated >12 years ago, by a study in which antagonists of glutamate and ACh receptors impaired performance on a delayed non-matching-to-sample task when given jointly, but not when each drug was administered alone [63]. The activation of NMDA receptors reduced ACh release, a mechanism that places local ACh release under the control of glutamatergic afferents [64]. Additional studies established the ACh–glutamate connection with regard to cerebral blood flow [65], cognition [66] and memory [67,68], using different combinations of specific inhibitors. More recently, using both immunostaining and electrophysiology, glutamate-mediated expression and regulation have also been demonstrated at the frog NMJ, a synapse innervated by a single cholinergic neuron [69]. In another model, metabotropic glutamate receptors have been shown to be expressed in rat striatal cholinergic interneurons [70], and their modulatory functions have also recently been demonstrated [71]. In cultured hippocampal neurons, excessive expression of AChE impairs glutamate-mediated synaptogenesis [72]. Interestingly, in this study, chronic inhibition of the peripheral anionic site of AChE – thought to mediate non-enzymatic roles through protein–protein interactions – impaired glutamate-mediated functions independently of cholinergic neuron activation. Finally, a transgenic model of chronic overexpression of synaptic AChE (TgS mice) showed upregulation of glutamate receptors in the prefrontal cortex (PFC) compared with control mice [22]. The capacity of the PFC to regulate ACh release in the posterior parietal cortex via glutamate-mediated and ACh-mediated prefrontal mechanisms has recently been demonstrated [73], establishing interactions of the cholinergic and glutamatergic systems in the stress-prone PFC. Inhibition of glutamate-mediated neurotransmission abrogated the hypersensitivity of ACh-mediated transmission to physostigmine induced by repetitive stress regimes [9], attesting to the interactions between ACh and glutamate activity in electrical signaling after stress.

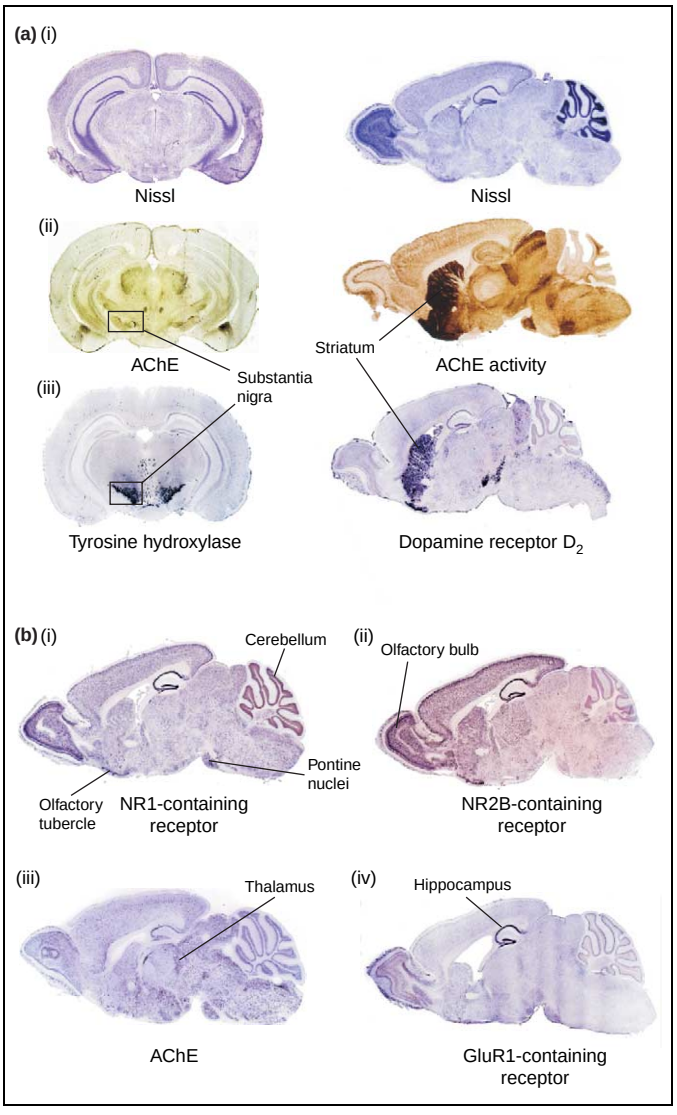

Figure 3. Correlation of AChE expression with the dopaminergic and glutamatergic systems. **(a)** The dopamine–ACh connection. Shown are coronal (left) and sagittal (right) mouse brain sections stained with Nissl staining (i), for AChE activity (ii) and for markers of dopaminergic neurons (iii). AChE activity correlates with high expression of markers of dopaminergic neurons in the striatum and the substantia nigra. **(b)** The glutamate–ACh connection. Shown are *in situ* hybridization images of sagittal mouse brain sections for NMDA-sensitive ionotropic glutamate receptors that include the NR1 subunit (i) or the NR2B subunit (ii), for AMPA-sensitive ionotropic glutamate receptors that include the GluR1 subunit (iv), and for AChE (iii). Brain regions where expression of AChE correlated with that of at least one additional glutamate receptor are indicated. Data provided under license by the Allen Institute for Brain Science (http://www.brainatlas.org/default.asp). All rights reserved.

progenitors in the developing murine brain suggests otherwise [33].

Although our focus here is on the CNS, stress-induced AChE-R overexpression is not limited to the brain. AChE-R mRNA transcript accumulates in many tissues during embryonic development, and after exposure to different stressors of intestinal epithelium cells, blood-cell progenitors or developing sperm [1]. The morphogenic activities attributed to AChE [1,32] are compatible with causal involvement of AChE-R in at least some of the cellular changes that are essential for the development of many different cell types and for their adaptation to stressful conditions. The concerted cascade that begins with altered post-transcriptional processing and leads to AChE-R accumulation in various cells and tissues might thus contribute to a complex physiological outcome.

Stress can induce an overwhelming feeling of fear; when that fear is out of proportion to its origin, it is defined as anxiety [34]. Anxiety-related disorders are the most common psychiatric disorders and are closely related both to stress [35] and to stimulation of cholinergic neurons [36]. A genetic study into a potential correlation between polymorphisms in the AChE–paraoxonase 1 (PON1) locus and anxiety scores revealed a predictive association between AChE–PON1 genotype and anxiety parameters [37]. Intriguingly, in healthy volunteers, the blood levels of soluble AChE-R monomers are inversely proportional to trait anxiety – that is, the tendency to become anxious that reflects a combination of demographic determinants (e.g. age, ethnic origin, gender and body–mass index) and experience-derived elements [37]. This further implies that the capacity to overproduce AChE-R in reaction to various stressors is an important determinant of ability to cope with anxious stimuli. In blood, AChE-R is rapidly inactivated [38], but it might be protected from both oxidative stress and inhibitory anti-cholinesterases by PON1 [39]. This explains why debilitating PON1 polymorphisms, which increase the risks of elevated state anxiety [37], also increase the risk of developing insecticide-induced Parkinsonism [40]. The freedom to overproduce AChE-R when needed thus mediates interactions of ACh signaling with both the glutamatergic and dopaminergic systems.

AChE variants in Alzheimer's disease, Parkinson's disease and myasthenia gravis

Alzheimer's disease (AD), Parkinson's disease (PD) and myasthenia gravis (MG) are the three best-studied neuropathologies associated with AChE alterations. AD is the most prevalent type of dementia in the elderly and is characterized by deposition of β-amyloid protein, which is processed by proteolytic cleavage of the β-amyloid protein precursor [41]. Forming of these deposits or plaques is characteristic of the early pathogenesis in AD. Cholinergic circuits are supposedly not impaired until later stages of AD [42]; nevertheless, recent evidence strongly suggests that AChE contributes to early plaque formation and pathogenesis [43]. This might suggest non-hydrolytic role(s) for AChE in the progression of AD. Most of the currently approved drugs for AD are anti-cholinesterases, which bind the active site of AChE and inhibit its action. However, active-site inhibitors might not affect the non-catalytic actions of AChE, which possibly partake in disease progression and severity. In addition, modified alternative splicing of AChE pre-mRNA induces AChE-R excess in AD [44]. Long-term intake of AChE inhibitors by individuals with AD selectively exacerbates these isoform-specific changes. Rivastigmine, for example, causes a selective increase in levels of AChE-R but not AChE-S [44]. Targeting a specific AChE isoform (e.g. by antisense or siRNA agents) could hence offer specific advantages. Alternative splicing of other transcripts has also been reported in AD. For example, a hypoxia-inducible form of presenilin 2 (PSEN2), which is generated by alternative splicing, is prevalent in the brains of individuals with AD

[45]. This implies that AD-related change(s) in general splicing-associated molecule(s) act upstream of either AChE or PSEN2.

PD is the most common neurodegenerative disorder that primarily affects motion control. PD involves degeneration of dopaminergic neurons in the substantia nigra, followed by loss of dopamine in the striatum. The rate-limiting enzyme in catecholamine synthesis, tyrosine hydroxylase (TH), marks clearly the main brain regions affected in PD (Figure 3a,iii). Because AChE activity is highest in the striatum (Figure 3a,ii), with substantial overlap between cholinergic and dopaminergic neurons, the cholinergic system is closely involved with PD pathology. AChE–PON1 interactions have been shown to increase the risk of anti-AChE-induced PD [40]. Moreover, exposure to AChE inhibitors, closed-head injury and psychological stress (all leading causes of PD) are associated with imbalanced AChE alternative splicing and AChE-R overexpression. That TgR mice, unlike TgS mice, seem to be neurologically protected suggests an adaptive role for AChE-R in aging and neurodegenerative diseases. High levels of soluble and diffusible AChE-R could potentially scavenge circulating and diffusing AChE inhibitors following exposure, thus acting as a protecting agent and alleviating toxicity symptoms, at least for a while. However, prolonged AChE-R excess could also lead to damage.

MG is an autoimmune disorder in which circulating autoantibodies against nicotinic acetylcholine receptors (nAChRs) lead to NMJ malfunction, characterized by progressive reductions in the amplitude of responses and by fluctuating weakness and fatigue of voluntary muscles. Treatment of MG currently involves cholinesterase inhibitors and often immune-directed treatment with either thymectomy or high-dose corticosteroids. AChE is thus directly related to both the pathophysiology and the treatment of MG [46]. Specifically, AChE-R, but not AChE-S, is overexpressed in the muscles of animals with experimental autoimmune MG. In these rats, antisense suppression of AChE-R resulted in: improved baseline compound muscle action potentials, measured by electrophysiological recordings; improved stamina, measured by treadmill exercise; and improved clinical scores, determined by weight gain of treated animals [21]. In Cynomolgus (macaque) monkeys (*Macaca fascicularis*), such treatment further suppressed neuronal pro-inflammatory cytokines [47], extending to the CNS Tracey's immunological concept of the suppression by ACh of inflammatory reactions [48].

In AD, PD and MG, AChE is directly involved in the pathophysiology of the disease, in the treatment of the disease, or both. Interestingly, splicing modulations always seem to have a chief role, leading to AChE-R accumulation. Targeted isoform-specific therapy could thus have profound implications for AChE-related neuropathologies.

Stress-induced changes in neuronal alternative splicing

Splicing aberrations have been reported in many diseases [49] and in aging [50], but the underlying molecular mechanism remains largely obscure. How does stress trigger alternative splicing of neuronal AChE and accumulation of AChE-R? To address this question, one should examine the combined consequences of stress on constitutive splicing (Box 2), alternative splicing and splicing factors.

Various stressful stimuli involve regulated, specific modulations in neuronal alternative splicing [51]. For example, perinatal manipulations and postnatal handling both selectively elevate levels of glucocorticoid receptor (GR) mRNA containing a hippocampus-specific exon that facilitates adaptation to modified stimuli [52]. Depending on neuronal depolarization, glucocorticoids also regulate splicing of the murine *Slo* gene (also known as *Kcnma1*), which encodes a brain K^+ channel [53]. Splice-site selection hence serves as a physiological adaptation to a change in the external conditions, while also contributing to the corresponding pathophysiological events. The fine balance between the splicing factors, more than the precise concentration of each specific protein, is the key regulatory determinant. Therefore, differential availability of splicing-related proteins probably alters the pattern of alternative splicing. Alternative splicing might thus be viewed as a beneficial adaptation strategy that modifies a finely tuned regulation process; alternatively, or in addition, its activation might induce adverse consequences of the modified processing of pre-mRNA [50].

The specific splicing of the glucocorticoid receptor, SLO K^+ channel and PSEN2 under stress supports the notion of adaptation, because alternative splicing in these instances facilitates beneficial stress responses, at least in the short term. Stress-induced changes in the alternative splicing of AChE pre-mRNA and the over-production of AChE-R are likewise beneficial in acute stress responses, because AChE-R downregulates stress-induced hyperarousal [8]. However, under prolonged stress

Box 2. Stress and constitutive splicing

Effects of stress on constitutive splicing were reported >20 years ago, with the discovery that a brief severe heat shock impaired the splicing process, leading to the accumulation of unspliced RNA products. This inhibition of splicing could be partly avoided in cells that were previously exposed to a mild heat shock, an adaptation process that involved the cellular activation of heat-shock proteins [74]. This first set of experiments suggested a controllable association of heat-shock-related proteins with components of the splicing machinery. Additional factors contributing to stress-induced splicing alterations include the family of heterogeneous nuclear ribonucleoprotein (hnRNP)-M proteins and the splicing-related protein 2H9, which participates in basal splicing processes and in the early stages of stress-induced splicing arrest [75,76]. Other studies showed that heat stress in HeLa cells disrupted the U4/U6·U5 complex of three small nuclear ribonucleoproteins (snRNPs) and inactivated a splicing factor associated with this complex [77]. Indeed, a heat-shock-induced shift from nuclear speckles to diffuse nuclear distribution of snRNP antigens was reported in mouse fibroblasts, whereas the splicing factor SC35 maintained its normal distribution [78]. Remarkably, spatial association between splicing factors and transcriptionally active genes in nuclear sites of transcription was intron independent, as evident by the association of both intron-rich HSP90α and intron-less HSP70 proteins with nuclear speckles upon exposure of cells to heat shock [79]. These findings indicate that splicing proteins have an intrinsic finely tuned capacity to target potential splice sites during cellular stress.

conditions, numerous physiological functions are adversely suppressed, with crucial consequences for the affected organism. Similarly, transgenic animal studies [23,27] and animal disease models [21,54] suggest that prolonged overproduction of AChE-R could take its toll.

In seeking splicing-related proteins that might be responsible, at least in part, for the mammalian post-stress effects, the general splicing factor SC35 was recently found to have expression patterns comparable to those of AChE-R in post-stress brain neurons (Figure 4). Moreover, SC35 shifted alternative splicing of AChE from AChE-S to AChE-R mRNA *in vitro* [55]. The splicing factor ASF/SF2 caused the opposite effect in cultured cells, driving splicing towards the AChE-S transcript (Figure 4). Recently, these two factors have been shown to influence alternative splicing of the glutamate receptor subunit 2 (GluR2), regulating the flip–flop isoform ratio of this protein [56]. An intriguing example of neuronal alternative splicing regulation comes from studies on neuro-oncological ventral antigen 1 (NOVA1), an RNA-binding protein that regulates alternative splicing of the $GABA_A$ receptor $\gamma 2$ subunit through direct binding to UCAU-rich intronic splicing enhancers. Importantly, NOVA1 is a master regulator of synapse-related transcripts [57] and in cases where NOVA levels falter (e.g. in the autoimmune disease paraneoplastic opsoclonus myoclonus ataxia, where anti-NOVA antigens are produced), abnormal neurological motor activity is observed, demonstrating the downstream consequences of perturbation of essential RNA-processing proteins.

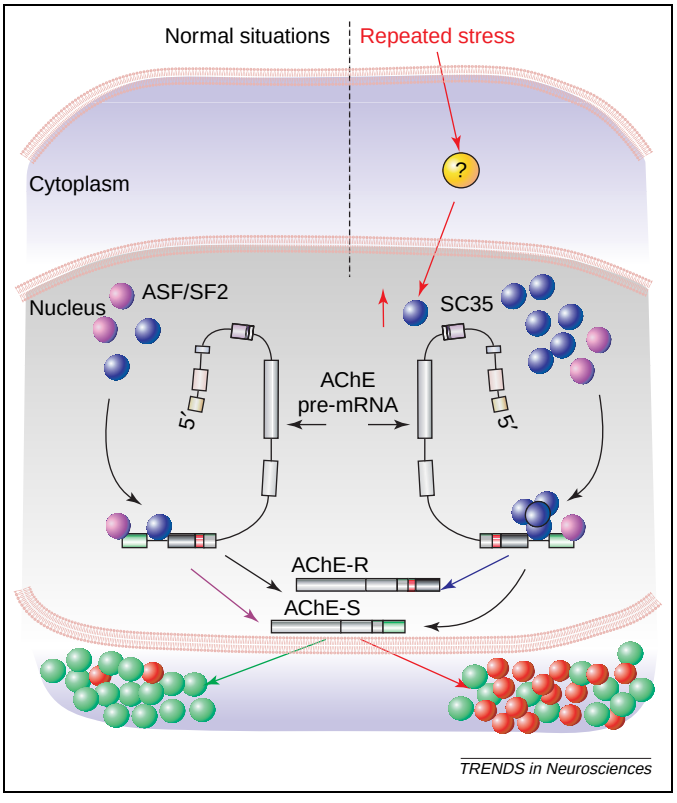

Figure 4. Proposed molecular mechanism for stress-induced alternative splicing of AChE. In normal situations (left), the balance between the different splicing factors, primarily ASF/SF2 (purple) and SC35 (blue), results in regulated splicing of AChE, yielding mostly AChE-S mRNA (purple arrow) and AChE-S protein (green). Repeated psychological stress in mice (right), results via an unknown mechanism in prolonged overexpression of SC35, which interacts with a specific exonic splicing enhancer, favoring production of AChE-R mRNA (blue arrow) and generating mostly AChE-R protein (red, bottom) [55].

Concluding remarks and future perspectives

The variety of AChE alternative transcripts highlights AChE as a complex molecule with a tightly regulated pattern of expression. The uniqueness of each variant is accentuated by its expression timing (e.g. during health or disease, or during normal or stressful situations), its expression compartment (e.g. in neurites or on synaptic membranes) and its interaction partners (e.g. PRiMA, GPI and RACK1). These three factors add an extra dimension to the intricate control of variant AChE proteins.

The recurrent involvement of AChE in various pathophysiological states, including neuropathologies and stress-related syndromes, places specific AChE variants as the main target of drug development and therapy for such disorders. The most frequently used compounds in such therapies are cholinesterase inhibitors (e.g. carbamates), which target the AChE active site and prevent the rapid degradation of ACh [58]. However, these drugs (or the chemically similar anti-cholinesterase insecticides [1]) occasionally have less desirable side effects [58], and their neurotoxicity – which most probably involves interactions of the cholinergic and glutamatergic systems [59] – pertains even at low doses [60]. Antisense-mediated degradation of AChE mRNA has been used successfully to prevent AChE-R overexpression and to alleviate symptoms in a closed-head injury model in mice [54], in a rat model of MG [21], and in mice experiencing enhanced stress-induced memory impairments and fear [28]. One of the immediate advantages of targeting the mRNA rather than the processed protein is that minute drug concentrations can be used, minimizing side effects. Variant specificity could also be used to modulate a specific protein partner rather than all AChE isoforms. With this in mind, it will be important to study the expression and roles of the recently identified 5′-spliced AChE transcripts and protein products [13] in AChE-related neuropathologies, and to identify their disease-specific variants. Molecular targeting of these novel proteins could have important implications for therapy. Small interfering RNAs (siRNAs), through the endogenous RNA interference (RNAi) machinery, can also be used to target specific AChE transcripts. Such RNAi-mediated AChE silencing has recently been used successfully to knockdown AChE expression in a human colon adenocarcinoma cell line [61], and effective systemic administration of siRNA has also been reported [62]. Characterization of the related transcripts of AChE, initial dissection of the molecular pathways involved and the emergence of new molecular tools might thus hold promise and hope for those seeking treatments for stress-related and AChE-related neuropathologies.

Acknowledgements

Our work is supported by the Israel Science Foundation 618-02-1, the European Community LSHM-CT-2003–503330 and European Alternative Splicing Network of Excellence- LSH-2004-1.1.5-3, the German–Israeli-Foundation (Grant 673), and Ester Neuroscience.

References

1 Soreq, H. and Seidman, S. (2001) Acetylcholinesterase – new roles for an old actor. *Nat. Rev. Neurosci.* 2, 294–302
2 Silman, I. and Sussman, J.L. (2005) Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. *Curr. Opin. Pharmacol.* 5, 293–302
3 Cousin, X. *et al*. (2005) Are there non-catalytic functions of acetylcholinesterases? Lessons from mutant animal models. *BioEssays* 27, 189–200
4 Quinn, D.M. (1987) Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. *Chem. Rev.* 87, 955–979
5 Behra, M. *et al*. (2002) Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. *Nat. Neurosci.* 5, 111–118
6 Xie, W. *et al*. (2000) Postnatal developmental delay and supersensitivity to organophosphate in gene-targeted mice lacking acetylcholinesterase. *J. Pharmacol. Exp. Ther.* 293, 896–902
7 Darvesh, S. *et al*. (2003) Neurobiology of butyrylcholinesterase. *Nat. Rev. Neurosci.* 4, 131–138
8 Kaufer, D. *et al*. (1998) Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature* 393, 373–377
9 Meshorer, E. *et al*. (2002) Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. *Science* 295, 508–512
10 Legay, C. *et al*. (1995) Developmental regulation of acetylcholinesterase transcripts in the mouse diaphragm: alternative splicing and focalization. *Eur. J. Neurosci.* 7, 1803–1809
11 Massoulie, J. (2002) The origin of the molecular diversity and functional anchoring of cholinesterases. *Neurosignals* 11, 130–143
12 Perrier, A.L. *et al*. (2002) PRiMA: the membrane anchor of acetylcholinesterase in the brain. *Neuron* 33, 275–285
13 Meshorer, E. *et al*. (2004) Combinatorial complexity of 5′ alternative acetylcholinesterase transcripts and protein products. *J. Biol. Chem.* 279, 29740–29751
14 Taylor, P. and Radic, Z. (1994) The cholinesterases: from genes to proteins. *Annu. Rev. Pharmacol. Toxicol.* 34, 281–320
15 Chandrasekharan, N.V. *et al*. (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc. Natl. Acad. Sci. U. S. A.* 99, 13926–13931
16 Sapolsky, R.M. (2003) Stress and plasticity in the limbic system. *Neurochem. Res.* 28, 1735–1742
17 Masuda, J. *et al*. (2004) Female rats living in small cages respond to restraint stress with both adrenocortical corticosterone release and acetylcholine release in the hippocampus. *Neurosci. Lett.* 358, 169–172
18 Tayebati, S.K. *et al*. (2004) Effect of treatment with the cholinesterase inhibitor rivastigmine on vesicular acetylcholine transporter and choline acetyltransferase in rat brain. *Clin. Exp. Hypertens.* 26, 363–373
19 Beck, K.D. *et al*. (2003) Stress interacts with peripheral cholinesterase inhibitors to cause central nervous system effects. *Life Sci.* 73, 41–51
20 Sternfeld, M. *et al*. (2000) Excess 'read-through' acetylcholinesterase attenuates but the 'synaptic' variant intensifies neurodeterioration correlates. *Proc. Natl. Acad. Sci. U. S. A.* 97, 8647–8652
21 Brenner, T. *et al*. (2003) The role of readthrough acetylcholinesterase in the pathophysiology of myasthenia gravis. *FASEB J.* 17, 214–222
22 Meshorer, E. *et al*. (2005) Chronic cholinergic imbalances promote brain diffusion and transport abnormalities. *FASEB J.* 19, 910–922
23 Cohen, O. *et al*. (2002) Neuronal overexpression of 'readthrough' acetylcholinesterase is associated with antisense-suppressible behavioral impairments. *Mol. Psychiatry* 7, 874–885
24 Perrier, N.A. *et al*. (2005) The readthrough variant of acetylcholinesterase remains very minor after heat shock, organophosphate inhibition and stress, in cell culture and *in vivo*. *J. Neurochem.* 94, 629–638
25 Abdel-Rahman, A. *et al*. (2002) Disruption of the blood–brain barrier and neuronal cell death in cingulate cortex, dentate gyrus, thalamus, and hypothalamus in a rat model of Gulf-War syndrome. *Neurobiol. Dis.* 10, 306–326
26 Antelman, S.M. *et al*. (1980) Interchangeability of stress and amphetamine in sensitization. *Science* 207, 329–331
27 Birikh, K.R. *et al*. (2003) Interaction of 'readthrough' acetylcholinesterase with RACK1 and PKCβ II correlates with intensified fear-induced conflict behavior. *Proc. Natl. Acad. Sci. U. S. A.* 100, 283–288
28 Nijholt, I. *et al*. (2004) Stress-induced alternative splicing of acetylcholinesterase results in enhanced fear memory and long-term potentiation. *Mol. Psychiatry* 9, 174–183
29 Orsini, C. *et al*. (2001) Pharmacological evidence of muscarinic-cholinergic sensitization following chronic stress. *Psychopharmacology (Berl.)* 155, 144–147
30 Aigner, T.G. (1995) Pharmacology of memory: cholinergic-glutamatergic interactions. *Curr. Opin. Neurobiol.* 5, 155–160
31 Chih, B. *et al*. (2005) Control of excitatory and inhibitory synapse formation by neuroligins. *Science* 307, 1324–1328
32 Nam, C.I. and Chen, L. (2005) Postsynaptic assembly induced by neurexin–neuroligin interaction and neurotransmitter. *Proc. Natl. Acad. Sci. U. S. A.* 102, 6137–6142
33 Dori, A. *et al*. (2005) Functional manipulations of acetylcholinesterase splice variants highlight alternative splicing contributions to murine neocortical development. *Cereb. Cortex* 15, 419–430
34 Weinberger, D.R. (2001) Anxiety at the frontier of molecular medicine. *New Engl. J. Med.* 344, 1247–1249
35 Strohle, A. and Holsboer, F. (2003) Stress responsive neurohormones in depression and anxiety. *Pharmacopsychiatry* 36(Suppl. 3), S207–S214
36 Battaglia, M. and Ogliari, A. (2005) Anxiety and panic: from human studies to animal research and back. *Neurosci. Biobehav. Rev.* 29, 169–179
37 Sklan, E.H. *et al*. (2004) Acetylcholinesterase/paraoxonase genotype and expression predict anxiety scores in health, risk factors, exercise training, and genetics study. *Proc. Natl. Acad. Sci. U. S. A.* 101, 5512–5517
38 Cohen, O. *et al*. (2003) Endotoxin-induced changes in human working and declarative memory associate with cleavage of plasma 'read-through' acetylcholinesterase. *J. Mol. Neurosci.* 21, 199–212
39 Bryk, B. *et al*. (2005) Inherited and acquired interactions between ACHE and PON1 polymorphisms modulate plasma acetylcholinesterase and paraoxonase activities. *J. Neurochem.* 92, 1216–1227
40 Benmoyal-Segal, L. *et al*. (2005) Acetylcholinesterase/paraoxonase interactions increase the risk of insecticide-induced Parkinson's disease. *FASEB J.* 19, 452–454
41 Citron, M. (2004) Strategies for disease modification in Alzheimer's disease. *Nat. Rev. Neurosci.* 5, 677–685
42 Small, D.H. (2004) Do acetylcholinesterase inhibitors boost synaptic scaling in Alzheimer's disease? *Trends Neurosci.* 27, 245–249
43 Inestrosa, N.C. *et al*. (1996) Acetylcholinesterase accelerates assembly of amyloid-β-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron* 16, 881–891
44 Darreh-Shori, T. *et al*. (2004) Long-lasting acetylcholinesterase splice variations in anticholinesterase-treated Alzheimer's disease patients. *J. Neurochem.* 88, 1102–1113
45 Sato, N. *et al*. (1999) A novel presenilin-2 splice variant in human Alzheimer's disease brain tissue. *J. Neurochem.* 72, 2498–2505
46 Schwendimann, R.N. *et al*. (2005) Management of myasthenia gravis. *Am. J. Ther.* 12, 262–268
47 Evron, T. *et al*. (2005) RNA-targeted suppression of stress-induced allostasis in monkey spinal cord neurons. *Neurodegenerative Dis.* 2, 16–27
48 Pavlov, V.A. and Tracey, K.J. (2005) The cholinergic anti-inflammatory pathway. *Brain Behav. Immun.* 19, 493–499
49 Stoilov, P. *et al*. (2002) Defects in pre-mRNA processing as causes of and predisposition to diseases. *DNA Cell Biol.* 21, 803–818
50 Meshorer, E. and Soreq, H. (2002) Splicing modulations in senescence. *Aging Cell* 1, 10–16
51 Shin, C. and Manley, J.L. (2004) Cell signalling and the control of pre-mRNA splicing. *Nat. Rev. Mol. Cell Biol.* 5, 727–738
52 McCormick, J.A. *et al*. (2000) 5′-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. *Mol. Endocrinol.* 14, 506–517
53 Xie, J. and McCobb, D.P. (1998) Control of alternative splicing of potassium channels by stress hormones. *Science* 280, 443–446
54 Shohami, E. *et al*. (2000) Antisense prevention of neuronal damages following head injury in mice. *J. Mol. Med.* 78, 228–236

55 Meshorer, E. *et al*. (2005) SC35 promotes sustainable stress-induced alternative splicing of neuronal acetylcholinesterase mRNA. *Mol. Psychiatry* 10, 985–997
56 Crovato, T.E. and Egebjerg, J. (2005) ASF/SF2 and SC35 regulate the glutamate receptor subunit 2 alternative flip/flop splicing. *FEBS Lett.* 579, 4138–4144
57 Ule, J. *et al*. (2005) Nova regulates brain-specific splicing to shape the synapse. *Nat. Genet.* 37, 844–852
58 Racchi, M. *et al*. (2004) Acetylcholinesterase inhibitors: novel activities of old molecules. *Pharmacol. Res.* 50, 441–451
59 Popke, E.J. (2003) From anticholinesterase toxicity to Alzheimer's disease: important interactions of cholinergic and NMDA receptor systems. *Toxicol. Sci.* 72, 185–187
60 Ray, D.E. (1998) Chronic effects of low level exposure to anticholinesterases – a mechanistic review. *Toxicol. Lett.* 102-103, 527–533
61 Park, S.E. *et al*. (2004) Acetylcholinesterase plays a pivotal role in apoptosome formation. *Cancer Res.* 64, 2652–2655
62 Soutschek, J. *et al*. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173–178
63 Ogura, H. and Aigner, T.G. (1993) MK-801 impairs recognition memory in rhesus monkeys: comparison with cholinergic drugs. *J. Pharmacol. Exp. Ther.* 266, 60–64
64 Metherate, R. and Ashe, J.H. (1995) Synaptic interactions involving acetylcholine, glutamate, and GABA in rat auditory cortex. *Exp. Brain Res.* 107, 59–72
65 Tsukada, H. *et al*. (1998) Interactions of cholinergic and glutamatergic neuronal systems in the functional activation of cerebral blood flow response: a PET study in unanesthetized monkeys. *Brain Res.* 796, 82–90
66 Li, H.B. *et al*. (1996) NMDA- but not AMPA-receptor antagonists augment scopolamine-induced spatial cognitive deficit of rats in a radial maze task. *Brain Res.* 725, 268–271
67 Gray, R. *et al*. (1996) Hippocampal synaptic transmission enhanced by low concentrations of nicotine. *Nature* 383, 713–716
68 Ciamei, A. *et al*. (2001) Effects of MK-801 and nicotine combinations on memory consolidation in CD1 mice. *Psychopharmacology (Berl.)* 154, 126–130
69 Pinard, A. *et al*. (2003) Glutamatergic modulation of synaptic plasticity at a PNS vertebrate cholinergic synapse. *Eur. J. Neurosci.* 18, 3241–3250
70 Bell, M.I. *et al*. (2002) Functional and molecular characterization of metabotropic glutamate receptors expressed in rat striatal cholinergic interneurones. *J. Neurochem.* 81, 142–149
71 Bonsi, P. *et al*. (2005) Modulatory action of metabotropic glutamate receptor (mGluR) 5 on mGluR1 function in striatal cholinergic interneurons. *Neuropharmacology* 49(Suppl. 1), 104–113
72 Dong, H. *et al*. (2004) Excessive expression of acetylcholinesterase impairs glutamatergic synaptogenesis in hippocampal neurons. *J. Neurosci.* 24, 8950–8960
73 Nelson, C.L. *et al*. (2005) Prefrontal cortical modulation of acetylcholine release in posterior parietal cortex. *Neuroscience* 132, 347–359
74 Yost, H.J. and Lindquist, S. (1986) RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell* 45, 185–193
75 Gattoni, R. *et al*. (1996) The human hnRNP-M proteins: structure and relation with early heat shock-induced splicing arrest and chromosome mapping. *Nucleic Acids Res.* 24, 2535–2542
76 Mahe, D. *et al*. (1997) Cloning of human 2H9 heterogeneous nuclear ribonucleoproteins. Relation with splicing and early heat shock-induced splicing arrest. *J. Biol. Chem.* 272, 1827–1836
77 Utans, U. *et al*. (1992) A splicing factor that is inactivated during *in vivo* heat shock is functionally equivalent to the [U4/U6.U5] triple snRNP-specific proteins. *Genes Dev.* 6, 631–641
78 Spector, D.L. *et al*. (1991) Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J.* 10, 3467–3481
79 Jolly, C. and Morimoto, R.I. (1999) Stress and the cell nucleus: dynamics of gene expression and structural reorganization. *Gene Expr.* 7, 261–270