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ACETYLCHOLINESTERASE: OLD QUESTIONS AND NEW DEVELOPMENTS

Topic Editors
Karl Tsim and Hermona Soreq

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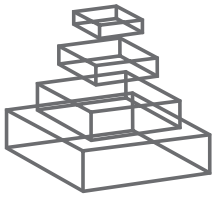

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ACETYLCHOLINESTERASE: OLD QUESTIONS AND NEW DEVELOPMENTS

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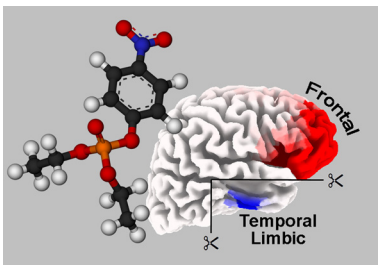

Image by Hermona Soreq.

Acetylcholinesterase (AChE) is a primary regulator of cholinergic signaling within and out of the central nervous system. It has been studied extensively for several decades, yet many basic questions about its regulation, composition and functions remain open. Thus, neither the full impact of AChE's activities on neuronal development, maintenance and functioning nor the reasons for why and how different AChE molecular forms are being synthesized and degraded are fully known yet, although recent evidence suggests the involvement of micro-RNA regulators in both of these aspects. The identification of the anchoring proteins, PRiMA and ColQ, stimulates studies on AChE's assembly and its specific localization in the brain and muscle. Additionally, non-cholinergic function(s) of AChE has been proposed as relevant both for neuronal differentiation and cognitive functioning, whereas transgenic engineering, recombinant production and structure-function analyses of this enzyme's variants with various inhibitors paved new avenues for the development of novel Alzheimer's disease therapeutics, agricultural pesticides and prophylactics in anticipation of warfare agent exposure.

The aims of this Research Topic is to provide a forum for experts in the field to critically discuss recent developments in AChE studies, initiate global critiques of the current views for resolving the fundamental questions in AChE research and promote the development of novel versions and uses of AChE-targeted agents. Topics of interest include but are not limited to: (i) post-transcriptional regulation of AChE; (ii) the non-cholinergic functions of AChE; and (iii) anti-AChE inhibitors and diseases.

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EDITORIAL
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Acetylcholinesterase: old questions and new developments

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The family of cholinesterases, including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), has been of significant interest to neuroscientists for several decades. Cholinesterases play an essential role in mediating neurotransmission in cholinergic synapses, which control the higher brain functions such as learning and memory; they also are causally involved in controlling nerve-muscle communication and muscle activities, and serve as an important component of the physiological differences characteristic of certain pathological conditions. A notable example is Alzheimer's disease, where premature death of cholinergic neurons has been the basis for drug development so that the leading therapeutic agents available to date are targeted to inhibit cholinesterase activities. AChE and BChE were initially identified as efficient hydrolases that possess the capacity to rapidly hydrolyze acetylcholine and butyrylcholine, respectively; and AChE presents more rapid and far more selective potency for such hydrolysis compared to BChE. Throughout the last 40 years, multiple studies of cholinesterases led to an exponential increase in the effort to explore their functions, structures and physiological roles in health and disease. The resultant findings strongly suggest vital roles for cholinesterases both in the nervous system and in other body systems, with non-neuronal functions such as the regulation of apoptosis and inflammation being a relatively new focus of interest. This vast interest, coupled with the rapid increase in the availability and power of different research tools allow far deeper understanding of the structure and functions of AChE and BChE in different disciplinary terms, including cellular and molecular biology, genetics and epigenetics, physiology, structure-function relationships at an atomic resolution level, the chemical mechanisms of enzymatic inhibition, pharmacology, drug development, disease therapy, etc. However, in spite of this massive multi-disciplinary effort, numerous critical and core questions of AChE and BChE are yet not fully answered, and some have never been addressed as yet. As two of the many enthusiastic scientists in this field, we believe that the current surge in cholinesterase research will further expand in the near future. Therefore, it has been an honor to co-edit this E-book, entitled "Acetylcholinesterase: old questions and new developments." This volume includes pertinent research articles by 11 leading groups, worldwide (Carvajal and Inestrosa, 2011; Chen et al., 2011; García-Ayllón et al., 2011; Hanin and Soreq, 2011; Bronicki and Jasmin, 2012; Durrant et al., 2012; Falugi and Aluigi, 2012; Gilboa-Geffen et al., 2012; Gnatek et al., 2012; Shehadeh Masha'our et al., 2012; Zhang and Greenberg, 2012). It covers the translational and post-translational modifications of cholinesterases, their developmental expression patterns in different species, their links to inflammation-related pathologies and their role in apoptotic processes. At the molecular level, one can find in this volume a reference to the microRNA regulation of cholinesterases as well as to the trans-acting factors governing the metabolism of cholinesterase mRNA. Also, the links of cholinesterases with inherited neuromuscular diseases as well as with Alzheimer's neuropathology and therapeutics is covered from both the physiological and structural points of view. Thus, this volume provides a glimpse into the most up-to-date research information in the diverse field of cholinesterases, as well as an important insight for outlying future research directions in this topic.

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Trans-acting factors governing acetylcholinesterase mRNA metabolism in neurons

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The most characterized function of acetylcholinesterase (AChE) is to terminate cholinergic signaling at neuron-neuron and neuro-muscular synapses. In addition, AChE is causally or casually implicated in neuronal development, stress-response, cognition, and neurodegenerative diseases. Given the importance of AChE, many studies have focused on identifying the molecular mechanisms that govern its expression. Despite these efforts, post-transcriptional control of AChE mRNA expression is still relatively unclear. Here, we review the trans-acting factors and cis-acting elements that are known to control AChE pre-mRNA splicing, mature mRNA stability and translation. Moreover, since the Hu/ELAV family of RNA-binding proteins (RBPs) have emerged in recent years as "master" post-transcriptional regulators, we discuss the possibility that predominantly neuronal ELAVs (nELAVs) play multiple roles in regulating splicing, stability, localization, and translation of AChE mRNA.

Keywords: Hu, nELAV, AChE, alternative splicing, mRNA stability, translation, micro-RNA

INTRODUCTION

Acetylcholinesterase (AChE) is one of the most thoroughly studied enzymes and is better known for its role in hydrolyzing acetylcholine (ACh) at central and peripheral nervous system (CNS and PNS) synapses (reviewed in Legay, 2000; Soreq and Seidman, 2001; Massoulie, 2002; Rotundo, 2003; Taylor, 2010). In addition to terminating cholinergic neurotransmission, detection of AChE prior to cholinergic synapse formation and in non-cholinergic neurons led to identification of functions in several cellular processes including neurite elongation (Koenigsberger et al., 1997), synaptogenesis (Sternfeld et al., 1998), cell adherence (Sharma et al., 2001), and apoptosis (Zhang et al., 2002) (reviewed in Jiang and Zhang, 2008; Paraoanu and Layer, 2008). The functional versatility of AChE is also implicated in neocortical development (Dori et al., 2005), cognition (Beeri et al., 1995), stress-response (Kaufer et al., 1998), neuronal tumors (Karpel et al., 1994) and neurodegeneration such as in Alzheimer's and Parkinson's diseases (reviewed in Meshorer and Soreq, 2006; Greenfield et al., 2008).

AChE's tissue-specific expression pattern and diverse roles are largely attributed to the generation of multiple splice variants from a single gene (Li et al., 1991; Meshorer et al., 2004). Classically, all AChE variants are categorized depending on their different C-terminal tails into $AChE_T$ (T-tailed), $AChE_R$ (R-read through) and $AChE_H$ (H-hydrophobic). In neurons and skeletal muscle $AChE_T$ interacts with either a collagenic tail termed ColQ, or with a proline-rich membrane anchor referred to as PRiMA. On the other hand, $AChE_H$ is primarily localized to erythrocytes and platelets, anchored to the plasma membrane via glycophospholipids. The typically low abundant variant found in several cell types, $AChE_R$, is a soluble monomer up-regulated during stress, AChE inhibition and organophosphate poisoning (reviewed in Zimmerman and Soreq, 2006). The importance of AChE for proper neuronal development and synaptic physiology, as well as its putative roles in brain pathologies, has gathered much attention over the last several decades toward delineating the molecular mechanisms that control its expression.

Neuronal transcription of AChE has first been reported during differentiation (Greene and Rukenstein, 1981, see also for example Deschenes-Furry et al., 2003), in response to stressors (Shapira et al., 2000; Meshorer et al., 2004) and in co-cultures with myotubes (Jiang et al., 2003). The murine *AChE* gene contains a complex 5′ regulatory region (5′RR) with alternate promoters and two enhancers (Mutero et al., 1995; Atanasova et al., 1999; Chan et al., 1999; Meshorer et al., 2004). Several transcription factors are now recognized to regulate AChE expression in neurons, including activating transcription factor-1 (ATF1) (Wan et al., 2000), glucocorticoid receptors (GR) (Meshorer et al., 2004), hepatocyte nuclear factor 3β (HNF3β) (Shapira et al., 2000) and ETS-like transcription factor (Elk-1) (Siow et al., 2010). At least 5 and 4 exon 1 (E1) variants have been identified in the mouse (mE1a–e) and human (hE1a–d) AChE 5′RR, respectively, with distinct promoters located upstream of each E1 (**Figure 1A**). Each leader exon generates a unique AChE 5′ untranslated region (UTR), which is presumably dependent on alternative promoter usage rather than alternative splicing. Although the purpose of these various 5′UTRs remains elusive, mE1e and hE1d encode a conserved (79%) amino acid chain that produces N-terminal extended AChE variants ($N\text{-}AChE_T$, $N\text{-}AChE_R$ and in principle $N\text{-}AChE_H$) (Meshorer et al., 2004). Interestingly, certain N-AChE variants have been shown to bind the cellular membrane, promote apoptosis in stress-induced cells and to associate with pathological markers in Alzheimer's brains (Kehat et al., 2007; Toiber et al., 2008, 2009).

In addition to transcription and distinct promote usage, an increasing amount of studies are demonstrating that

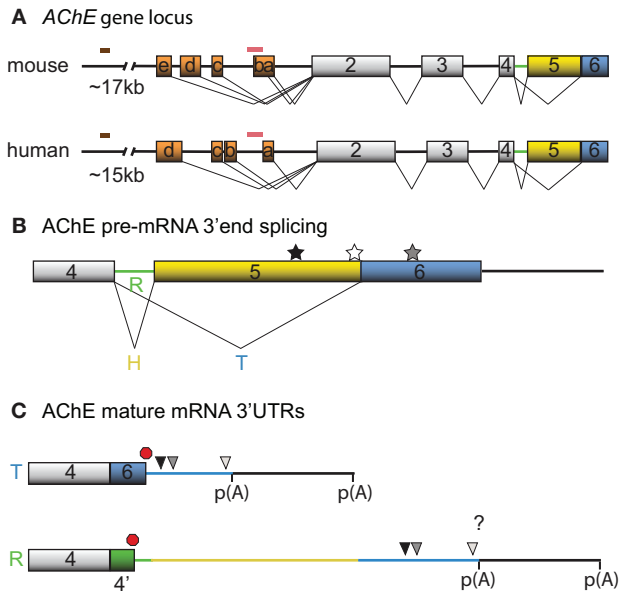

FIGURE 1 | Schematic diagrams depicting the mouse and human *AChE* genes, alternative splicing options and cis-element locations. **(A)** Mouse and human *AChE* genes, including the leader exons identified in brain tissues. Noteworthy is the 5′ regulatory region that harbors alternate exons 1a–e in mouse and 1a–d in human (orange boxes), distal enhancer glucocorticoid response element (brown line) and proximal muscle-specific enhancer (pink line). Gray boxes and black lines represent constitutive exons and introns, respectively. **(B)** Alternative splicing at the 3′ end of AChE pre-mRNA produces tissue-specific R (green), H (yellow) and T (blue) variants. Approximate locations of the U-rich regulatory sequence, SC35 and ASF/SF2 splicing factor binding sites are denoted by black, white, and gray stars, respectively. **(C)** The 3′ untranslated regions (3′UTRs) of mature T and R AChE variants and the alternative polyadenylation-dependent extended regions. The $AChE_H$ variant is not shown since it is not significantly expressed in neurons. Symbols represent the PBE (black triangle), ARE (gray triangle), miR-132 binding (white triangle), translation stop (red octagon), and polyadenylation signal (p(A)) sites. 4′ signifies a pseudo-intron that contains the $AChE_R$ translation termination site. Whether E6 and the downstream region are included in $AChE_R$ transcripts is unknown (?).

post-transcriptional mechanisms are equally important for AChE expression. However, our understanding of the trans-acting factors and cis-acting elements involved in these processes is still limited. Here, we review the work describing the currently identified RNA-binding proteins (RBPs), microRNAs (miRs) and RNA motifs that regulate AChE pre-mRNA splicing, mRNA stability, and translation. Given the established neuronal pleiotropic effects of one family of RBP, namely the principally neuronal ELAVs (nELAVs), we also speculate on their involvement in AChE mRNA metabolism at multiple steps.

REGULATION OF AChE PRE-mRNA SPLICING

Recent high throughput sequencing revealed that more than 90% of human genes undergo alternative splicing (for review see Kalsotra and Cooper, 2011). In accordance with this finding, AChE pre-mRNA is subjected to alternative splicing at the 3′end, which produces $AChE_T$ or $AChE_R$ in neurons. The terminal sequence of the *AChE* gene contains a 5′ splice donor site downstream of exon 4 (E4) and two 3′ splice acceptor sites, one upstream of E5 (proximal) and the other upstream of E6 (distal). When splicing to either of the two downstream acceptor sites is inhibited, the intron (4′) and exons downstream of E4, are included into the mature transcript producing $AChE_R$. Conversely, splicing to the distal acceptor site incorporates E6 into the mature mRNA and generates $AChE_T$ (reviewed in Meshorer and Soreq, 2006) (**Figure 1B**).

Although little is known regarding the splicing factors involved, two serine/arginine-rich (SR) auxiliary splicing factors, SC35 and ASF/SF2, have been found to inversely affect $AChE_R$ versus $AChE_T$ expression (Meshorer et al., 2005). In particular, *in vitro* experiments with an AChE minigene in COS-1 and HEK293 cells unveiled that SC35 promotes alternative splicing of $AChE_R$ transcripts whereas ASF/SF2 seemed to favor expression of $AChE_T$. Furthermore, immunohistochemistry and fluorescent in situ hybridization (FISH) experiments detected a correlation between SC35 protein and $AChE_R$ mRNA expression during embryonic brain development and adult stress-response. However, some discordance between SC35 protein and $AChE_R$ mRNA levels in developing and post-stress cortex were apparent (Meshorer et al., 2005), indicating that other trans-acting factors may also govern splicing of precursor AChE mRNAs. In agreement with this view, Guerra et al used an AChE minigene and sequence mutagenesis to uncover various putative motifs, predominantly located in the E5 non-coding region, that influence production of the R/H/T splice variants in skeletal muscle cells (Guerra et al., 2008).

STABILITY-DETERMINANTS OF AChE mRNA

Post-transcriptional regulation of AChE resulting in increased mRNA stability was initially documented in Neuro2a (N2a) cells under the control of the thyroid hormone T3 (Puymirat et al., 1995) and in differentiating P19 embryonal carcinoma cells (Coleman and Taylor, 1996). Moreover, increased stability of AChE mRNA through an unknown mechanism has been observed during calcium-dependent apoptosis (Zhu et al., 2007). Screening the $AChE_T$ transcript sequence for cis-acting elements that might regulate mRNA stability revealed the presence of an AU-rich element (ARE) in the 3′UTR and a CU-rich sequence in E2 (Cuadrado et al., 2003; Deschenes-Furry et al., 2003). Based on the current understanding of AChE alternative splicing, it would appear that the R transcript also contains these motifs. However, whether the ARE-surrounding sequence that is encoded by E6 is also present in $AChE_R$ remains controversial (Guerra et al., 2008; Camp et al., 2010; Hanin and Soreq, 2011; Marrero et al., 2011) (**Figure 1C**). In either case, the existence of an ARE indicates RBP-dependent regulation of AChE mRNA stability. AChE transcripts are also susceptible to alternative polyadenylation that preferentially produces a shorter 3′UTR in mature neurons with an expected altered mRNA stability (**Figure 1C**) (Li et al., 1991).

One of the most extensively studied families of mRNA stabilizing RBPs, termed the Hu/ELAV family, consists of the ubiquitously expressed HuR (ELAVl1) and the predominantly neuronal (known as nELAVs) HuB (ELAVl2), HuC (ELAVl3) and HuD (ELAVl4) (reviewed in Bolognani and Perrone-Bizzozero, 2008;

Hinman and Lou, 2008; Pascale et al., 2008). Brain developmental and spatial expression patterns of Hu/ELAV members are partially distinct and their amino acid sequences are moderately conserved in certain regulatory regions, suggesting that these RBPs are not completely functionally redundant (Okano and Darnell, 1997; Hambardzumyan et al., 2009). Hu/ELAVs contain two RNA-Recognition Motifs (RRMs) which enable binding to AREs in the 3′UTR and a third RRM that interacts with the poly(A)-tail and other proteins (Abe et al., 1996; Ma et al., 1997; Kasashima et al., 2002; Fialcowitz-White et al., 2007). Recent studies indicate that both ARE and non-ARE sequences located in 5′UTRs, exons and introns are also targeted by Hu/ELAVs (Bolognani et al., 2010; Lebedeva et al., 2011; Mukherjee et al., 2011; Uren et al., 2011). Through these binding mechanisms, Hu/ELAVs can regulate almost all aspects of mRNA metabolism including splicing, polyadenylation, nuclear export, stabilization, localization, and translation (reviewed in Hinman and Lou, 2008; Pascale et al., 2008—and see below). Since several nELAV mRNA targets encode proteins that have essential roles ranging from neurogenesis to synaptogenesis, nELAVs act as "master" post-transcriptional regulators of neuronal development, maintenance, and function (Bolognani et al., 2010 and for review see Deschenes-Furry et al., 2006; Pascale and Govoni, 2012).

The lack of positive correlation between transcription rate and AChE mRNA levels (see for example Boudreau-Lariviere et al., 2000; Angus et al., 2001) together with the presence of an ARE motif in the AChE transcript prompted us several years ago, to initiate a series of studies to determine whether nELAVs control AChE mRNA stability. Using several complementary approaches, we were able to show that HuD binds to the ARE-containing region in the 3′UTR and enhances AChE mRNA abundance following its ectopic expression in PC12 cells (Deschenes-Furry et al., 2003). Around the same time, the group of Cuadrado et al used an *in vitro* RNAse protection assay to establish that HuD binds to the AChE ARE and CU-rich sequence. In a congruent experiment to ours, they were able to demonstrate that overexpression of HuD is sufficient to increase AChE mRNA expression in N2a cells (Cuadrado et al., 2003). Moreover, similar findings were obtained in a skeletal muscle differentiation model when we demonstrated that HuR also binds to the AChE 3′UTR thereby stabilizing the transcript (Deschenes-Furry et al., 2005).

In subsequent work, we ascertained that the regulation of AChE mRNA stability by HuD also occurs *in vivo*. To this end, we conducted experiments using two distinct models. First, we made use of transgenic mice overexpressing HuD in regions of the brain. In these animals, we detected through in situ hybridization and RT-PCR experiments, an increase in AChE levels in brain regions showing high levels of transgene expression. Furthermore, RNA immunoprecipitation experiments confirmed *in vivo* an interaction between the HuD transgene product and endogenous AChE transcripts. In a second approach, axotomy of neurons of the spinal cervical ganglion (SCG) produced a concomitant decrease in AChE mRNA and HuD protein levels, as well as a clear reduction in their interaction. Importantly, the decrease in AChE mRNA abundance could be rescued by localized overexpression of HuD delivered via a viral vector (Deschenes-Furry et al., 2007). Together, these *in vitro* and *in vivo* studies highlight the critical importance of HuD in controlling AChE mRNA stability (**Figure 2**) during neuronal development and following injury. Whether these functions extend to the other nELAVs is not presently known.

LOCALIZATION AND TRANSLATION OF AChE TRANSCRIPTS

Transport of mRNAs into neurites necessitates a heterogeneous and dynamic assembly of proteins, known as ribonucleoprotein (RNPs) particles, onto 3′UTR cis-elements (**Figure 2**). RNP

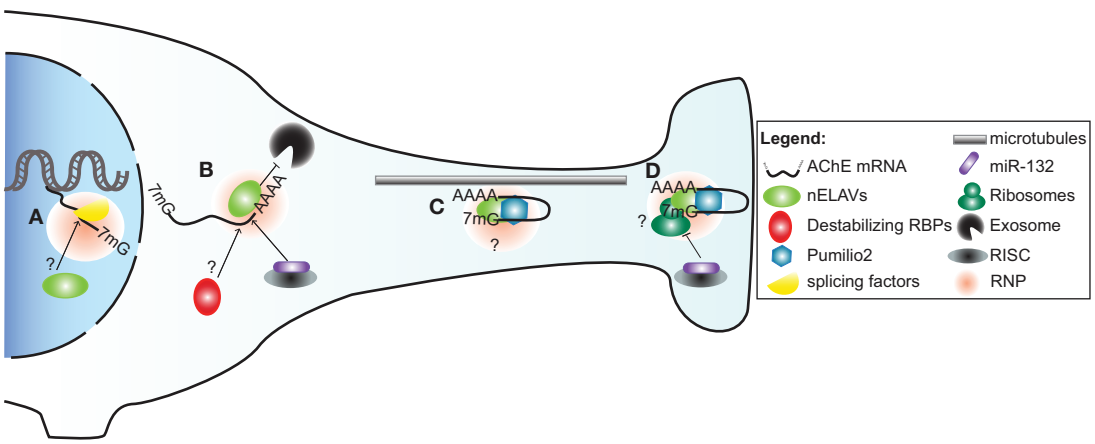

FIGURE 2 | A model illustrating post-transcriptional regulation of AChE expression and localization in neurons by trans-acting factors. (A) Alternative splicing of AChE pre-mRNA is controlled by SC35 and ASF/SF2 general splicing factors. As part of a ribonucleoprotein particle (RNP), nELAVs might also bind to cis-elements within the AChE pre-mRNA to regulate alternative splicing. **(B)** AChE mRNA is stabilized by HuD and possibly other nELAVs. Stabilization of AChE mRNA could depend on nELAVs outcompeting destabilizing RBPs and/or RISC loaded miR-132, thereby preventing exosome-mediated mRNA degradation. **(C)** RNPs, conceivably containing nELAVs and Pumilio 2 (Pum2), transport translationally repressed AChE transcripts along microtubules into neurites. **(D)** At the synaptic terminal, AChE translation might be promoted by nELAVs or inhibited by Pum2 or RISC loaded miR-132.

components stabilize the transcript, inhibit translation during transport and promote/repress protein synthesis at the synapse (reviewed in Wang et al., 2010a; Liu-Yesucevitz et al., 2011). In a potentially analogous mechanism, translocation of $AChE_R$ mRNAs into neurites correlates with increased AChE activity following physiological stress, AChE inhibition or corticosterone treatment (Meshorer et al., 2002). Collectively with the discovery of augmented $AChE_R$ transcript levels in cortical neurites subsequent to AChE inhibition (Kaufer et al., 1998), these findings support the notion that post-transcriptional events regulate $AChE_R$ mRNA translocation and possibly local translation at the neurite terminal (Meshorer and Soreq, 2006).

In agreement with this view, a study by Marrero et al has recently identified Pumilio2 (Pum2) as a regulator of AChE translation (Marrero et al., 2011). Pum2 is a member of the PUF family of RBPs that bind to distinct Pumilio binding elements (PBEs) in 3′UTRs of mRNAs and inhibit their translation (reviewed in Quenault et al., 2011). Using protein-RNA binding assays, the authors demonstrated that Pum2 specifically binds to a PBE element in the 3′UTR and consequently represses AChE translation (Marrero et al., 2011). Although these studies were conducted in skeletal muscle, Pum2 is also found in the nervous system, where it controls synaptic function and dendrite outgrowth (Vessey et al., 2010). Furthermore, disruption of Pum2 impairs long-term memory in Drosophila while its ablation produces numerous behavioural defects in mice (Dubnau et al., 2003; Siemen et al., 2011). Together, these studies suggest that Pum2 may inhibit AChE translation during transport and at synaptic termini in neurons (**Figure 2**).

MicroRNA REGULATION OF AChE EXPRESSION

Stability and translation of mRNAs are also under the influence of miRs, which are short (∼23 nt) single-stranded non-coding RNAs. MiRs function by imperfectly binding to target sequences, usually in the 3′UTR, thereby promoting transcript degradation and/or translational silencing when loaded into the miR-induced silencing complex (miRISC) (Berezikov, 2011). Recently, Shaked et al. used a reporter assay along with binding site point mutation in cultured CHO cells to demonstrate that miR-132 targets the AChE 3′UTR. The authors then elaborated on these findings *in vivo* by showing that miR-132 overexpression and locked nucleic acid (LNA)-mediated reduction decreased and increased, respectively, AChE catalytic activity in bone marrow cells (Shaked et al., 2009).

MiR-132 is also abundant in the brain implying that AChE is subjected to this regulatory event in neurons (Cheng et al., 2007). Indeed, a very recent study revealed that transgenic mice overexpressing $AChE_R$ transcripts lacking the 3′UTR contain elevated miR-132 levels which correlate with decreased $AChE_T$ mRNA abundance in the hippocampus (Shaltiel et al., 2012). However, it remains to be tested whether miR-132 induces AChE mRNA translational inhibition, degradation or both (**Figure 2**). Additional bioinformatics analysis of 3′UTR sequences further identified at least 47 and 81 putative miR sites in $AChE_T$ and $AChE_R$, respectively (Hanin and Soreq, 2011); though the functionality of these sites requires validation. Amongst these potential sites, at least 13 miRs, for example miR-124, -125b, -194 and -214, are of greater interest because they have been implicated in neuronal development, regeneration, and/or brain diseases (Saba and Schratt, 2010; Hanin and Soreq, 2011).

IMPLICATION OF Hu/ELAVs IN REGULATING AChE mRNA METABOLISM AT MULTIPLE STEPS

It is now well established that members of the Hu/ELAV family of RBP can exert multiple effects on RNA metabolism by assuming several distinct, yet complementary functions. Thus, the multifunctional nature of Hu/ELAVs in controlling the fate of mRNAs suggests that these RBPs regulate more than stability of AChE transcripts. In support of this idea, a recent compilation of high throughput data implicates HuR in coupling pre-mRNA alternative splicing to mRNA stability. More specifically, nascent HuR targets that contain both intronic and 3′UTR binding sites were found to be more stable than those that do not (Mukherjee et al., 2011).

In view of this, a U-rich sequence in the AChE E5 non-coding region was demonstrated to control splicing to the distal 3′ splice site to favor $AChE_T$ expression in skeletal muscle (Guerra et al., 2008). Interestingly, the Hu/ELAV family has been shown to control pre-mRNA splicing by binding to AU- and U-rich sequences (reviewed in Hinman and Lou, 2008). For example, one study demonstrated that nELAVs regulate splicing of the calcitonin/calcitonin gene-related peptide (CGRP) pre-mRNA in a neuron-specific manner by outcompeting TIA/TIAR splicing factors for U-rich intronic binding sites (Zhu et al., 2006). Various Hu/ELAV members were shown to similarly bind and control splicing of Fas (Izquierdo, 2010), Neurofibromatosis type 1 (NF1) (Zhu et al., 2008), Ikaros (Bellavia et al., 2007) and even HuD pre-mRNAs (Wang et al., 2010b). In most of these cases Hu/ELAVs were found to function as splicing repressors with the only exception being a splicing enhancement role on HuD pre-mRNA (Wang et al., 2010b). Based on their established nuclear functions, nELAVs could thus regulate splicing of AChE 3′ alternative exons by competing for the U-rich site within the E5 non-coding region (**Figures 1B and 2**).

In addition to governing pre-mRNA splicing, compelling evidence supports a role for nELAVs in regulating both localization and translation of ARE-bearing transcripts in neurites (reviewed in Pascale et al., 2008). For instance, two ARE-containing HuD target mRNAs that are both involved in axon outgrowth namely, GAP-43 and Tau, colocalize with HuD protein and polysomes in growth cones during neuronal differentiation (Smith et al., 2004; Atlas et al., 2007). Co-immunoprecipitation and *in vitro* binding assays revealed that HuD interacts with a motor protein, KIF3A, and directly binds to a microtubule component, MAP1B (Aronov et al., 2002; Fujiwara et al., 2011). Strengthening the notion of nELAV-mediated translation, HuD was shown to enhance cap-dependent protein synthesis and PC12 cell neurite extension by binding to eIF4A and the poly(A)-tail of transcripts (Fukao et al., 2009). Hu/ELAV members are also capable of repressing translation through a mechanism that is unclear but appears to depend on the target transcript and/or the cellular context (Kullmann et al., 2002; Meng et al., 2005). Based on these findings, it is conceivable that nELAVs regulate both AChE mRNA stability and translation in neurons, in a manner analogous to that previously

observed for the mRNA targets GLUT1 and NOVA-1 (Jain et al., 1997; Ratti et al., 2008).

CONCLUDING REMARKS

Despite extensive research into the regulation of AChE expression, only a handful of studies have precisely described some of the molecular mechanisms that control AChE expression, particularly at the post-transcriptional level. Currently, our knowledge of trans-acting factors and cis-acting elements that affect AChE pre-mRNA splicing, mature mRNA stability, transport and translation is rather limited. Nevertheless, some progress has been made over the years and increasing evidence suggests that RBPs (SC35, ASF/SF2, HuD and Pum2) and at least one miR (miR-132) play integral roles in controlling AChE expression in cholinergic and non-cholinergic neurons under various physiological contexts (see above). Based on their implications in neurogenesis and synaptic plasticity, trans-acting factors may also regulate AChE expression during learning and memory (Quattrone et al., 2001; Mellios et al., 2011; Siemen et al., 2011; Tognini et al., 2011; Apostolatos et al., 2012). Under such circumstances, post-transcriptional events might differentially regulate $AChE_T$ versus $AChE_R$ at multiple levels, since the expression patterns and functions of these variants are often contrasting (reviewed in Meshorer and Soreq, 2006). Control of mRNA stability, transport and local translation would facilitate temporal and spatial expression of AChE variants at growth cones and mature synapses and thus contribute to the multifaceted biochemical, morphological and physiological modifications necessary for neuronal differentiation, synaptic plasticity and stress-response. Conversely, altered abundance and/or function of trans-acting factors, such as nELAVs and consequently AChE, may be directly or indirectly involved in neurodegenerative pathogenesis, namely Alzheimer's disease (Berson et al., 2008; Amadio et al., 2009). In light of the emerging role of post-transcriptional events governing AChE metabolism, it is now clear that a thorough knowledge of all mechanisms controlling AChE expression and localization is necessary if the ultimate goal is to better understand neuronal development and function including neurogenesis, stress-response, cognition, neurodegeneration, as well as other brain diseases and injuries.

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Interactions of AChE with Aβ aggregates in Alzheimer's brain: therapeutic relevance of IDN 5706

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Acetylcholinesterase (AChE; EC 3.1.1.7) plays a crucial role in the rapid hydrolysis of the neurotransmitter acetylcholine, in the central and peripheral nervous system and might also participate in non-cholinergic mechanism related to neurodegenerative diseases. Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive deterioration of cognitive abilities, amyloid-β (Aβ) peptide accumulation and synaptic alterations. We have previously shown that AChE is able to accelerate the Aβ peptide assembly into Alzheimer-type aggregates increasing its neurotoxicity. Furthermore, AChE activity is altered in brain and blood of Alzheimer's patients. The enzyme associated to amyloid plaques changes its enzymatic and pharmacological properties, as well as, increases its resistant to low pH, inhibitors and excess of substrate. Here, we reviewed the effects of IDN 5706, a hyperforin derivative that has potential preventive effects on the development of AD. Our results show that treatment with IDN 5706 for 10 weeks increases brain AChE activity in 7-month-old double transgenic mice (APP_{SWE}–PS1) and decreases the content of AChE associated with different types of amyloid plaques in this Alzheimer's model. We concluded that early treatment with IDN 5706 decreases AChE–Aβ interaction and this effect might be of therapeutic interest in the treatment of AD.

Keywords: Alzheimer's disease, amyloid plaques, acetylcholinesterase, AChE–Aβ interactions, Aβ neurotoxicity, AChE activity, APP–PS1 transgenic mice, IDN 5706

INTRODUCTION

Alzheimer's disease (AD) is characterized by progressive memory and cognitive impairment and the cerebral accumulation of extracellular amyloid plaques and intra-neuronal neurofibrillary tangles (NFTs) in areas of brain involved in learning and memory (Ballard et al., 2011). Amyloid plaques are extracellular deposits of aggregated amyloid-β (Aβ) peptide, surrounded by dystrophic neurites and reactive glial cells. Aβ peptide is the main constituent of senile plaques and the major neurotoxic agent (Li et al., 2010). Intra-neuronal NFTs consist largely of hyper phosphorylated twisted filaments of the microtubule-associated protein tau (Lee et al., 2001). Synaptic pathology is an early marker of both, AD and aging, with decreased dendritic spine density, degeneration of neurites, neuronal loss, and cortical atrophy (Knobloch and Mansuy, 2008).

Original neurochemical findings in AD brains pointed out to disturbances of acetylcholine metabolism and led to the formulation of the "cholinergic hypothesis" of AD. This hypothesis suggests that there is a loss of cholinergic neurons in the basal forebrain of AD patients (Bartus et al., 1982; Bartus, 2000). The deficiency of cholinergic projections in AD has been linked to the buildup of Aβ and *tau*. Acetylcholinesterase (AChE; EC 3.1.1.7) and choline acetyltransferase activities decreases, while Na^+-dependent high-affinity choline uptake increases, perhaps due to compensatory mechanisms (Slotkin et al., 1994; Bissette et al., 1996; Shinotoh et al., 2000; DeKosky et al., 2002). Presynaptic α7 nicotinic acetylcholine receptors are essential for cognitive processes, and their levels increase in early AD, decreasing later on (Ikonomovic et al., 2009). The levels of muscarinic acetylcholine receptors, or receptor coupling, are reduced in the brains of patients with AD. However, pharmacological stimulation of the postsynaptic muscarinic type 1 acetylcholine receptors activates protein kinase C, favoring the processing of amyloid precursor protein (APP) that does not yield an amyloidogenic fragment (Nitsch, 1996; Farias et al., 2004). According to the cholinergic hypothesis, the impairment of cognitive functions and the behavioral disturbances that affect patients with AD are in part due, to cortical deficiencies in cholinergic neurotransmission (Bartus et al., 1982; Dumas and Newhouse, 2011). AD is associated with an early and severe depletion of cholinergic innervations. AChE activity is lower in most regions of AD brains, but it is increased within and around amyloid plaques (Geula and Mesulam, 1989b; Beach et al., 2000). The different molecular forms of AChE are altered in AD, showing a decrease in the tetrameric AChE G_4 isoform localized at central synapses (Xie et al., 2010), while the minor light forms (dimers G_2 and monomers G_1) increase (Atack et al., 1983; Saez-Valero et al., 1999). Interestingly, the activity of the light forms appears to increase in the most severely affected cases (Arendt et al., 1992). Some studies indicate that the level of an amphiphilic monomeric form of AChE is increased in the brains of transgenic mice which produce the human Aβ protein (Sberna et al., 1998), and in the brain and cerebrospinal fluid (CSF) of rats which received intra-cerebral-ventricular injections of the Aβ peptide (Saez-Valero et al., 2002). So far, the precise nature of

this subset of G_1 species which increase in AD brains remains unclear, however this minor species can be distinguished from other brain AChE forms (including tetramers but also from other monomeric AChE isoforms), by its unusual lectin-binding pattern and the lack of binding to anti-AChE antibodies (Saez-Valero et al., 2000; Garcia-Ayllon et al., 2007). Some cholinergic deficits have been shown to appear in transgenic mouse model reproducing preclinical and early stages of amyloid pathology. Region-specific modifications in AChE activity were reported in APP_{696LD} (London V642I mutation) transgenic mice with Aβ plaques, being decreased in subiculum but increased in the dentate gyrus, a CA_1 sub-region of the hippocampal formation (Bronfman et al., 2000). AChE activity was unchanged in APP_{751SWE} and APP_{695SWE} transgenic mice despite extensive Aβ plaques (Apelt et al., 2002; Boncristiano et al., 2002). However, when specific AChE isoforms were taken into account, the activity of an abnormally glycosylated G_1 version increased in cortical extracts of APP_{695SWE} mice, whereas the activity of the tetrameric G_4 AChE was unchanged (Fodero et al., 2002).

AChE activity in the blood and plasma has also been measured to assess the pathophysiology of AD. Plasma AChE activity levels are increased in AD patients, which correlates with an increase in the light AChE species ($G_1 + G_2$) which are the major species in human plasma, whereas tetramers, that are normally only presents in trace amounts, are slightly decreased in AD plasma (Garcia-Ayllon et al., 2010). Plasma AChE is likely to have multiple cellular origins including cells from the brain. Thus, we can hypothesize that the increase observed in AD plasma may be associated with the particular increase in the light AChE species characterized in AD brain (Arendt et al., 1992; Saez-Valero et al., 1999). Blood AChE and butyrylcholinesterase (BuChE) activities have been studied as markers for Alzheimer's. AD patients have lower AChE activity in lymphocytes compared to control subjects. In contrast, erythrocyte AChE activity is higher in patients with vascular dementia and is reduced in sporadic AD. Low ChE activity in lymphocytes is the best discriminator for AD. Both globular forms are subnormal. Because it is already low at very early stages of AD, AChE could be helpful as an early biomarker of differential diagnosis for the follow-up of patients during their early stages of cognitive impairment before a clinical dementia is established (Inestrosa et al., 1994; Von Bernhardi et al., 2005). Blood and plasma are easily accessible in comparison to CSF, together with specific and sensitive assays for AChE detection, therefore this enzyme could be used as clinical marker in the development of AD (Garcia-Ayllon et al., 2010).

IS THERE A ROLE FOR AChE IN THE PATHOGENESIS OF NEURODEGENERATIVE DISEASES?

Previous studies have demonstrated that AChE and BuChE are present in amyloid plaques *in vivo* and are associated to a cholinergic deficit (Mesulam and Geula, 1994; Geula and Mesulam, 1995; **Figure 1**). Moreover, AChE promotes $A\beta_{1–40}$ fibril formation, in fact, AChE forms macromolecular complexes with the growing amyloid fibrils, and is incorporated into senile-like plaques *in vitro* (Alvarez et al., 1997, 1998). In this context, studies with synthetic $A\beta_{1–40}$ *in vitro* have shown that this peptide aggregates and forms amyloid fibrils similar to the filaments found in the brains of AD patients (Morgan et al., 2004). Different mutations of Aβ were used to see its effect in the formation of aggregates. For example, the single mutation Val18 → Ala induces a significant increase on α-helical content in Aβ, and dramatically diminishes fibrillogenesis (Soto et al., 1995). However, the substitution of Glu22 → Gln found in hereditary cerebral hemorrhage with amyloidosis of the Dutch type, yields a peptide with increased ability to form amyloid fibrils (Soto et al., 1995). In fact, AChE had little effect on the aggregation of the highly amyloidogenic Dutch variant (Inestrosa et al., 1996). However, when the $A\beta_{val118 \rightarrow Ala}$ was incubated with AChE, a significant increase in the amyloid fibrils was observed (Inestrosa et al., 1996; Inestrosa and Alarcon, 1998). Previous investigations have shown that wild-type $A\beta_{1 \rightarrow 40}$ is able to bind AChE, while the Dutch variant $A\beta_{Glu22 \rightarrow Gln}$ is not (Muñoz and Inestrosa, 1999). These data are correlated with previous observations that indicate that the presence of different types of Aβ peptide differentially affects AChE–Aβ interactions (Inestrosa and Alarcon, 1998). These studies indicated that AChE, but not BuChE increases the final yield of Aβ fibrils. In this context, an *in vitro* study, demonstrated that BuChE acts as a negative modifier of the Aβ aggregation process, and it is also capable of suppressing the facilitation of amyloid fibril-formation enhanced by AChE. So, BuChE may has acquired an inverse role to that of AChE in the pathogenesis of AD (Diamant et al., 2006).

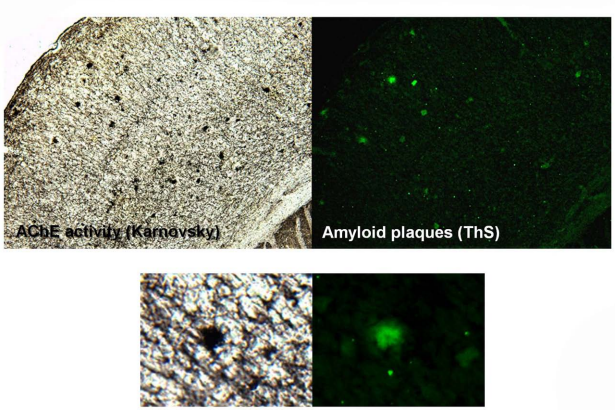

FIGURE 1 | AChE activity in amyloid plaques in the brain of APP–PS1 mice. Studies carried out in the presence of iso-OMPA, an inhibitor of BuChE, in the mouse cerebral cortex. A single amyloid plaque shows its association to AChE activity.

AChE A NUCLEATION FACTOR FOR Aβ AGGREGATION, AND THE ROLE OF ITS PERIPHERAL ANIONIC SITE ON Aβ AGGREGATION

In 1996, we discovered that AChE was able to accelerate the assembly of $A\beta_{1–40}$ into Alzheimer's fibrils by decreasing the lag phase of the peptide aggregation, suggesting a role of AChE as a chaperone for $A\beta_{1–40}$ assembly into oligomers of a high structural complexity (Inestrosa et al., 1996). These results suggested that the enzyme was acting through two possible mechanisms. First, it might increase the seeds necessary for the nucleation step and second, it may stimulate fibril elongation (Harper et al., 1997; Inestrosa et al., 2005a,b; **Figure 2**). When the formed amyloid was evaluated with

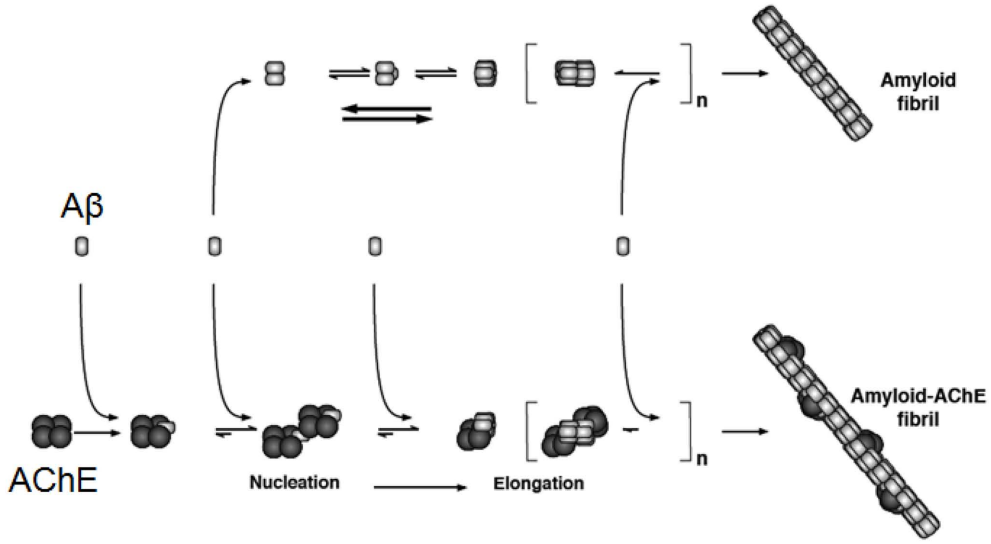

FIGURE 2 | AChE acts on the Aβ aggregation and then in the formation of amyloid fibrils. The incorporation of AChE occurs at early steps of Aβ aggregation, acting as a nucleation factor or seed, then it acts in the elongation of the amyloid fibrils. Evidence for the tight binding of the enzyme with the formed amyloid fibrils was obtained using biochemical as well as immunocytochemical techniques, using an anti-AChE antibody conjugated with gold and examined using a transmission electron microscope. The original data was published by Alvarez et al. (1998).

thioflavin-S (ThS) plus AChE activity, it became apparent that the enzyme was strongly associated with amyloid deposits, exactly as described by Mesulam and Geula, for the senile plaques in AD patients (Geula and Mesulam, 1989a). In this case, at least part of the enzyme became tightly associated to the amyloid fibril, as it was shown by electron microscopy, using a monoclonal antibody conjugated with gold particles that nicely decorated growing amyloid fibrils (Reyes et al., 1997). To test this idea *in vivo* a double transgenic mice which express both human APP_{SWE} and human AChE was generated by Brimijoin, Younkin, and Soreq. In these hybrid transgenic mice, AChE promotes plaque accumulation supporting the notion of its causal involvement with the fibril-formation process (Rees et al., 2003, 2005).

The kinetic and pharmacological properties of AChE–amyloid complex have been examined, and the data show that the kinetic parameters of the enzyme change (Geula and Mesulam, 1989a). The K_m and V_{max} values for AChE associated to amyloid were higher than those for the free enzyme. Similarly, for the AChE–$A\beta_{Glu22 \rightarrow Gln}$ complex and AChE–$A\beta_{Val18 \rightarrow Ala}$ complex, the K_m values were elevated compared with the enzyme alone. When kinetic studies were carried out under varying pH conditions, AChE associated to either the wild-type or the mutant Aβ peptides was more resistant to low pH. Similarly, AChE associated in AChE–Aβ complexes was more resistant to the incubation at high substrate concentrations (Alvarez et al., 1998; Inestrosa and Alarcon, 1998; Inestrosa et al., 2005b). Furthermore, biochemical studies have indicated that senile-plaque-associated AChE is only partially extracted using collagenase digestion, heparin, or high-salt buffers plus detergents (Nakamura et al., 1990; Kalaria et al., 1992; Alvarez et al., 1998), indicating that either different molecular forms are involved, or alternative some changes occur in the biochemical properties of the globular subunit.

Pharmacological studies of AChE associated to amyloid showed that AChE in these conditions also appears more resistant to inhibition by anti-AChE agents as observed with both active site inhibitors such as tacrine, edrophonium, and BW284c51, and with peripheral anionic site blockers, such as propidium and gallamine (Inestrosa and Alarcon, 1998). In almost all cases, a higher inhibitor concentration was required to obtain the same level of inhibition observed with the free enzyme. Overall, the AChE–$A\beta_{Val18 \rightarrow Ala}$ complex showed the largest differences with respect to the free enzyme, suggesting that it has greater degree of interaction with AChE than the other more amyloidogenic Aβ peptides. Contrastingly the complexes AChE–$A\beta_{Glu22 \rightarrow Gln}$ and AChE–$A\beta_{1–42}$ were the least affected of all complexes studied. These data are consistent with the idea that the association of AChE with Aβ fibrils leads to changes in its enzymatic properties, in the absence of any pathological alteration of the enzyme (Inestrosa and Alarcon, 1998).

It is well know that AChE possesses two binding sites for the neurotransmitter acetylcholine, *the active center site* that is located at the bottom of a 20-Å gorge and *the peripheral anionic binding site* (PAS) that is rich in hydrophobic residues and is located at the rim of the gorge on the surface of the enzyme (Sussman et al., 1991). When aggregation experiments were carried and repeated in the presence of AChE inhibitors (AChEIs) directed against the two different sites, it turned out that only the PAS inhibitors were able to block the effect of AChE on amyloid formation (Alvarez et al., 1998). The PAS inhibitors, propidium and fasciculin, were able to prevent the effect of AChE on Aβ aggregation process (Bartolini et al., 2003; Inestrosa et al., 2008). On the other hand, the amyloid aggregation in the presence of edrophonium, an active site inhibitor of AChE, showed no effect on the role of AChE in this capacity to accelerate Aβ assembly into Alzheimer's fibrils

(Inestrosa et al., 2008). Further studies, we were able to identify a 3.5-kDa peptide located close the PAS region which was able to mimic effect of the whole AChE enzyme in its capacity to stimulate the Aβ aggregation (De Ferrari et al., 2001). Moreover, structural studies of AChE showed how the regulation of catalysis by PAS ligands (propidium, decidium, and gallamine) offers information on the residues that interact with other molecules and which could participate in the nucleation process of amyloid fibrils (Bartolini et al., 2003; Inestrosa et al., 2008). To understand the mechanism of the AChE–Aβ interaction Vaux and co-workers have studied a 14 residue peptide named $AChE_{586–599}$, which corresponds to a region within the C-terminal oligomerization domain of human AChE (Jean et al., 2008). The region encompassing $AChE_{586–599}$ shares homology with Aβ and possesses high propensity for conversion to non-native β-strand, a property associated to amyloidogenicity (Cottingham et al., 2002; Greenfield et al., 2008; Belli et al., 2011). Analysis of stabilizing or destabilizing effects of residue substitutions on the amyloid assembly of $AChE_{586–599}$ has provided evidence for the critical role of specific side-chain interactions in the stabilization of nascent aggregates and for the position dependence of these side-chains upon polymerization and fibril formation. Consistently with the experimental observations and assembly models for other amyloid systems, they have proposed a model for $AChE_{586–599}$ assembly in which a steric-zipper formed through specific interactions (hydrophobic, electrostatic, cation-π, SH-aromatic, metal chelation, and polar–polar) would maintain the β-sheets together. The dissection of the specific molecular recognition driving $AChE_{586–599}$ amyloid assembly has provided further knowledge on such poorly understood and complicated process, which could be applied to protein folding and the targeting of amyloid diseases (Belli et al., 2011).

DIMERIC TYPE OF AChE INHIBITORS DIRECTED AGAINST THE ACTIVE AND THE Aβ SITE OF THE ENZYME

The current standard of care for mild to moderate AD includes treatment with AChEIs to improve cognitive function (Hardy and Selkoe, 2002; Terry and Buccafusco, 2003; Ballard et al., 2011). Several classes of AChEIs such as donepezil, rivastigmine, and galantamine were developed to treat AD (Colombres et al., 2004), and currently constitute the only FDA approved therapeutic approach. The NMDA antagonist memantine, has also been shown to improve cognitive function and reached the market in 2004 (Cummings et al., 2006). Nevertheless AChEIs, even valuable in improving the patient's quality of life, represent only symptomatic and palliative tools that slow down the progression of the disease. Blockade of PAS by specific inhibitors has emerged as promising disease-modifying therapeutic strategies for AD. Based on these assumptions, the dual binding AChEIs, that are molecules able to interact simultaneously with both, the catalytic and the peripheral binding sites of the enzyme, emerged as valuable tools to pursue a disease-modifying approach (Colombres et al., 2004; Muñoz-Torrero, 2008). In this regard, several classes of dual binding site AChEIs have been developed and proved to be endowed with a strong inhibitory activity due to the increased capability to interact with both bindings sites of the target (Muñoz-Torrero and Camps, 2006). Some recent examples include benzophenone-based derivatives bearing a [benzyl(methyl)amino]methyl moiety (Belluti et al., 2009), Xanthostigmine derivatives (Belluti et al., 2005) and novel huprine derivatives with inhibitory activity toward Aβ aggregation and formation (Viayna et al., 2010). Owing to the simultaneous activity against AChE and amyloid formation and aggregation, dimeric type of AChEIs might attack AD on multiple fronts, with a better therapeutic outcome. Together with coumarin derivatives (Piazzi et al., 2008), and tacrine based heterobivalent ligands (Camps et al., 2009) they are able to act both at the acetylcholine site and at the amyloid formation triggering site. To further support this strategy, Shen and co-workers reported the discovery of novel dual inhibitors of AChE and BACE-1, which demonstrated not only *in vitro* enzyme inhibitory potency and cellular activity, but, more importantly, *in vivo* functional efficacy (Zhu et al., 2009). This strategy, embodied by single chemical entities able to simultaneously modulate multiple targets involved in the neurodegenerative cascade, has proven particularly fruitful in recent years and has led to the discovery of several promising anti-AD drug candidates.

AChE INDUCES THE AGGREGATION OF THE CELLULAR PRION PROTEIN

Prion disease, such as the Creutzfeldt–Jakob disease (CJD) in human and bovine spongiform encephalopathy (BSE), can be transmitted by an infectious process which involves the prion protein (PrP). The most remarkable feature of PrP is its ability to be folded into two isoforms, PrP^{C} (C, cellular form) being the native protein and PrP^{Sc} (Sc, scrapie form) being the pathological conformation (Prusiner, 1998; Varela-Nallar et al., 2006). During the pathogenesis of prion disease there is a conformational conversion from PrPC to PrPSc consisting of a drastic alteration of the structure, as well as of the biochemical properties of the protein. Aβ-positive senile plaques in AD brains commonly contain PrP deposits; while sporadically Aβ-positive senile plaques have also been identified in prion diseases such as CJD and Gerstmann–Sträussler–Scheinker (GSS) disease (Miyazono et al., 1992; Hainfellner et al., 1998). On the other hand, a decrease in the in CFS levels of AChE from patients with CJD has been demonstrated, suggesting that an alteration in the cholinergic system also occurs in some prion diseases (Silveyra et al., 2006). Based on the common features between PrP and Aβ, it has been shown that AChE is able to induce the aggregation of the peptide deduced from PrP sequence spanning residues 106–126 ($PrP_{106–126}$), the hydrophobic segment involved in PrP protein aggregation as has been previously described in a similar way for Aβ protein (Pera et al., 2006), through the PAS region of AChE (Inestrosa et al., 2008). The role of the peripheral site of AChE accelerating the assembly of $PrP_{82–146}$ was demonstrated using propidium iodide (Pera et al., 2009), a specific inhibitor of the PAS region of AChE (Inestrosa et al., 1996; Bartolini et al., 2003). It has been extensively demonstrated that propidium iodide can also inhibits the AChE-induced Aβ aggregation. This study showed that AChE acts as a nucleating factor increasing not only the formation of new oligomers, but also fibril formation. A similar effect has been observed with huprine

derivatives X, Y, and Z (Clos et al., 2006; Pera et al., 2006), which is spite of being active site AChEIs have been shown to interfere with the binding of ligands to the peripheral site of the enzyme (Camps et al., 2000). Therefore, inhibitors of the PAS region of AChE could be relevant as potential anti-Aβ and PrP aggregation drugs.

AChE INCREASES THE NEUROTOXICITY OF Aβ AGGREGATES

Considering that the presence of senile plaques in the brain of aging individuals does not necessarily lead to symptoms of AD (Katzman et al., 1988), the presence of AChE in some critically located amyloid plaques could play a key role in triggering the cytotoxic events that occur around mature plaques in AD (Mesulam, 2004). *In vitro* assays on PC12 cells showed that aggregates of AChE–$A\beta_{1-40}$ complexes were more toxic than those of $A\beta_{1-40}$ and that neurotoxicity depends on the amount of AChE bound to the complexes, suggesting that AChE may play a key role in the neurodegenerative changes observed in Alzheimer brain (Muñoz and Inestrosa, 1999). In this context, previous results showed that Aβ–AChE complexes are more toxic than the Aβ fibrils alone on rat hippocampal neurons. In fact, neurons treated with Aβ–AChE complexes showed a much disrupted neurite network compared to neurons treated with Aβ (Alvarez et al., 1998). Other *in vivo* study showed that the hippocampal injection of AChE–Aβ complexes results in the appearance of some features reminiscent of Alzheimer-like lesions in rat brain (Reyes et al., 2004). The early events triggered in neurons in response to Aβ peptide have been largely studied. Aβ oligomers/fibrils induce intracellular calcium deregulation that leads to apoptosis through mitochondrial dysfunction, by direct interaction with isolated mitochondria or by indirect association with the neuronal membrane (Kim et al., 2002; Abramov et al., 2004). One of the earliest effect of Aβ–AChE complexes was the increase in intracellular calcium, which leads to the loss of the mitochondrial membrane potential (Dinamarca et al., 2010). Disruption of intracellular homeostasis of Ca^{2+} by channels opening has been extensively proposed as a mechanism of Aβ neurotoxicity (Mattson et al., 1992; Laferla, 2002). Aβ–AChE complexes and Aβ treatment have different effects over the mitochondrial membrane potential. Our studies indicated that Aβ–AChE complexes affected $\Delta\Psi_{mit}$ more than Aβ alone; also, we observed that the mitochondrial membrane potential was compromised in a non-reversible manner even when the calcium increase was reversed after wash out. On the other hand, previous studies from our laboratory indicated that lithium (a pharmacological activator of Wnt signaling) protects hippocampal neurons against Aβ peptide and Aβ–AChE complex neurotoxicity (Dinamarca et al., 2010). Additionally, we found that pre-incubation with the Wnt-7a ligand prevents the increase in cytosolic calcium induced by Aβ (Quintanilla et al., 2005). These studies suggest that the activation of Wnt signaling prevent the toxic effects of Aβ–AChE complexes (Inestrosa et al., 2008), consistent with this possibility a "synaptic form" of AChE induces tau phosphorylation and activation of glycogen synthase kinase-3β (GSK-3β, a component of the Wnt/β-catenin signaling pathway). These effects were prevented by GSK-3β and AChE inhibition (Toiber et al., 2008). In this context it is interesting to mention that Huperzine A, a lycopodium alkaloid extracted from the chinese folk medicine, *Huperzia serrata*, a reversible and selective inhibitor of AChE, activates Wnt/β-catenin signaling and enhances the non-amyloidogenic pathway in a transgenic mouse model of AD (Wang et al., 2011).

IDN 5706 A POTENTIAL DRUG AGAINST AD

Previously, we have shown that hyperforin, the active molecule for the anti-depressant activity of St. John's Wort (*Hypericum perforatum*; Griffith et al., 2010), reduces the behavioral alteration induced by intra-hippocampal injection of Aβ aggregates, an acute rat model of AD (Dinamarca et al., 2006). Tetrahydrohyperforin (IDN 5706), a semi synthetic derivative of hyperforin with higher stability and increased oral bioavailability (Cerpa et al., 2010) also shown some neuroprotective properties. Previous studies in our laboratory indicated that IDN 5706 was able to reduce memory impairments, as well as neuropathological markers in 12-month-old APP–PS1 mice treated with 2 mg/kg IDN 5706 for 1 month (Cerpa et al., 2010). Even more, a reduction in the size of ThS positive plaques was observed by this treatment. Interestingly, we have previously demonstrated that IDN 5706: (a) releases AChE from the Aβ aggregates, and (b) inhibits AChE–Aβ interaction *in vitro* and *in vivo* (12-month-old APP–PS1 mice treated with 2 mg/kg IDN 5706; Cerpa et al., 2010). In young APP–PS1 mice, IDN 5706 improves memory and prevents the impairment of synaptic plasticity, inducing a recovery of long-term potentiation, prevented the decrease in synaptic proteins in hippocampus and cortex, decreased levels of tau hyperphosphorylation, astrogliosis and total forms of Aβ (Inestrosa et al., 2011). Moreover, we have shown that *in vitro*, hyperforin is able to disaggregate pre-formed fibrils into protofibrils and amorphous material (Dinamarca et al., 2006). Taking in consideration our previous study in AD aged and young mice (Cerpa et al., 2010; Inestrosa et al., 2011), it is apparent that IDN 5706 has anti-amyloidogenic actions *in vitro* and *in vivo*.

EFFECT OF IDN 5706 ON THE ESTERASE ACTIVITY PRESENT IN Aβ PLAQUES IN A MOUSE MODEL OF AD

A major issue in AD research is to find some new therapeutic drugs which decrease Aβ aggregation and inhibit AChE with dual specificity, being directed to both the active and "peripheral" sites (De Ferrari et al., 2001; Inestrosa et al., 2008). For these reasons, we investigate the effect of IDN 5706, in those activities. IDN 5706 also inhibits the aggregation of $A\beta_{1-40}$, delaying the nucleation phase. When we checked the amount of soluble Aβ peptide after the aggregation assay, IDN 5706 decreased the amount of Aβ peptide in the sediment fraction increasing the amount of soluble Aβ. Furthermore, we evaluated whether the anti-aggregation property of IDN 5706 was stronger or weaker than the pro-aggregating effect of the AChE. To explore this point we evaluated the stability of the Aβ fibrils formed in the absence or the presence of the AChE incubated with IDN 5706. The hyperforin derivative was able to disassemble a 50% of the Aβ fibrils, but only a 15% of the AChE–Aβ fibrils after 5 h incubation, suggesting that the fibrils formed in the presence of AChE have a more stable arrangement (Dinamarca et al., 2008). Then, the effect of this compound in the AChE activity was evaluated. Toward this aim, aliquots were taken at different

time points and the pellet (fibrils) was separated from the soluble fraction by centrifugation. As expected, at initial time of incubation with IDN 5706, most of the enzyme activity is found in the pellet fraction, but after 2 h of incubation of the AChE–Aβ aggregates with IDN 5706, the enzyme decreases in the pellet fraction and started to increase in the soluble fraction. After 4 h incubation the enzyme is found in both pellet and soluble fraction in similar amount (Dinamarca et al., 2008). This data showed that IDN 5706 is able to disaggregate the AChE from the AChE–amyloid complexes *in vitro*.

PRESENCE OF AChE IN AMYLOID PLAQUES OF A DOUBLE APPswe + PS1 TRANSGENIC MICE

A decrease of AChE in the brain appears to be a consistent finding in AD brain (DeKosky and Scheff, 1990). We observed similar finding in APP–PS1 mice brains compared with *wild-type* mice. Moreover, IDN 5706 treatment increases AChE activity in the brains of APP–PS1 mice injected with 4 and 6 mg/kg (**Figure 3A**) and also increases the BuChE activity of APP–PS1 brain in mice injected with 6 mg/kg of IDN 5706 (**Table 1**). The specific neuroprotective effect of IDN 5706 might be related to the increases in the AChE activity in total brain protein extracts, suggesting a neuroprotective effect on cholinergic and cholinoceptive neurons. Moreover, we observed AChE activity associated to amyloid plaques (**Figure 3B**, left panel) visualized by the Karnovsky reaction for AChE (**Figure 3B**, right panel) in APP–PS1 mice treated with iso-OMPA an inhibitor for BuChE activity. Such amyloid plaques from mice brain are similar to those observed in patients with AD (Tago et al., 1986; Geula et al., 1994).

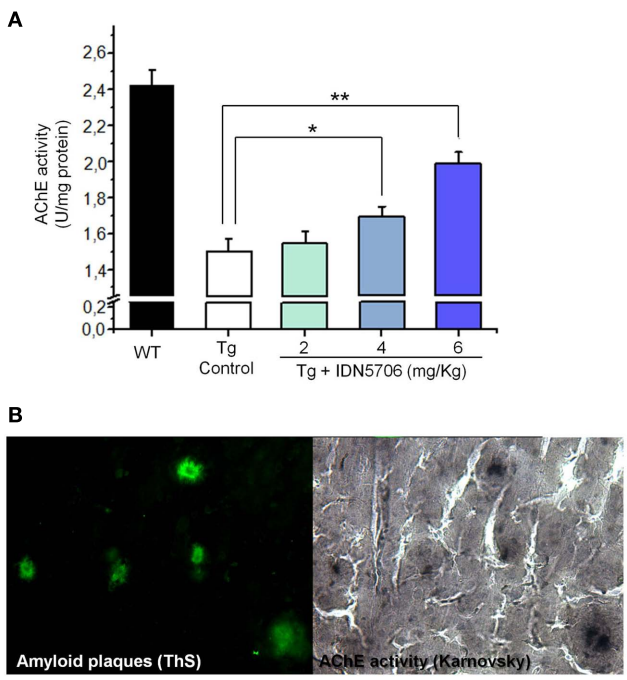

FIGURE 3 | IDN 5706 increases AChE activity in whole brain extract of APP–PS1 transgenic mice. Effects of IDN 5706 on AChE activity from brains of transgenic mice injected i.p. with vehicle solution or 2, 4, and 6 mg/kg IDN 5706 three times a week per 10 weeks **(A)**. AChE activity in amyloid plaques from brain of APP–PS1 mice in the presence of iso-OMPA **(B)**. $*p < 0.05$; $**p < 0.01$.

Table 1 | IDN 506 increases BuChE activity in brains of double transgenic APP–PS1 mice.

Treatment	BuChE activity (U/mg protein)
WT	0.91 ± 0.02
Tg control	0.58 ± 0.04
Tg IDN 2 mg/kg	0.61 ± 0.04
Tg IDN 4 mg/kg	0.69 ± 0.05
Tg IDN 6 mg/kg	0.71 ± 0.03*

$*p < 0.05$. Statistical significance between APP–PS1 mice control and APP–PS1 mice treated with IDN 5706. Comparison of BuChE activity from brains of wild-type mice, APP–PS1 mice treated with control vehicle solution and APP–PS1 mice treated with 2, 4, and 6 mg/kg IDN 5706 three times a week per 10 weeks.

EFFECT OF IDN 5706 ON AChE ACTIVITY ASSOCIATED TO AMYLOID PLAQUES

Since AChE associated with amyloid and its activity correlates with amyloid plaque toxicity (Alvarez et al., 1998; Reyes et al., 2004; Dinamarca et al., 2010), we carried out an analysis of amyloid plaques positive for AChE activity. Brains from control and treated APP–PS1 animals were stained for AChE with the method of Karnovsky (Tago et al., 1986) and amyloid plaques were revealed by ThS staining. In APP–PS1 mice most of the amyloid plaques were positive for AChE in cortex (**Figure 4A**), however, the percentage of AChE-positive plaques in relation to the total amount of ThS positive plaques in the cortex were decreased in IDN 5706 treated mice (**Figure 4B**), suggesting that in addition to the decreased number of amyloid plaques, there is a decrease in the association of AChE with the amyloid plaques present in IDN 5706 treated APP–PS1 mice.

We have also determined AChE activity in two types of amyloid plaques, the diffuse (Type I) and compact (Type II) plaques present in control transgenic mice (**Figure 5A**, left panels), as well as in mice treated with IDN 5706. Under this condition AChE activity was reduced (**Figure 5A**, right panels). Quantification of the number of different type of amyloid plaques positive for AChE with the Karnovsky reaction revealed that treatment with IDN 5706 decreases the amount of AChE activity in type II plaques however, no effect was observed in type I plaques (**Figure 5B**). These preliminary results suggest a rather specific effect of IDN 5706 on the association of AChE with Aβ aggregates. Previous studies from our laboratory indicate a key role for AChE in the neurotoxicity of amyloid plaques (Alvarez et al., 1998; Chacon et al., 2003; Reyes et al., 2004; Dinamarca et al., 2010). Taken together, our data indicates that IDN 5706 might be considered as a possible therapeutic agent for AD treatment.

CONCLUSION

As discussed, AChE is able to accelerate amyloid formation of at least two different macromolecules: the Aβ peptide and the PrP. In addition pro-aggregating effect of the enzyme depends on the intrinsic amyloidogenic properties of the peptide used.

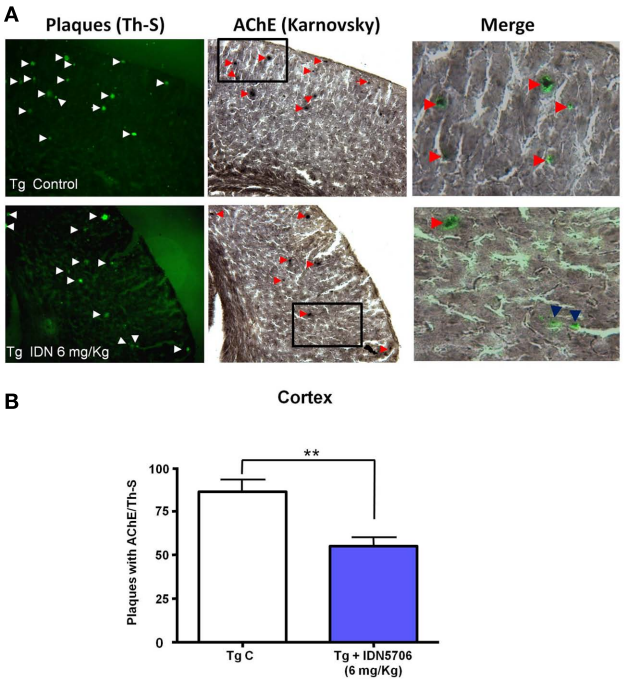

FIGURE 4 | IDN 5706 decreases amyloid plaques positive for AChE activity in APP–PS1 mice. (A) Cortical slices obtained from APP–PS1 mice stained for ThS for amyloid plaques and AChE activity with the Karnovsky method. **(B)** Quantification of the percentage of amyloid plaques with AChE activity in the cortex of APP–PS1 brain of mice injected i.p. with vehicle solution or 6 mg/kg IDN 5706 three times a week per 10 weeks. $*p < 0.05$.

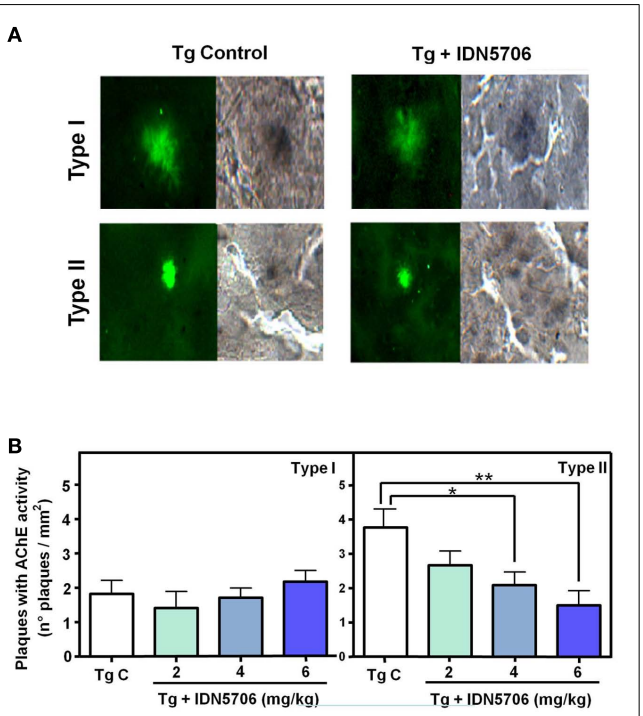

FIGURE 5 | IDN 5706 reduces the amount of different types of amyloid plaques containing AChE activity. (A) Detection of amyloid plaques (ThS) positives for AChE activity in vehicle and IDN 5706 (6 mg/kg) treated APP–PS1 mice brain. Plaques were morphologically classified as Type I (diffuse plaques) and Type II (compact plaques). **(B)** Quantification of the total number of the different types of plaques positive for AChE activity in brain from APP–PS1 mice injected i.p. with vehicle solution or 2, 4, and 6 mg/kg IDN 5706 three times a week per 10 weeks. $*p < 0.05$; $**p < 0.01$.

In AD patients, AChE activity is altered in the brain and in the blood, and co-localized with senile plaques. AChE associated to amyloid plaques showed changes in biochemical and pharmacological properties, as well as an increase in the neurotoxicity of the AChE–Aβ complexes. The AChE effect on amyloid aggregation is sensitive to drugs that block the PAS of the enzyme, suggesting that new and specific AChEIs might well provide an attractive therapeutic possibility for AD treatment. IDN 5706, an hyperforin derivative, prevents the development of the disease in a transgenic mice model of AD (Inestrosa et al., 2011). Interestingly, we have previously demonstrated that IDN 5706 releases AChE from the Aβ aggregates and inhibit AChE–Aβ interactions *in vitro* and *in vivo*. In summary, our findings indicate that IDN 5706 decreases AChE–Aβ interaction and this effect might be of therapeutic interest for the treatment of AD.

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Serum cholinesterases are differentially regulated in normal and dystrophin-deficient mutant mice

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The cholinesterases, acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) (pseudocholinesterase), are abundant in the nervous system and in other tissues. The role of AChE in terminating transmitter action in the peripheral and central nervous system is well understood. However, both knowledge of the function(s) of the cholinesterases in serum, and of their metabolic and endocrine regulation under normal and pathological conditions, is limited. This study investigates AChE and BChE in sera of dystrophin-deficient *mdx* mutant mice, an animal model for the human Duchenne muscular dystrophy (DMD) and in control healthy mice. The data show systematic and differential variations in the concentrations of both enzymes in the sera, and specific changes dictated by alteration of hormonal balance in both healthy and dystrophic mice. While AChE in *mdx*-sera is elevated, BChE is markedly diminished, resulting in an overall cholinesterase decrease compared to sera of healthy controls. The androgen testosterone (T) is a negative modulator of BChE, but not of AChE, in male mouse sera. T-removal elevated both BChE activity and the BChE/AChE ratio in *mdx* male sera to values resembling those in healthy control male mice. Mechanisms of regulation of the circulating cholinesterases and their impairment in the dystrophic mice are suggested, and clinical implications for diagnosis and treatment are considered.

Keywords: acetylcholinesterase, butyrylcholinesterase, mouse serum, *mdx*, muscular dystrophy, testosterone, orchidectomy, H-P-G axis

INTRODUCTION

The presence of cholinesterase activity in serum was already described by Dale in 1914 (Dale, 1914). It was later found that this activity was due to the two acetylcholine (ACh)-hydrolyzing enzymes (Mendel and Rudney, 1943), acetylcholinesterase (AChE), and butyrylcholinesterase (pseudocholinesterase, BChE), that could be distinguished by their substrate specificity (Mendel and Rudney, 1943) and differential sensitivity to selective inhibitors (Austin and Berry, 1953). By now, both enzymes have been well-characterized in terms of structure and function (Massoulié, 2002; Massoulié et al., 2008). AChE is abundant in the nervous system and muscles, where it is clearly responsible for controlling the duration of cholinergic transmission (Katz and Miledi, 1973). Additional functions have been ascribed to both enzymes in normal development of the nervous system and in altered physiological or pathological conditions due to stress, inflammation, and neurodegenerative diseases, (e.g., Alzheimer's disease) and their treatment (for reviews, see e.g., Layer and Willbold, 1995; Darvesh et al., 2003, 2010; Ballard et al., 2005; Dori and Soreq, 2006; Meshorer and Soreq, 2006; Massoulié et al., 2008; Vogel-Höpker et al., 2012). Moreover, the soluble form of BChE in mammalian serum may serve as a safeguard against the diffusion of ACh into the bloodstream and/or against orally ingested toxic compounds, since it reacts with a broad range of substrates (Lockridge and Masson, 2000; Masson and Lockridge, 2010). In fact, serum BChE has been used as an indicator of hepatic, renal, and thyroid diseases and as a marker for pesticide toxicity. It is therefore important to understand how the levels and distribution of the ChEs are regulated. Such knowledge may have therapeutic consequences for the treatment of diseases modulated by ChE levels. However, little attention has been paid to the metabolic, and particularly to the hormonal, regulation of BChE or of AChE. Several lines of evidence suggest that such regulation may exist. Thus, rat serum BChE was shown to be under endocrine regulation by gonadal steroids and by growth hormone (GH) (Illsley and Lamartiniere, 1981; Lamartiniere, 1986). Furthermore, systematic changes in AChE/BChE ratios have been established during normal development. Thus, whereas AChE activity in rat muscle increased concomitantly with decrease in BChE, resulting in an inversion in ratio of the amounts of the two enzymes in adult muscle (Berman et al., 1987), an opposite change was observed in mouse serum, leading to a high BChE/AChE ratio post-puberty (Oliver et al., 1992). However, this was not the case for the serum of the *mdx* dystrophin-deficient mutant mouse, an animal model for Duchenne muscular dystrophy (DMD). In the *mdx* mouse, as in DMD patients, a point mutation in the *dystrophin* gene results in synthesis of a non-functional truncated protein, so that full-length dystrophin is absent from skeletal and cardiac muscles, as well as from certain neural cell populations in the

brain (Sicinski et al., 1989; for a review see Muntoni et al., 2003), with devastating, and eventually fatal, consequences in humans. In *mdx* sera, AChE activity did not decline with the onset of puberty as in normal mice sera, but stayed at a high level (Oliver et al., 1992), which might indicate impaired hormonal regulation. In the present study we found not only that AChE activity was elevated but that BChE was drastically diminished in the *mdx* sera. We went on to show that in normal mouse serum BChE levels are subject to endocrine regulation by the androgen, testosterone (T) in male mice, whereas AChE levels are not influenced. We further examined whether the systematic differences in the levels of both ChEs in *mdx* sera were due to impaired endocrine regulation, and if hormonal manipulation can restore the AChE/BChE ratio to that in normal mouse serum.

MATERIALS AND METHODS

ANIMALS

Male *mdx* and control (*wt*) mice from the same mouse strain (C57BL/10ScSn-D^{mdx} and C57BL10Sn, Jackson Laboratory, Bar Harbor, ME) were bred in our animal facility. Animals were maintained and treated according to an approved protocol of the Ethics Committee (IACUC) of the Hebrew University Medical School. The Hebrew University is an AAALAC internationally accredited institute. All mice were maintained under a controlled photoperiod (lights on 07:00–19:00). Food and water were provided *ad libitum*. Body weights were monitored.

SURGICAL MANIPULATIONS

Bilateral orchidectomy

Exteriorized testes and epididymis of 19–21 week mice were removed under anaesthesia (0.45 mg ketamine/0.05 mg xylazine per ml saline solution, 0.9 ml/mouse, i.p.). Briefly, the spermatic cord was clamped with a hemostat. Then, the distal portions of the vas deferens and spermatic artery were severed. Once homeostasis had been achieved (∼5 min), the spermatic cord was unclamped, and the incision was closed. All control animals underwent sham surgery.

Testosterone implants

This procedure was kindly provided by Dr. Robert A. Steiner (U. Washington, Seattle, WA). Immediately after orchidectomy (or sham surgery), testosterone capsules (T, 20–25 mg/pellet), were implanted subcutaneously (about halfway down the mouse's back) via a small incision at the base of the neck. Then the skin was sutured and the animal was allowed to wake up. The T pellet was designed to achieve normal physiological levels. The dose was chosen based on previous research, which established that it would produce significant androgenic actions at target tissues, i.e., spermatogenesis in gonadotropin-deficient mice (Singh et al., 1995; Lindzey et al., 1998). All untreated animals received empty (sham) capsules.

COLLECTION OF BLOOD AND SEPARATION OF PLASMA

Submandibular vein withdrawal for ChE measurements

Blood samples for the measurement of circulating ChE concentrations were collected *via* heparinized capillaries from punctured submandibular vein of lightly sedated mice (ketamine hydrochloride/xylazine, ∼0.1 ml/mouse, i.p.). Blood was drawn during the period of 10:00–12:00 am from control and castrated animals of age ∼21 weeks. It should be noted that prior to blood collection, all mice were acclimated for 2 weeks under a new controlled photoperiod (lights on 09:00–21:00).

Orbital sinus withdrawal for creatine kinase measurements

Creatine kinase (CK) levels in circulation were used as a convenient index to confirm the genotype of the animals (see below). Animals were anesthetized (chloral hydrate solution, 0.05 g/ml, 0.1 ml/10 g body weight, i.p.) and 0.5 ml of blood was drawn from the orbital sinus *via* heparinized capillaries.

Sera

After blood collection, samples were allowed to clot for 30 min at room temperature (RT), and then centrifuged (Eppendorf 5415 at 1250 rpm, 15 min, RT). The separated plasma was stored at −80°C. Sera from male rhesus macaque were a generous gift from Dr. Tony Plant (U. Penn., Pittsburgh, PA) and bovine sera were kindly donated by Dr. Joel Zeron (SION A.I. Center & Breeding Ltd., Shikmim, Israel).

DETERMINATION OF ChE ACTIVITIES IN SERA

Spectrophotometric method

ChE activity was measured in microplates by the colorimetric method of Ellman et al. (1961) in 1.0 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in 0.1 M Tris, pH 7.6, containing 0.5 mM acetylthiocholine iodide (ATCh) or butyrylthiocholine iodide (BTCh) (Sigma, St. Louis, MO). Selective inhibitors were used to distinguish between AChE and BChE as described below. The increase in absorbance at 412 nm was followed for 12–15 min at 31–34°C using a PowerWave$_x$340 Model microplate reader (Bio-Tek Instruments, Inc, Winooski, VT). Activities were expressed as ΔOD units per minute.

Radiometric method

ChE activity was assayed by a radiometric method, monitoring the ^{3}H-acetate generated during acetylcholine hydrolysis (Johnson and Russell, 1975). This method is of higher sensitivity than the Ellman's technique (see above) thus allowing for better resolution when measurement of variations in the low AChE activities of mouse sera was required. Briefly, samples were incubated in 0.1 ml of 1 mM of ^{3}H-acetylcholine iodide (^{3}H-ACh, PerkinElmer Life Sciences) in 0.1 M NaCl and 0.01 M Tris buffer (pH 7.4). The reaction was stopped as required by addition of 0.1 ml 1 M chloroacetic acid/0.5 M NaOH/2 M NaCl, followed by addition of 4 ml scintillation fluid composed of 90% QuickSafe N (Zinsser Analytic, Frankfurt, Germany) and 10% isoamyl alcohol. The reaction product, [^{3}H]-acetate, was extracted into the organic phase and counted in a Tri-CARB 2900TR liquid scintillation counter (Packard BioScience, Meriden, CT). Selective inhibitors were used to distinguish AChE and BChE activities (see below).

Selective ChE inhibitors

Tetra-isopropyl-pyrophosphoramide (0.1 mM, iso-OMPA, Sigma) and 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one

dibromide (0.01 mM, BW284c51, Burroughs Wellcome Co.) were used to selectively inhibit BChE and AChE activities, respectively (Austin and Berry, 1953). Samples were pre-incubated with either inhibitor for 20 min at RT and then assayed for ChE activity in the presence of the inhibitors using either the colorimetric or radiometric method.

DETERMINATION OF CREATINE KINASE (CK) ACTIVITY

Circulating CK values, expressed as units of enzyme activity per liter of serum sample (U/L), were used as an index to identify the genotype of the animals (*mdx* or *wt*). CK values in blood samples drawn from orbital sinuses were determined using a standard enzymatic method and an automated clinical analyzer.

STATISTICAL ANALYSES

Data were collected for samples from individual animals in all experiments. Mean values were obtained by averaging measurements obtained for a group of 4–10 animals for a particular experimental day (as indicated in each experiment). The significance of differences between groups was determined by One-Way ANOVA (parametric with Tukey's multiple comparison post-test) for all groups, or by Kruskal–Wallis (non-parametric) tests, depending whether or not the data showed normal Gaussian distribution. Comparison between two groups was by non-paired *t*-test with unequal variances (our usual case). All tests were available in the GraphPad Prism4 (GraphPad Inc.), Statistica10 (StatSoft) and Microsoft Office Excel Professional Software. The difference between groups was considered significant when ($p < 0.05$).

RESULTS

POST-PUBERTAL EFFECTS OF DYSTROPHY ON BODY WEIGHT

Although the *mdx* mouse was reproductively competent (thus, T was present and functional), its postnatal growth was hindered compared to that of normal controls. The *mdx* mice displayed lower and more variable body weight compared to the controls, with the phenomenon being more pronounced in *mdx* females than in males (not shown). However, the body weight stabilized by 11 weeks of age at levels not significantly different from those of the control mice (**Figure 1**). The oscillatory shape of the curve was consistent with the finding that *mdx* mouse muscles undergo cycles of degeneration and regeneration until most muscles stabilize by 10 weeks (Settles et al., 1996). The mice we used were therefore aged 19–22 weeks, at which age their body weights are stable, and the majority of muscle fibers no longer display transient degeneration (Karpati et al., 1988).

SERUM ChE ACTIVITIES IN NORMAL AND DYSTROPHIC MICE

Mouse serum contains BChE and AChE, both of which hydrolyze ATCh. Total ChE activities in sera of male *mdx* mice were at least 15% ($p < 0.01$) below those in normal mice sera (**Figure 2A**), indicating reduced levels of AChE and/or BChE in the *mdx* sera. AChE activity, which was found to be very low in sera of normal mice, was significantly elevated in the *mdx* sera (**Figure 2A**, detailed below). In contrast, BChE levels, assayed on BTCh, were found to decrease in male *mdx*-sera to 64–67% of the levels in *wt* sera (**Figure 2B**, $p < 0.002$). Similar results were observed when BChE was assayed on ATCh, with AChE activity being

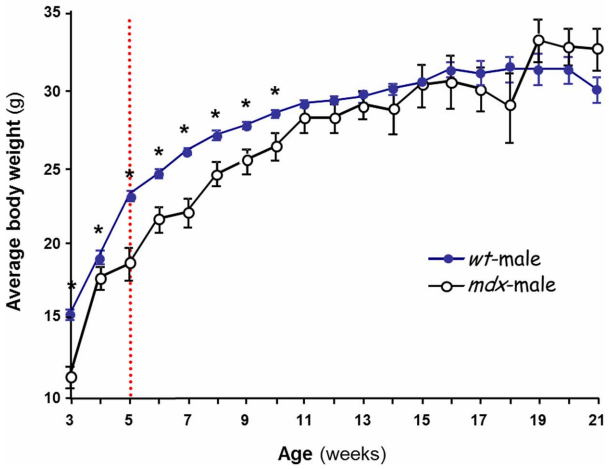

FIGURE 1 | Postnatal changes in body weight in male mice. The effect of strain on body weight is shown. Several litters of *C57BL/10J* (*wt*, •) and *mdx* (○) *male* mice were weighed over 3–21 weeks. Each data point is the mean ± SEM ($n = 6$–34). The dashed line at ~5 weeks marks initiation of puberty in the *wt*. *$p < 0.05$ between *wt* and *mdx* of the same age, showing that *mdx* mice gain less weight than *wt* during postnatal development but reach normal level by 11 weeks.

selectively inhibited by BW284c51 (**Figure 2A**). Since AChE levels in normal mouse serum are very low, the more sensitive radiometric assay was used to confirm the results obtained using the colorimetric Ellman procedure (**Figures 2C** and **D**), showing that AChE activity in *mdx*-sera indeed increased by 50–80% over the level in control *wt* sera ($p < 0.05$), confirming an earlier report (Oliver et al., 1992). BChE activity was shown, also by the radiometric assay, to decrease in *mdx*-sera to 61–76% of *wt* values (**Figure 2D**, $p < 0.001$). Since BChE is the major ChE form in mouse serum (>70%), the large drop in BChE was responsible for the overall reduction in total ChE activity observed in *mdx* sera. However, the decrease in BChE levels, taken together with the increase in AChE levels, in *mdx* sera (as shown in **Figures 2C** and **D**), reduced the BChE/AChE ratio from 2.3 ± 0.2 in the *wt*-sera to 1.3 ± 0.5 in *mdx*-sera. Similar results were found for adult female *mdx*-mice and are summarized elsewhere (Anglister et al., 2008).

As an internal control, CK levels in sera were measured to validate the genotype of the animals (*mdx* compared to *wt*). Consistent with the 3–14-fold elevation reported by Glesby et al. (1988), the male *mdx* mice used in our studies showed variable, but consistently increased, CK levels in sera, *viz*., 9.9 ± 1.4 ($\times 10^3$ U/L, mean ± SEM) in *mdx* mice, compared to 1.3 ± 0.3 in controls (**Figure 2E**). These differences were highly significant ($p < 0.001$).

ENDOCRINE REGULATION OF NORMAL MOUSE SERUM BChE ACTIVITY

To determine whether the abnormal BChE/AChE ratio in *mdx* sera is due to hormonal influences, we first investigated regulation by the androgen, T, of ChEs in the sera of adult male *wt* mice.

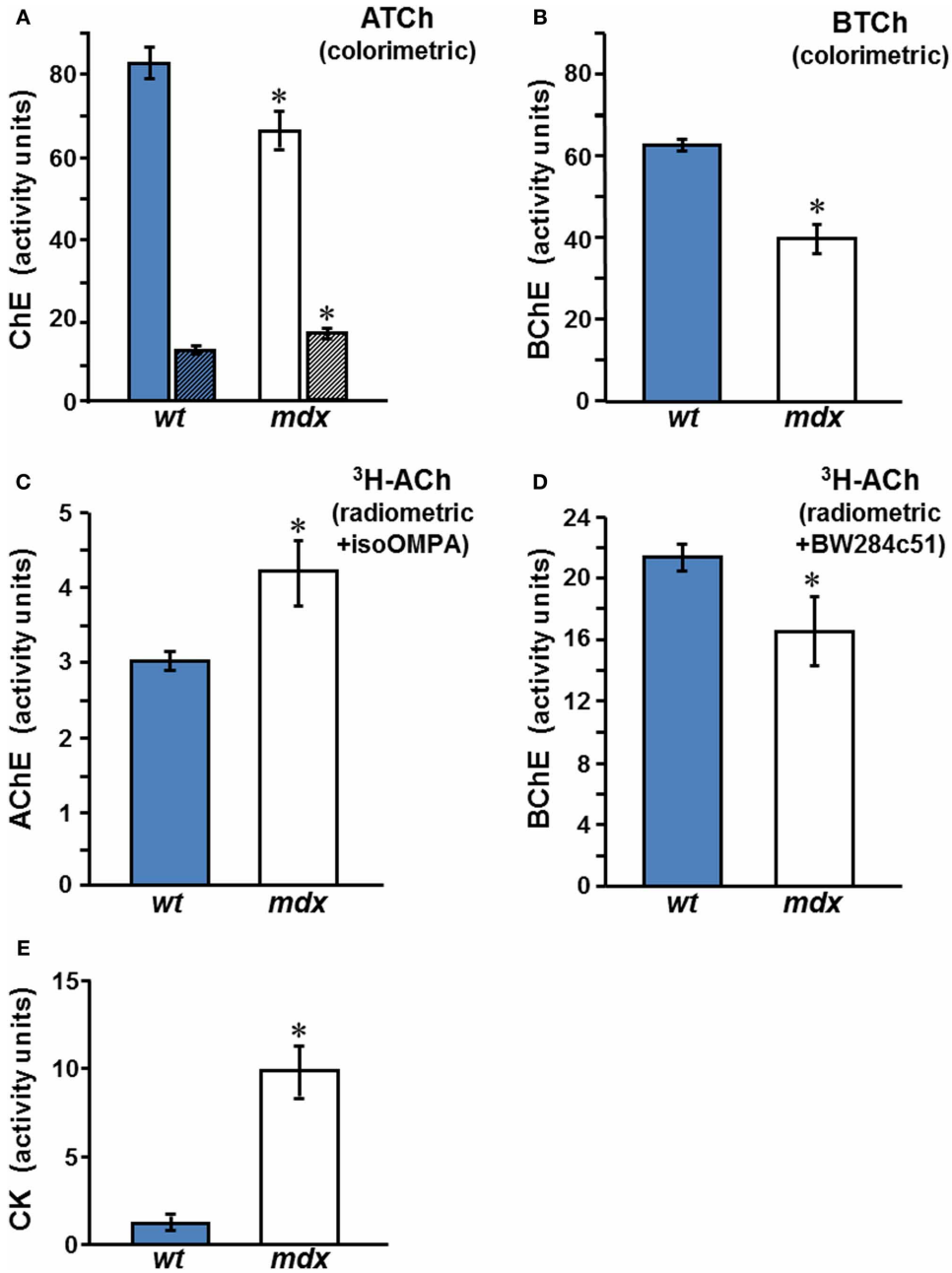

FIGURE 2 | Circulating ChE activities in *wt* and *mdx* mice. ChE activities in the sera of adult male *wt* (*C57Bl/10J*) ■ and *mdx* □ mice were assayed by: **(A,B)** Measurement using the Ellman colorimetric method, with ATCh as substrate and the selective inhibitors, iso-OMPA, for measurement of AChE (striped columns), or BW284c51, for measurement of BChE (plain columns) **(A)**; BChE activity was also measured using BTCh **(B)**; **(C,D)** The radiometric method with ^{3}H-ACh as substrate and with iso-OMPA for AChE **(C)**, or with BW284c51 for BChE **(D)**. **(E)** CK levels in the sera of *wt* and *mdx* mice. Activity values represent the mean ± SEM, of samples from 6 to 8 mice per group for ChEs and of 12 animals per group for CK. Activities in *mdx* sera differed from *wt* significantly: The BChE level in *mdx* sera was reduced compared to *wt* ($^*p < 0.01$ in **B** and < 0.05 in **D**), while AChE in *mdx* sera was elevated compared with *wt* ($^*p < 0.05$ in **A,C**). As expected, CK levels in *mdx* sera were elevated, compared to those in the *wt* sera (**E**, $^*p < 0.001$).

Sera from orchidectomized 21 week adult male *wt* mice were analyzed for ChE activities (**Figure 3**). Gonadectomy raised BChE activities in adult male mice serum by 20–37% ($p < 0.001$), whether measured on BTCh, or on ACh in the presence of a selective AChE inhibitor (**Figures 3A** and **B**, respectively).

T-replacement in adult *wt* males, performed concomitantly with castration, abolished the increase caused by castration, restoring BChE activity to the levels observed in the sera of intact males (**Figures 3A** and **B**). Thus, a high basal level of BChE activity is found in the sera of agonadal males (in the

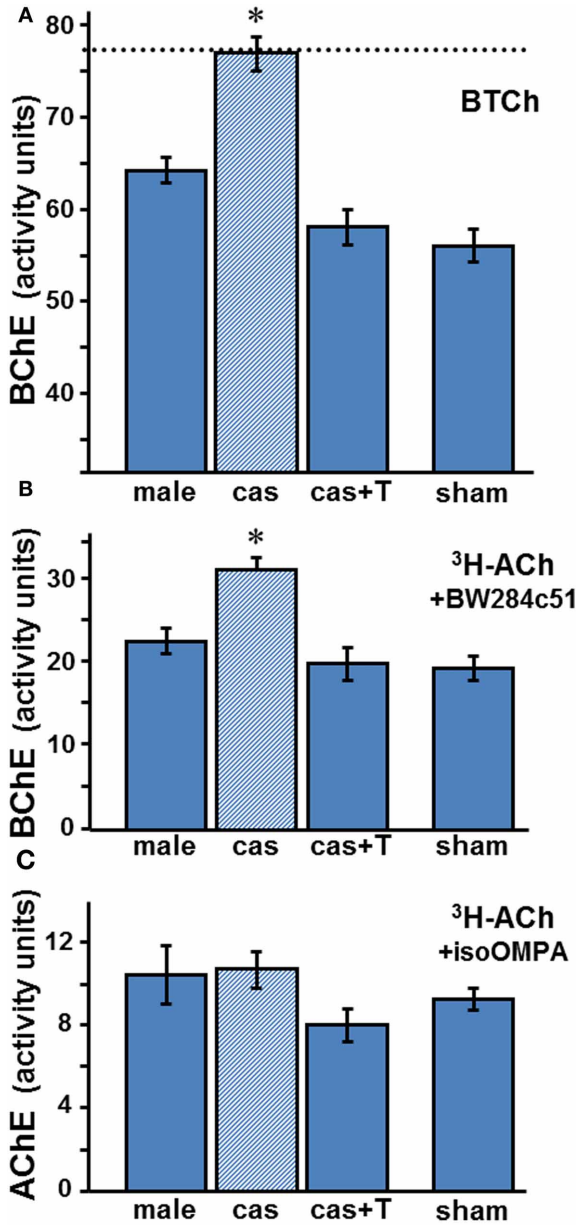

FIGURE 3 | Effect of orchidectomy and T-replacement appears to be on BChE, not on AChE. Activities in *wt* sera of BChE **(A,B)** and AChE **(C)** were determined colorimetrically **(A)** or radiometrically **(B,C)** with appropriate substrates and selective inhibitors as described in the legend to **Figure 2**. Values are the mean ± SEM for 6–8 samples per group (sham group, $n = 3$). BChE levels in gonadectomized animals (dashed line in **A**) differ significantly from those in intact, T-treated and sham animals: Data were analyzed by One-Way ANOVA, showing statistical significance in **A** ($p \ll 0.0002$) and in **B** ($p \ll 0.0001$), with Tukey's *post-hoc* revealing significant differences (∗) between castrated male (cas, shaded column) and the other three groups: intact male (male) ($p < 0.0003$), castrated with T-replacement (cas + T) ($p < 0.0002$) or sham ($p < 0.0002$). All other comparisons showed insignificance ($p > 0.05$): male compared to cas + T ($p = 0.086$), or to sham ($p = 0.07$), and cas + T compared to sham ($p = 0.92$). Similarly, *post-hoc* test in **B** showed significant difference between cas and the other groups: male ($p < 0.001$), cas + T ($p < 0.0004$), and sham ($p < 0.001$), which were very similar. No significant changes were observed in AChE activity **(C)**.

Table 1 | Effect of gonadectomy on serum BChE and AChE levels in adult male *wt* mice.

wild-type	*n*	BChE	AChE	BChE:AChE
No surgery	5	1830–2350	790–1010	2.3 ± 0.2
Castrate	8	2770–3870	730–1460	3.0 ± 0.1
Castrate + T	5	1570–2550	530–980	2.5 ± 0.2
Sham	3	1660–2130	870–1040	2.1 ± 0.1

*The radiometric data presented in this table (in dpm units) are averaged and presented as histograms in **Figures 3B** and **C**.*

absence of T), and both endogenous and exogenous androgen down-regulate the enzyme.

Whereas castration of the male *wt* markedly raised the BChE level in the serum, that of AChE (measured with ACh in the presence of iso-OMPA) did not change significantly (**Figure 3C**; $p = 0.41$). The ranges of values recorded radiometrically, with ACh and the respective selective inhibitors of BChE and AChE, in sera of male adult *wt* mice under the different treatment regimes, are shown in **Table 1**. The data show clearly that BChE activities were increased from a low level (1830–2350 arbitrary assay units) to a high level (2770–3870 units) after orchidectomy, and reverted to the base level after T-implant. In contrast, the AChE activities observed in adult male mice sera fell in a similar range (530–1460 arbitrary units), whether the mice were intact or castrated, with or without T-implant. These results indicate a specific and selective negative androgen modulation of BChE, but not of AChE. Furthermore, the large increase in BChE after castration resulted in an increased ratio of BChE to AChE activities from 2.3 ± 0.2 in sera of intact *wt* males to 3.0 ± 0.1 in castrated *wt* males (**Table 1**).

EFFECTS OF ORCHIDECTOMY AND T-REPLACEMENT ON SERUM BChE LEVELS IN ADULT *mdx* MICE

We went on to examine whether orchidectomy raises BChE levels also in the sera of *mdx* mice, and whether, as a consequence, the high agonadal baseline ChE levels seen in *wt* mice sera can be reached. The data presented in **Table 2** and **Figure 4** reveal low levels of BChE in the sera of control adult *mdx* males (32.2–48.6 assay units). Although levels increase substantially after gonadectomy (48.7–61.9 assay units), by an average of 35%, the BChE deficiency observed in untreated *mdx* mice is sustained

Table 2 | Effect of gonadectomy in elevating serum BChE activity in normal and dystrophic mice.

	wt	*mdx*	*p*
No surgery	56.1–68.2 (8)	32.2–48.6 (5)	<0.002
Castrate	68.0–83.3 (6)	48.7–61.9 (4)	<0.002
Castrate + T	50.1–62.3 (6)	34.0–46.2 (5)	<0.001
Sham	51.6–57.8 (3)	40.8–44.8 (3)	<0.006

*Activities relate to the same animals and assay conditions as for the data displayed in **Figure 4**.*

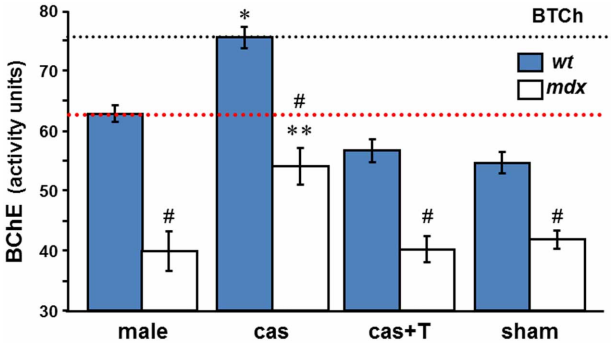

FIGURE 4 | T-regulation of BChE activity in male *mdx* sera. BChE activities were measured colorimetrically with BTCh as substrate in sera of intact, castrated, castrated with T-replacement, and sham operated *wt* ■ and *mdx* □ mice, (as in **Figure 3A**). Values, mean ± SEM for samples taken from 4 to 8 mice per group (sham group, $n = 3$). *$p < 0.001$ between BChE levels in castrated *wt* and in intact, T-treated, and sham mice sera (of either *wt* or *mdx* strains). **$p < 0.05$ (detailed in **Table 3**) between BChE levels in castrated *mdx* and in intact, T-treated, and sham *mdx* mice sera. #Significant difference between BChE level in *mdx* and *wt* in all the experimental groups (*p*-values are listed in **Table 2**). The dashed black and red lines indicate BChE levels in agonadal and gonadal *wt* sera, respectively.

(**Figure 4**): thus, the agonadal "baseline" level in *mdx* mice was significantly below the baseline level in *wt* agonadal mice (29%, $p < 0.002$). It was slightly lower than the level in control *wt* mice (14%, $p = 0.06$) or in sham-operated *wt* mice. As in *wt* mice, T-replacement reversed the effect of orchidectomy on BChE activity. Thus, after orchidectomy, the BChE in *mdx* circulation may almost reach the gonadal baseline level of BChE activity in the serum of intact male *wt* mice, but not the agonadal baseline level in the serum of castrated *wt* mice.

T-REGULATION OF SERUM AChE ACTIVITIES IN mdx MICE

We went on to examine the effects of orchidectomy and T-replacement on AChE levels in the sera of *mdx* mice. Specifically, we checked whether orchidectomy of *mdx* mice, with or without T-implant, affected serum levels of AChE. As documented in **Table 3**, the mean AChE activity in the sera of intact *mdx* mice was 1260 ± 120 units, as compared to 790 ± 70 units in the castrated mice. Despite the great variability in AChE levels, castration appears to reduce AChE activity by >30%, $p < 0.02$). Interestingly, the mean value for agonadal *mdx* mice was not different from that for gonadal *wt* mice ($p = 0.2$).

T-implant into agonadal *mdx* mice resulted in a mean serum AChE activity of 930 ± 180, not significantly different from the value for agonadal *mdx* mice lacking the T-implant ($p = 0.40$). This value is also very similar to the level in the gonadal *wt* mice ($p = 0.89$), but lower than the average value for gonadal *mdx* mice, although a statistically significant difference could not be demonstrated due to the large standard errors within the two groups, resulting in $p = 0.11$. Furthermore, the values obtained for the small group of "sham" *mdx* mice analyzed, were intermediate, not being significantly different from either the values for sera of intact *mdx* and intact *wt* mice ($p = 0.34$ and 0.39, respectively). These limitations prevent us from drawing definite conclusions concerning the effect of orchidectomy on serum AChE in *mdx* mice, although orchidectomy decreases AChE levels. However, the large increase in BChE levels, coupled with the apparent decrease in AChE levels, in the agondal *mdx* mice, results in an increase in the BChE/AChE activity ratio from 1.2 ± 0.5 in the male *mdx*-sera to 2.5 ± 0.4 after castration (**Table 3**), a ratio similar to that in *wt*-sera (2.3 ± 0.2, **Tables 1** and **3**).

SPECIES-SPECIFIC AND DEVELOPMENTAL VARIATIONS IN CIRCULATING ChEs

Fetal calf serum is known to be a good source of AChE (Ralston et al., 1985). It occurred to us that bovine serum might serve as a good system for testing postnatal down-regulation of serum AChE, and for examining, in parallel, the regulation of BChE, assuming BChE to be a serum component common to all species. We therefore compared ChE activities in fetal bovine sera and in post-pubertal (adult) bull sera. Consistent with the literature, we observed a high level of AChE activity, >96% of total ChE, in fetal bovine sera. Interestingly, AChE activity was >10-fold lower in adult bull sera (**Table 4**). However, whereas in adult mouse serum, BChE was the major ChE component, as in several

Table 3 | Changes in BChE and AChE activities in the sera of adult *wt* and *mdx* adult male mice before and after orchidectomy, and after T-implant.

	wt	*mdx*			
	Male	**Male**	**Castrate**	**Castrate + T**	**Sham**
Total ChEs	3700 ± 100**	3000 ± 90	3340 ± 220	2880 ± 120	2940 ± 270
BChE	2140 ± 90	1440 ± 70	1970 ± 110*	1540 ± 130	1350 ± 90
AChE	910 ± 40	1260 ± 120#	790 ± 70	920 ± 140	1070 ± 140
BChE/AChE	**2.3 ± 0.2**	**1.2 ± 0.5**	**2.5 ± 0.4**	**1.7 ± 0.3**	**1.3 ± 0.2**

*Data shown refer to the same animals as those used in **Figure 4**.*

**BChE in castrated mdx sera is significantly elevated compared with mdx male ($p < 0.02$), castrated with T implant ($p < 0.05$) and sham sera (<0.01).*

***BChE in wt male sera is significantly higher than in mdx male ($p < 0.001$), castrated with T implant ($p < 0.006$) and sham sera ($p < 0.001$).*

#AChE in mdx male serum is significantly higher than in castrated mdx ($p < 0.02$) and in wt male sera ($p < 0.05$).

Table 4 | Species-specific variations in circulating ChE activities.

	Bovine		Macaque	Mouse	
	Fetal	**Adult**	**Rhesus**	***wt (C57BL)***	***mdx***
Total ChE (no inhibitor)	5020	470	2240	3500 ± 210	3260 ± 270
BChE (+BW)	190	100	1990	2140 ± 90	1650 ± 220
AChE (+iso-OMPA)	4830	370	250	910 ± 40	1260 ± 130
BuChE/AChE	**0.04**	**0.27**	**3.9 ± 0.4**	**2.3 ± 0.2**	**1.3 ± 0.5**
AChE U/mL*	1.2–1.7	0.11	0.081	0.24	0.35

*All sera were from male animals. ChE activities were determined by radiometric assay with ACh and the respective inhibitors, and presented as units in 125 μL serum. Macaque and bovine values were obtained from pooled sera of three animals each. The values observed for bovine sera were confirmed by Drs. B. P. Doctor and H. Saxena (Walter Reed Army Institute of Research, Silver Spring, MD, pers. communication). *AChE U/mL values represent activities calculated in enzyme units (U = μmoles ACh hydrolyzed/min).*

other species (Ecobichon and Corneau, 1973; Chatonnet and Lockridge, 1989), in bovine sera it was the minor ChE component, with very low activity (**Table 4**). Although BChE activity in fetal and adult bovine sera was similar, its fraction of the total ChE activity rose from fetal to adult (∼4–25%) due to the big drop in AChE activity. Because of the striking difference in ChE composition of mouse and bovine sera, we decided to examine the ChE composition of the sera of rhesus (RHS) monkeys, which are closely related to humans and for which many endocrinological studies have been performed, including examination of the effects of androgens. As seen in **Table 4**, in adult RHS serum, as in mouse serum, BChE is the major component (89%).

DISCUSSION

In the present study we explored the regulation of serum ChEs in normal and dystrophic mice. Several findings were made: (1) While there is more BChE than AChE activity in mouse serum, as in the sera of several other mammals (reviewed by Chatonnet and Lockridge, 1989), the value of the BChE/AChE ratio for the sera of *mdx* mice is about half of the ratio for the sera of *wt* mice; (2) While AChE levels increased slightly in the *mdx* sera (Oliver et al., 1992), BChE levels decreased by >30%, resulting in an overall decrease in ChE activity of ∼20% in *mdx* as compared to *wt* sera; (3) Although orchidectomy elevated levels of BChE activity in the sera of both strains, levels in *mdx* sera remained lower than in *wt* sera; (4) T-replacement reversed the effect of orchidectomy on BChE activity in both *wt* and *mdx* sera; (5) T-modulation was specific to serum BChE, not significantly affecting levels of AChE; (6) Orchidectomy did not increase AChE activities in either strain. These findings are consistent with involvement of the hypothalamic-hypophysial-gonadal (H-P-G) axis in the impairment of BChE activity in *mdx* sera.

The regulation of mouse serum ChEs, either AChE or BChE, via the (H-P-G) axis, has not been previously explored. Our results demonstrate that testosterone (T) down-regulates the expression of BChE in sera of mice, confirming the early observations on male rat sera that the total ChE content was reduced by circulating T (or increased by estrogen in female), not as a result of a direct interaction of the steroid with the enzyme but via higher level regulation (Everett and Sawyer, 1946; Sawyer and Everett, 1946, 1947; Illsley and Lamartiniere, 1981).

HOW CAN T MODULATE LEVELS OF SERUM BChE?

The target of testosterone (or estrogen) that produces modulation of serum BChE levels is not known. It may be in the hypothalamus, in the pituitary, or perhaps in a target organ that produces serum BChE, such as the liver (Leeuwin, 1965), together with pituitary factors. Experiments on hypophysectomized rats have demonstrated that pituitary mediation is required for both androgen and estrogen action (Everett and Sawyer, 1946; Illsley and Lamartiniere, 1981): while sex steroids do modulate ChE activity from a basal level, either due to suppression by androgen in males, or due to stimulation by estrogen in females, their action is abolished by hypophysectomy. It has also been shown in rats that hypophysectomy (Illsley and Lamartiniere, 1981; Edwards and Brimijoin, 1983) or lesion of the arcuate nucleus in the hypothalamus of males (Lamartiniere, 1986) reduces levels of GH and increases levels of ChE in the serum. The effects on serum ChEs were shown to be reversed by exogenous administration of GH after either type of surgery. Thus, both T and GH appear to be negative modulators of rat serum BChE in male rats. Moreover, they can act synergistically, since their levels are inter-related: the hypothalamus regulates the release of pituitary hormones (e.g., GH) and gonadotropins that, in turn, drive the gonads to produce T and estrogen, *viz.*, the H-P-G axis. In addition, T itself influences GH secretion, and GH in turn reduces serum BChE levels; thus, T can also reinforce down-regulation of BChE via GH.

WHERE CAN T AND GH EXERT THEIR INFLUENCES ON SERUM BChE?

Serum BChE in adults is apparently produced in the liver (Silver, 1974). Indeed, GH has been shown to modulate several sex-differentiated hepatic enzymes, including serum ChE (Lamartiniere, 1981, 1986). Since hypophysectomy abolishes rat liver estrogen receptors and GH restores them, GH may also restore responsiveness of the liver to circulating sex steroids, and result in androgen or estrogen modulation of serum ChE. A similar mechanism could apply to GH regulation of other potential target organ for serum BChE production. In considering the liver as the source for serum BChE, it should be taken into account that, the liver secretes BChE monomers and

dimers (Perelman et al., 1990), not the G_4 tetramer which is the major isoform in serum (Lockridge et al., 1979). Furthermore, we have preliminary evidence that T, castration, and T-replacement after castration, alter predominantly the level of G_4 BChE in mouse serum. Thus, it appears that the BChE isoforms produced/secreted by the liver are not the isoform present in serum and modulated by the hormones. This unresolved issue, as well as the influence of T and/or GH on BChE isoforms in serum, liver, and other tissues need to be revisited and further investigated.

IS H-P-G AXIS (AND GH) CONTROL OF SERUM BChE IMPAIRED IN *mdx* MICE?

It became apparent in this study that the *mdx* mice, that lacked dystrophin, had less BChE in their sera (both before and after orchidectomy), relative to *wt* controls. Since T and GH have both been found to be negative modulators of BChE, it is possible that the intact *mdx* mice secrete higher levels of T and/or GH than the *wt* mice. Consistent with this suggestion, *mdx* mice exhibit hypertrophy of somatotroph cells in the pituitary, and slightly elevated pituitary GH in adult females (Anderson et al., 1994). The reduced levels of BChE we show in castrated *mdx* mice compared to castrated *wt* are also likely to be due to gender-specific effects of GH at the hypothalamic-pituitary level, and not to a direct effect of T, since castration has eliminated T from the system. Further studies will be needed to determine whether the perturbation is at the pituitary or hypothalamic level.

Further correlation of GH levels with dystrophin deficiency comes from studies on DMD patients that reported mitigation of the severity of the dystrophy in GH-deficient patients (Zatz et al., 1981a,b), or after treatment with a GH inhibitor (Zatz et al., 1986). Another report provided some evidence for an altered hypothalamic-hypophyseal axis (Zaccaria et al., 1989). This is consistent with the observation referred to above, of high GH levels in *mdx* sera (Anderson et al., 1994). In addition, insulin growth factor-1 (IGF-1), which is secreted in correlation with GH, is elevated in *mdx* plasma (De Luca et al., 1999). Altogether, dystrophin deficiency is likely to be associated with impaired H-P-G-regulation.

REMOVAL OF GONADAL CONTROL NORMALIZES THE BChE/AChE RATIO IN MALE *mdx* SERA

Consistent with Oliver et al. (1992), it became apparent in this study that *mdx* mice had more AChE in their sera (prior to orchidectomy), than *wt* controls. Elevated levels of serum AChE were also reported for dystrophic chickens (Lyles et al., 1980) and for other animal dystrophies (Skau, 1985), as well as for fetal calf serum (Ralston et al., 1985). Oliver et al. (1992) proposed that the increased AChE levels in the sera of the dystrophic mice reflect increased release of muscle AChE relative to release in *wt* controls. Our finding that post-pubertal castration of adult *mdx* males normalizes their serum ChE levels by elevating BChE and, perhaps reducing AChE, and thus normalizing the BChE:AChE ratio, suggests that the *mdx* sera BChE levels are not just a reflection of muscle degradation but are under control of the gonads and of the aforementioned higher centers. Moreover, in addition to T, there is likely to be an additional factor involved in the impaired down-regulation of AChE and up-regulation of BChE in *mdx* sera. This seems likely since although the BChE level increased after castration, it did not reach the agonadal *wt* level, and T-replacement did not reverse the effect on *mdx* serum AChE. Before one can extrapolate from our results in mice to human patients, one has to prove that gonadal regulation of BChE occurs in higher primates.

POSTNATAL MATURATION OF SERUM BChE/AChE RATIO

Because, in *mdx* sera, AChE failed to decrease upon maturation and BChE was low in the adult, we considered the possibility that *mdx* mice are locked in a pre-adult stage and retain some embryonic regulatory properties. It was shown in mouse sera that in the embryo AChE predominates, while in the adult BChE is the major ChE species (Oliver et al., 1992). Surprisingly, we report here strong postnatal down-regulation of AChE in bovine serum, with only small amounts of BChE in both fetal and adult serum relative to other species (**Table 4**). However, we found BChE to be the major ChE component in sera of adult normal *wt* mice and rhesus monkeys (**Table 4**), as has been reported for many animals (Ecobichon and Corneau, 1973; Chatonnet and Lockridge, 1989). Indeed, while human 12th week fetal serum may contain 40% AChE (Hahn et al., 1993), adult human plasma contains >99% BChE (Brimijoin and Hammond, 1988). Higher primates, such as the rhesus macaque, should provide an appropriate model for further studies on the regulation of both AChE and BChE in human serum.

While in the present study we have shown that the AChE in mouse serum is not regulated by circulating T, it should be noted as mentioned, that AChE decreased ∼2-fold in the serum of normal mice, prior to puberty. During that time, a progressive increase in AChE coincident with a reduction in BChE occurred in muscle resulting in inversion in the amounts of the two enzymes in adult muscle (Berman et al., 1987). Thus it may still be that the decrease in serum AChE and increase in muscle AChE are due to one or more postnatal triggers, e.g., humoral factor(s) discharged before the episodic T release at puberty (∼5 weeks). In this context, it should be noted that BChE precedes AChE expression in embryonic neural development of birds (Layer, 1991; Layer and Willbold, 1994) and mammals (Koenigsberger et al., 1998).

One needs to bear in mind, considering the various roles attributed to ChEs in development, during normal function and in pathological conditions, that modulation of their levels may both reflect and influence normal processes or existing impairments. In the context of the present study, the abnormal BChE/AChE ratio in the serum of dystrophic mice, as compared to normal, may be developed as a tool for easy detection of dystrophy, provided similar observations are made for DMD or for other human dystrophies.

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Early appearance and possible functions of non-neuromuscular cholinesterase activities

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The biological function of the cholinesterase (ChE) enzymes has been studied since the beginning of the twentieth century. Acetylcholinesterase plays a key role in the modulation of neuromuscular impulse transmission in vertebrates, while in invertebrates pseudo cholinesterases are preeminently represented. During the last 40 years, awareness of the role of ChEs role in regulating non-neuromuscular cell-to-cell interactions has been increasing such as the ones occurring during gamete interaction and embryonic development. Moreover, ChE activities are responsible for other relevant biological events, including regulation of the balance between cell proliferation and cell death, as well as the modulation of cell adhesion and cell migration. Understanding the mechanisms of the regulation of these events can help us foresee the possible impact of neurotoxic substances on the environmental and human health.

Keywords: acetylcholinesterase, butyrylcholinesterase, cell communication, development, organophosphates, pre-nervous activity

INTRODUCTION

Neurotransmission systems are formed by sets of molecules which cooperate for the efficiency and control of the intercellular communication in both the nervous central (CNS) and peripheral (PNS) systems.

Since the 1970s, reports are available in the literature, suggesting that the highly organized synaptic neurotransmission may derive from evolution of ancestral pre-nervous (Buznikov et al., 1972) and non-nervous cell-to-cell communication systems, present since the prokaryotic cells (Stacy et al., 2011), and eukaryotic protists. In these last unicellular organisms, molecules belonging to the cholinergic, GABAergic and nitric oxide-based systems were found and related to the cell-to-cell communication driving the mating behavior (Delmonte Corrado et al., 1999, 2002). In paramecia, AChE activity was localized at the cell surface and along the ciliary rows (Delmonte Corrado et al., 1999). This activity was also found along the ciliated epithelia of stigmas of ascidian zooids, larval trochi, and generally at the basis of ciliated epithelia.

In addition, the expression and activity of acetylcholinesterase (AChE, E.C. 3.1.1.7) has been found during gametes interaction (Piomboni et al., 2001; Angelini et al., 2004, 2005; Falugi et al., 2008), embryonic development, in temporal and spatial windows related to differentiation and cell migration (Drews, 1975; Fluck et al., 1980; Falugi, 1993).

In addition, AChE can function as a regulator of inflamed tissues thanks to its role of support to apoptosis (Zhang et al., 2002; Zhu et al., 2008) which is the main objective of anti-tumor therapies.

The previously listed functions can be considered pre-nervous because they are based upon the catalytic effect of AChE enzyme on ACh and consequently on the ionic modulation of ACh receptors. On the contrary, the role played by AChE in cell adhesion (reported by Inkson et al., 2004; Bigbee and Sharma, 2004; for human and mammalian cultured cells) and in cell migration, including the elongation of axons toward their target (Giordano et al., 2007) is not exerted through acetylcholine cleavage. Instead, these effects are due to cell-to cell and cell-matrix interactions, through the affinity of membrane-bound AChE molecules to laminin-1, identified previously as an *in vitro* AChE ligand and, to a lesser extent, to collagen IV and to AChE itself (Johnson and Moore, 2007).

Nevertheless, the possible role of the cholinergic system in non-nervous cell-to-cell communication is still controversial. In this review, we will follow the history of cholinergic-related molecules in non-nervous and pre-nervous structures, and will outline the possible relationships between the exposure to cholinesterase inhibitors and developmental anomalies.

THE CHOLINERGIC SIGNALING SYSTEM

The cholinergic cell-to-cell communication is performed by a coordinated set of molecules, formed by the signal molecule, acetylcholine (ACh) and its muscarinic and nicotinic receptors, its biosynthetic enzyme, choline-acetyltransferase (ChAT, E.C. 2.3.1.6), the specific ACh lytic enzyme, acetylcholinesterase (AChE, E.C. 3.1.1.7), and other less specific choline esterases: butyryl-cholinesterase (BChE, E.C. 3.1.1.8) and propionyl-cholinesterase (PChE, E.C. 3.1.1.8), together called pseudocholinesterases (ChE). These are able to cleave ACh (acetylthiocholine iodide) at a minor rate compared to their specific substrates, butyrylthiocholine iodide (BTChI) and propionylthiocholine iodide (PTChI). In the past 20 years their role of implementing or substituting the tasks of AChE when its activity is impaired by inhibitors has been researched (Robitzki

et al., 1998; Geyer et al., 2008; Pezzementi and Chatonnet, 2010). Actually, the pseudo-cholinesterase activities increase in case of stress due to the exposure to cholinomimetic drugs during chick development (Angelini et al., 1998; Aluigi et al., 2005) or to cholinesterase inhibitors in cultured cells possibly as a homeostatic response to AChE activity failure.

In most marine invertebrates, and/or in some tissues of marine invertebrates, PChE activity is preeminent compared to the others (see **Figure 13**). Actually, AChE and BChE are thought to be the result of a gene duplication event early in vertebrate evolution, soon after the appearance of gnatostomes (Massoulié et al., 1993; Pezzementi et al., 2011).

ACETYLCHOLINESTERASE AS A MODULATOR OF THE CHOLINERGIC COMMUNICATION SYSTEM

The enzyme AChE removes ACh from the receptors, in order to make them able to respond to the following impulses. AChE inhibition, such as the one caused by exposure to nerve weapons or to neurotoxic pesticides, would prevent ACh receptors rescue and cause enormous damages, blocking all the functions depending from ACh reception. The magnitude of the effect is due to the amount of blocked receptors, which depends on the body mass of the target organisms. In general, the impairing of AChE activity may generate in specific animal models the increase of central cholinergic tone, inducing the behavioral analogy of depression (Sklan et al., 2004). This implies a correlation between the correct functioning of AChE and human health, including neurodegenerative diseases.

NON-NEUROMUSCULAR ROLES OF THE MOLECULES RELATED TO THE CHOLINERGIC SYSTEM

In the first half of the last century, Youngstrom (1938) reported the presence of the enzyme activity in the embryos of three amphibian species long before the onset of the nervous system. In these cases, ChE activity increases since the 2–4 stage cells up to the beginning of tadpole motility. In *Ambystoma punctatum*, Sawyer (1943) observed the same behavior.

Whittaker et al. (1977) found cholinesterase since the first cleavage in the cells of the muscular genealogy of ascidian embryos. As the ascidian embryos develop according to the mosaic type, such localization has to be considered as a case of chemical differentiation preceding the structural one, rather than a pre-nervous activity, that generally is involved in instructive cell to-cell communication. On the contrary, the epithelial localization of AChE activity in the ascidian embryo and larva, reported by Minganti and Falugi (1980) should be considered an ancestral neuromuscular-like activity, because it is involved in the shrinkage of the tail epithelial cells during metamorphosis, and is enhanced by acetylcholine exposure (Coniglio et al., 1998).

A number of research groups found active ChE localized in extra-neuromuscular sites of different animal embryos, including invertebrate (Drews, 1975), and vertebrate embryos (Drews, 1975; Fluck et al., 1980; Minganti and Falugi, 1980; Minganti et al., 1981), and in developing buds of vegetal organisms (Raineri and Modenesi, 1986, 1988). They suggested that this AChE activity might be related to "embryonic" functions: cell migration (Drews, 1975) and cell-to-cell communication related to positional information exchange, such as the one between apical ectodermal ridge (AER) and mesenchyme during the limb development (Drews, 1975; Falugi and Raineri, 1985). In the limb bud, active AChE diffuses from the AER to the underlying mesenchyme, with focal points in the pre-cartilage cell clusters, and disappears in the core of early cartilage nodules (**Figure 1**).

ChE ACTIVITY IN DEVELOPMENTAL EVENTS

All the instructive signaling in developmental events is mediated by cell-to-cell communication: in particular, molecules related to neurotransmitter systems play a relevant role in modulating messages mediated by ion fluxes or ion intracellular changes. Buznikov and colleagues (Buznikov et al., 1972, 1996; Buznikov, 1980, 1990; Buznikov and Shmukler, 1981; Buznikov et al., 2001) detailed the history of classic neurotransmitter involvement (biogenic amines, acetylcholine, nitric oxide, and GABA) in early developmental events from fertilization and segmentation to organogenesis of highly specialized structures (Buznikov, 1990; Buznikov et al., 1996). They found evidence that developmental changes in this functional activity are involved from the stage of maturing oocytes through neuronal differentiation. This scheme reflects not only the spatial-temporal sequence of these changes, but also the genesis of neurotransmitter functions, from "protosynapses" represented by interacting gametes and cleaving embryos to the development of specialized synapses belonging to the neuromuscular system.

Professor Minganti's group (Minganti et al., 1975, 1981; Minganti and Falugi, 1980) advanced the hypothesis that the embryonic localization of ChE activities might be related to its role in the modulation of intracellular dynamics responsible for cell-to-cell communication. These assumptions were confirmed by experiments on a number of models, including chordates and high vertebrates (Minganti et al., 1981). The demonstration of these functions was achieved only years later using ChE inhibition bioassays, either with specific inhibitors such as BW284c51, iso-OMPA, and physostigmine, (Sigma, IT), or with organophosphate and carbamate anti-ChE compounds. In all the biological systems investigated, including human stem cells, AChE and BChE activities were found to perform different tasks. BChE is involved in the regulation of cell proliferation, while AChE is involved in cell differentiation (Sperling et al., 2008). This is characterized by decreased proliferation and increased cell death. ChE inhibitors also cause changes in intracellular calcium concentration and calcium-related membrane permeability as demonstrated by chlortetracycline (CTC) and merocyanine potentiometric dyes (Aluigi et al., 2010). Electrical events were always present in the cells and tissues characterized by the presence of AChE and/or ChE activities, and were always affected by either AChE inhibitors or cholinomimetic agents.

THE SPATIAL-TEMPORAL WINDOWS

The distribution of active ChE molecules occurs from fertilization, cleavages, gastrulation, etc., according to successive temporal windows, and in different expression fields, whenever and wherever cell-to-cell interaction takes place. The fields of expression of these molecules are more and more restricted as development proceeds, and in differentiated structures are only expressed in the "mature" neurotransmission sites.

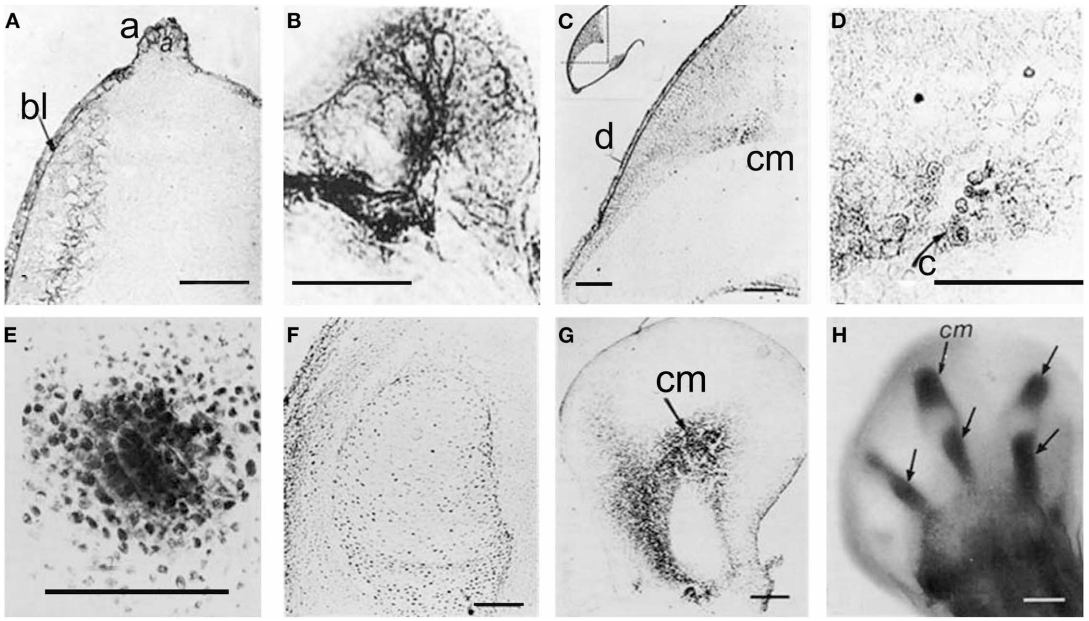

FIGURE 1 | Karnovsky and Roots 81964 reaction, showing AChE activity by a dark precipitation. (A) Forty-eight hours stage, longitudinal section, 15 μm thick; **(B)** AER, same stage; **(C)** Seventy-two hours stage, showing that from the AER and dorsal ectoderm AChE activity is diffused to focal point where the pre-cartilage cells begin to differentiate **(D)**; **(E)** Pre-cartilage mesenchyme condensation; **(F)** Five days stage, no AChE activity is present inside the cartilage core. **(G,H)** BChE activity in pre-cartilage buds. a, AER; bl, basal layer; C, cartilage; cm, cartilage matrix; d, dorsal; e, ectoderm; m, mesenchyme [from Falugi and Raineri (1985)].

Sperm-egg interaction

In gametes, the presence and localization of AChE activity was investigated by several researchers. In sperms a number of cholinergic molecules and receptors were localized in sites which suggest their involvement in the regulation of propulsion and fertilizing ability. Nelson (Nelson, 1964, 1978; Nelson et al., 1970) related the localization in the flagellum to the regulation of sperm motility. In fact, sperms present ChE activity (AChE) at the surface of the acrosome, in flagella (Chakraborty and Nelson, 1976; Cariello et al., 1986) and in the neck region. In addition, a high number of reports showing bioassays with AChE and ChE inhibitors shed light on the relevant function of ChEs in sperm swimming and fertilizing ability (Perry, 2008; Okamura et al., 2009), including epidemiological studies in humans professionally exposed to pesticides (Yucra et al., 2008).

Concerning ovogenesis, AChE activity has been localized in ovogonia at different maturation stages in several animal species: in sea urchins (**Figure 2A**), ascidians (Cangialosi et al., 2006), copepods etc. The AChE protein was localized around the nuclei in early maturation stages and close to the oocyte surface (**Figure 2A**). In the same sites AChE activity was localized by electron microscopy in the perinuclear cistern, in the Golgi vesicles and, at maturation, in vesicles close to the surface, (**Figure 2B**) including the cortical granules (Falugi et al., 1993). This active AChE is important as it cooperates to prevent polyspermy. In fact, activated sperms release the acrosomal granule (**Figure 4A**) in order to pass through the egg envelope, and expose the post-acrosomal membrane, containing ChAT (**Figure 4B**, Angelini et al., 2004). This causes the synthesis of ACh that binds to the nicotinic receptors in the egg membrane (characterized by Ivonnet and Chambers, 1997). The small Na^+ entrance caused by the excitation of nicotinic AChR produces a small membrane depolarization (described by Epel, 1980), that causes membrane permissivity to the sperm-egg fusion. If it is maintained by exposure to eserine or to nicotine (Baker and Presley, 1971; Angelini et al., 2004), such low depolarization allows polyspermy.

In this moment AChE, that has been synthesized in great amount and stored inside the cortical granules and other membranous vesicles (**Figures 3A–C**), is released into the perivitelline space (**Figure 4C**), where it cooperates with the fast block to polyspermy, by immediately removing ACh from its receptors localized in the oolemma. After the release of the cortical granules, AChE activity is present for a short time at the membrane of the fertilization cone (**Figures 3D–F**).

Early embryonic development and differentiative events

In differentiating stem cells, such as NT2 and pre-adipocytes (Aluigi et al., 2010, 2009), the presence of AChE molecules around the nuclei and in the cytoplasm was shown since the stage 0 by immunohistochemical methods and was confirmed by the presence of specific mRNA. The ChE activity was differently distributed, showing that part of the AChE molecules present in the cells are not active and other ChE active molecules non-immunoreactive to the AChE specific antibody are present in the cytoplasm (**Figures 5E,F**).

During segmentation, ChE activity is localized at the surface of blastomeres facing each-other (**Figures 5A,B**). This also appears to be true for the NT2 cultured cells in the proliferative stage (**Figures 5C,D**). At these early stages (cleavage divisions) Buznikov and Shmukler (1978) and Shmukler (1981, 1993)

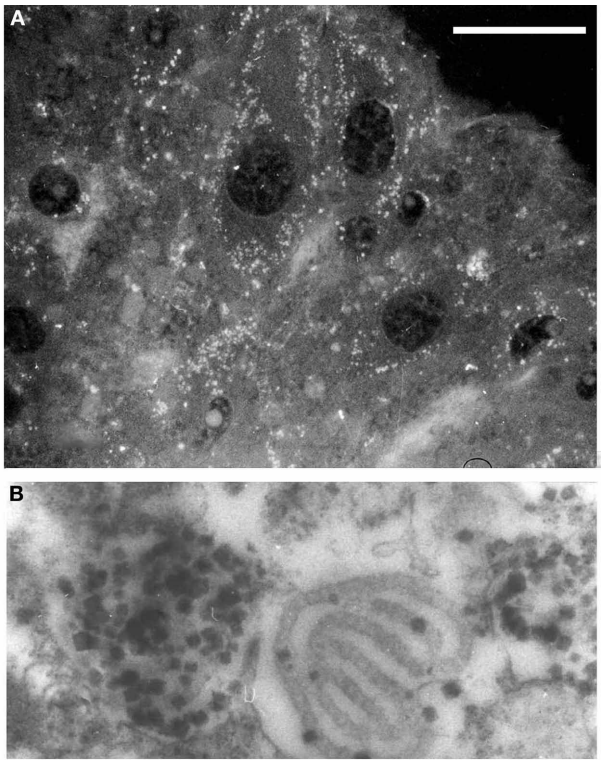

FIGURE 2 | (A) AChE-positive vesicles in sea urchin ovarian eggs. 5 μm section, antibody anti AChE (Sigma, IT); bar equals 20 μm. **(B)** Electron microscopy image, showing AChE activity localized in cortical vesicles and granules in unfertilized eggs (×20.000).

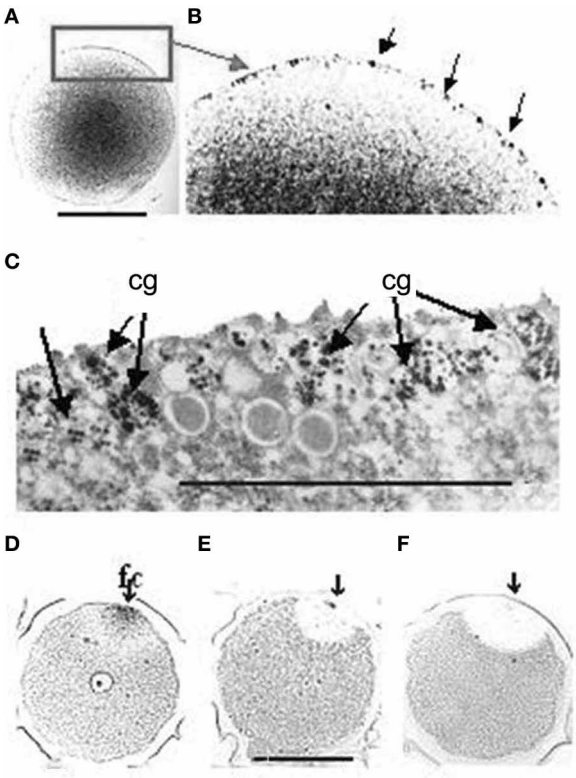

FIGURE 3 | (A) AChE reaction is localized in membranous granules at the periphery of the egg cell. **(B)** Higher magnification of the cortical layer, corresponding to the rectangular area in **A**. The arrows point to some of the positive granules. **(C)** TEM image of the same granules. The AChE reaction (dark staining) is reduced to the surface of the fertilization cone after fertilization. **(D,E,F)** The arrows show the point of sperm entry. Bars equal 50 μm. f, fertilization cone.

reported the presence of a serotoninergic cell-to-cell dialogue between blastomeres and put it in correlation with a pre-nervous membrane signal reception. This suggests a cooperation between prenervous transmitter mechanisms similar to nervous ones in establishing a precocious positional information. Shmukler et al. (1981) also reported a time-dependent passage of positional information: if separated soon after cleavage, the majority of blastomeres remain totipotential, if separated little bit later (about 10 min after blastomere post-division adhesion), the blastomeres further develop with partial cleavage pattern.

A wide expression of acetylcholinesterase is found during gastrulation of invertebrate and vertebrate embryos except crustaceans, round worms and insects, probably because the mosaic development of these organisms does not need informational inputs. In vertebrates the Hensen's node and the consequent chord-mesoderm present intense AChE activity since their first organization (**Figure 6A**).

The successive AChE localization is found in the somites and in the rhombomeres, and is a precursor of the real neural function of the enzyme. From the whole bodies of metameric somites, AChE activity is progressively restricted to the myotomes. In the dorsal chord AChE remains active for a long time, extending to the neural floor plate long after the neural tube closure.

At this stage, an intense exchange of inductive messages is still present between the dorsal chord and the floor plate. These messages have a crucial role in building the neural tube architecture, as demonstrated by Placzek et al. (1993) by transplantation experiments. The localization of AChE activity in these interacting structures is shown in **Figures 6B,C**. During organogenesis the enzyme activity is localized in the head, heart, and limbs and, later, in the feather buds of the dorsal skin (**Figure 7**).

Cell migration

Drews (1975) first reported the presence of AChE activity in migrating cells of sea urchin, amphibian, and chick embryos during the gastrulation events. The same localization was reported by (Fluck et al., 1980; Fluck and Shih, 1981) in *Oryzias latipes* mesoderm cell cultures. After these discoveries, the presence of AChE activity is considered as a good marker of migrating neural crest cells (Le Douarin, 1986) and of poly ingressing epiblast cells during gastrulation (Weinberger et al., 1984). The exposure to ChE inhibitors, such as eserine, was able to impair the movement of these structures, affecting skeletal rods elongation in sea urchin (Ohta et al., 2009) and causing the formation of cardia bifida in vertebrate embryos (**Figure 8**).

The functional mechanism of AChE in cell movement can be explained by the fact that AChE contains affinity sites to

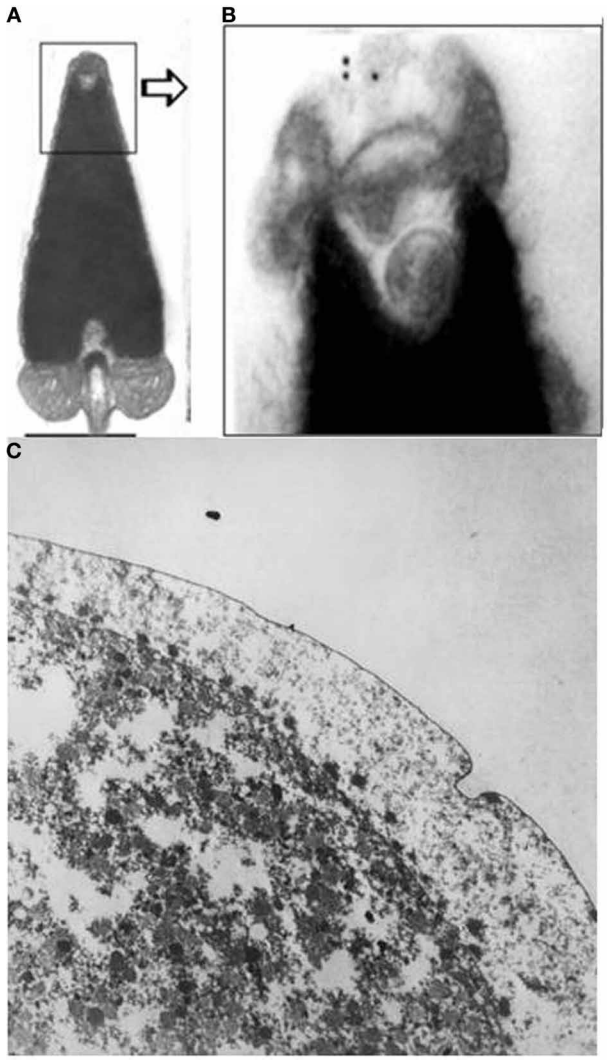

FIGURE 4 | (A,B) ChAT activity in the sperm acrosome (immunogold), electron microscopy images (Angelini et al., 2004; original images **(A)** bar = 0.5 μm; **(B)** ×20,000): **(C)** The arrows show some of the AChE-positive cortical granules exocytosized into the perivitelline space at fertilization (×20,000). OsO_4 was not used for fixation, in order to maintain the activity of AChE.

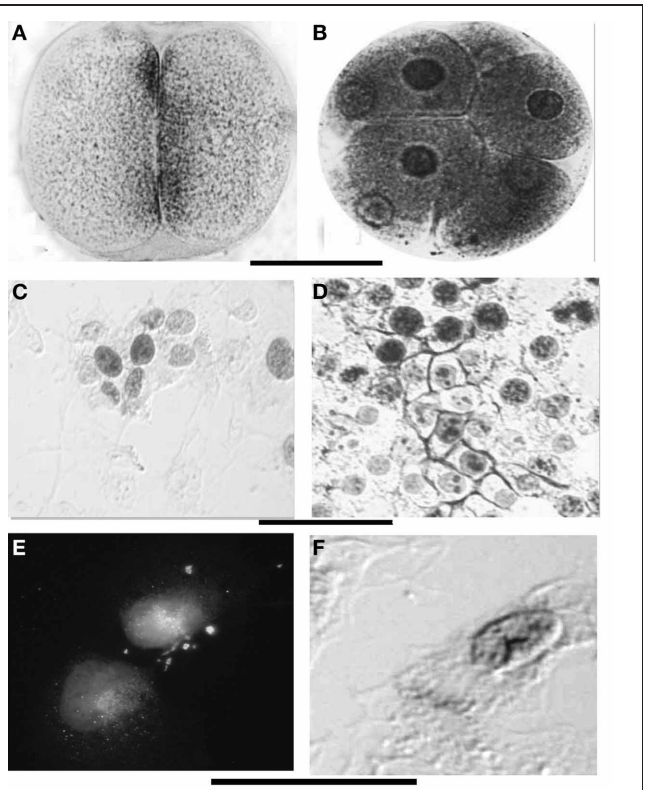

FIGURE 5 | (A,B) ChE localization at the membranes and perinuclear envelopes in sea urchin early embryos. **(C,D)** Show the similar aspect in cultured N-TERA2-D1 (teratocarcinoma cells), native **(C)** and activated by retinoic acid exposure **(D)**. *In toto* staining, Karnovsky and Roots (1964) with modified fixation; **(E)** Immunoreactivity to mammalian AChE antibody shows perinuclear localization of the protein, **(D)** AChE activity is present around the nuclei and at the periphery of the cells (arrow). Bars equal 50 μm **(A–D)** and 12 μm **(E,F)**.

cell membrane or to matrix components, such as laminin and fibronectin (Bigbee and Sharma, 2004; Anderson et al., 2008). ChE molecules might directly interact with fibronectin, that is localized (**Figure 9B**) in the migration pathways during morphogenetic movements of both invertebrate and vertebrate embryos. The link between AChE (**Figure 9A**) and matrix components in these structures might be exerted through association with structural proteins, such as ColQ or PRiMA (described by Massoulié et al., 2008; Liang et al., 2009).

CELLULAR EVENTS PRESENTING PRE-NERVOUS AChE ACTIVITY

Inflammation and balance between apoptosis/cell proliferation

Recently, inflamed cells and tissues were found to present a greater amount of ACh, (Wessler and Kirkpatrick, 2008), accompanied by AChE expression (de Oliveira et al., 2012) and activity, compared to the healthy ones. A similar increase of AChE activity was found in wound healing tissues (Falugi et al., 1994). The ACh activity enhancement is known to trigger cell proliferation and block apoptosis, by the activation of nicotinic and muscarinic receptors (Thunnissen, 2009). The homeostatic increase of AChE, on the contrary, was demonstrated to increase apoptosis (Zhang et al., 2002; Mor et al., 2008b). For this reason, AChE activity seems to be a check point between proliferation (and possibly cancer promotion) and apotosis in inflamed tissues.

In cultured cells, cDNA microarray studies showed that the most statistically significant pathways affected by AChE inhibition were related to cellular death and cell proliferation (Catalano, 2007). Aluigi et al. (2010) supplied evidence that AChE inhibition may affect the balance between cell differentiation and proliferation of NTera2-D1 (NT2) cells in a dose-dependent way, from the early stages of differentiation, both in control and in retinoic acid exposed cells. NT2 cells showed a time-dependent increase of cell death, caused by exposure to AChE inhibitors, showing a number of features characteristic of apoptosis, including membrane and mitochondrial potential changes and caspase

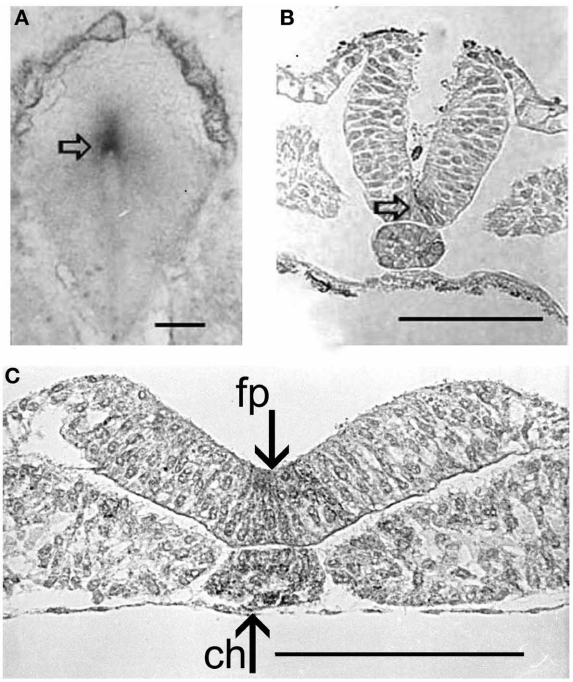

FIGURE 6 | AChE activity (arrows) (Karnovsky and Roots, 1964) in the Hensen's node of a 24 h chick embryo (A), and in the chord and neural floor plate (empty arrow) along further development (B,C). fp, loor plate; ch, chord. Bars equal 100 μm.

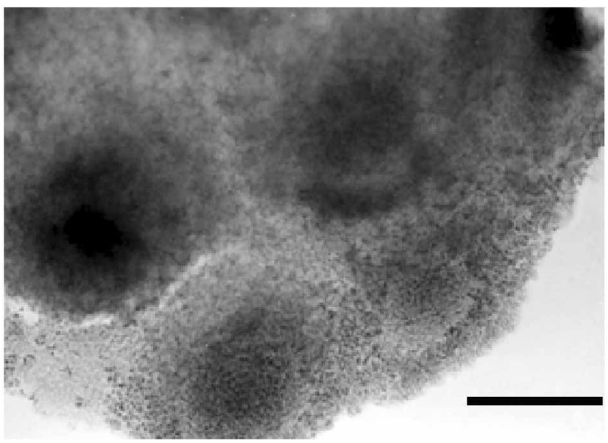

FIGURE 7 | *In toto* dorsal skin of a 6 day chick embryo. The dark staining reveals AChE activity by the Karnovsky and Roots (1964) method. Bar equals 100 μm.

cascade activation. Since the NT2 cells possess acetylcholine receptors, the regulatory pathway on apoptosis may be exerted by AChE through the modulation of ACh present at the receptorial sites. In this case, the role of AChE should be considered as a pre-nervous, or even nervous activity, as it is related to the cleavage of ACh, and to the consequent effect on nicotinic receptors.

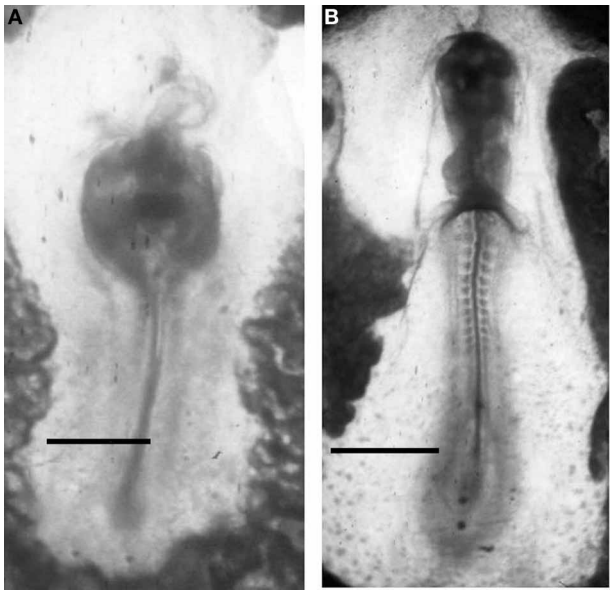

FIGURE 8 | ChE inhibition in 48 h chick embryos, exposed to 10^{-5} M diazinon prevents anterior closure of the heart (the head is not developed) (A). Control at the same incubation stage **(B)**. Bars equal 200 μm.

CHOLINESTERASE ACTIVITY INHIBITION

Most of the information about the functions of ChE molecules in development has been achieved through bioassays carried out using anti-ChE molecules, such as BW 284c51, physostigmine (eserine), tetraisopropyl pyrophosphoramide (Iso-OMPA), etc. In addition, a huge amount of information has been obtained from exposure to organophosphate (OP) and carbamate (CB) insecticides. These experiments were performed on the basis of applied research, with the aim to understand their impact on environmental and human health. While BW284c51 and iso-OMPA are inhibitors discriminating between AChE and BChE, the neurotoxic pesticides are less specific.

OP is the general name for the esters of phosphoric acid. These are the basis of a number of pesticides and insecticides used worldwide and poured into the environment in the amount of thousands tons every season. The OP compounds were actively studied during the Second World War as nerve agents to be used as weapons, and after the ban of DDT and other chlorine derivatives were widely marketed as neurotoxic insecticides (see Karczmar, 1970, for a thorough review).

These compounds and their formulate derivatives are used for a wide range of aims: from insect to small vertebrate pest control, at concentrations proportional to the body mass of the target organisms.

The OPs can affect the activity of all the ChE molecules, including true AChE and pseudocholinesterases, but not their expression, as it is demonstrated in **Figure 10**.

In blood the effects of such compounds may last a long time, because the AChE activity may be inhibited up to several months, as it was demonstrated in the case report (Romero et al., 1989)

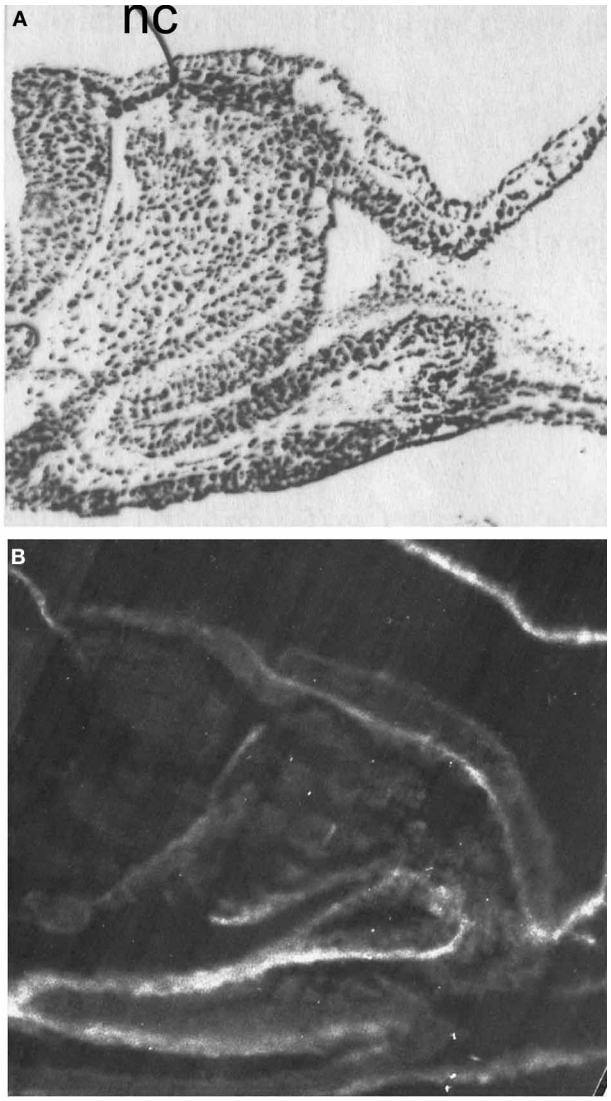

FIGURE 9 | Distribution of AChE activity (A) and fibronectin-like molecules (B). AChE activity is revealed by dark staining, (Karnovsky and Roots, 1964); Fibronectin is revealed by immunofluorescent reaction. Resin sections, 5 μm; nc, neural crest. Bar equals 100 μm.

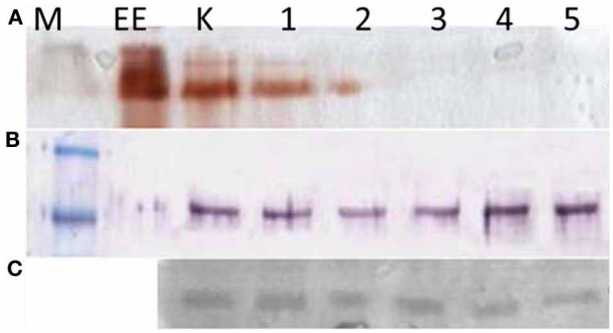

FIGURE 10 | Blood lymphocytes and monocytes isolated from buffycoats of different donors. (A) Electrophoretic revelation of ChE activity, by the Karnovsky and Roots (1964) method; **(B)** AChE immunoreactive molecules ($M = 82$ kDa). These are present in all the lanes, but inactive in the 3, 4, 5 lanes. **(C)** CDF/LIF ($M = \sim$27–30 kDa), the cholinergic differentiation factor expression also seems scarcely affected. M, molecular mass; EE, 15 units of electric eel AChE; K, control; 1, BW 10^{-4} M; 2, iso-Ompa 10^{-4} M; 3, chlorpyriphos 10^{-5} M; 4, chlorpyrifos oxon 10^{-5} M; 5, carbaryl 10^{-5} M.

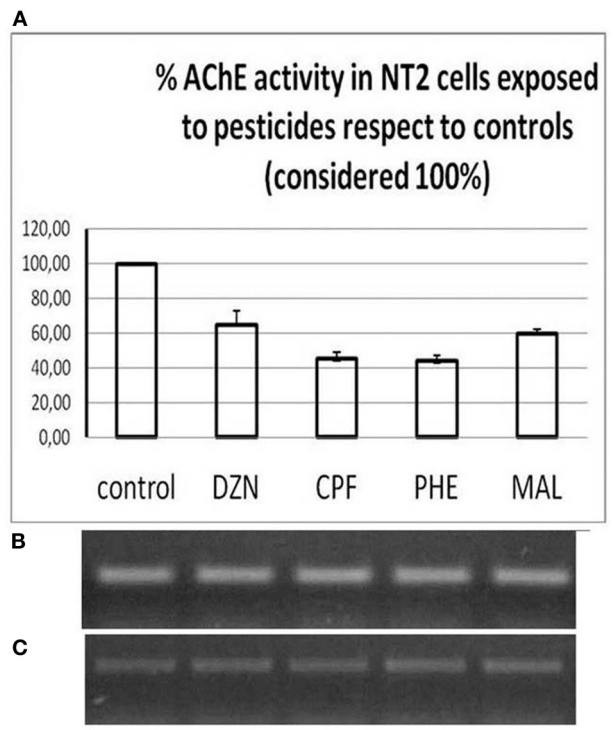

FIGURE 11 | NT2 cells exposed to 10^{-5} M inhibitors. (A) Changes in AChE activity; **(B)** Corresponding AChE mRNA (538 bp) amounts; **(C)** Actin expression (259 bp). DZN, diazinon; CPF, chlorpyrifos; PHE, phenthion; MAL, malathion.

resulted by monitoring a group of exposed farmers. In that case, pesticides were also able to pass through the placental barrier of a pregnant woman, causing embryonic anomalies.

The same aspect is revealed in human NT2 cells exposed to 10^{-4} M pesticides (**Figure 11**):

Moreover, the toxic potential varies considerably among the different pesticides, as demonstrated by Zoltan Rakonczay (SENS-PESTI report, 2006), and their effect is different among different organisms and also among different tissues. Rakonczay and Papp (2001) found that after an acute (4 h) treatment with an irreversible cholinesterase OP inhibitor, metrifonate, the activities of both AChE and BChE were inhibited in the rat brain cortex and hippocampus, while no significant changes were present in the other parts of the brain, such as olfactory bulb, or in the choline acetyltransferase activity in all three brain areas.

DEVELOPMENTAL OP TOXICITY TARGETS

Reproductive (Nelson, 1990) and developmental facts (Chanda and Pope, 1996; Aluigi et al., 2005, 2008, 2010) were demonstrated by maternal, embryonic or differentiating cells exposure to OP pesticides. The effects were dose-dependent as shown

by *in vitro* fertilization and morulae formation experiments (Ducolomb et al., 2009).

The developmental anomalies occurring after exposure to cholinergic drugs generally regard tissues and organs where, in normal conditions, AChE activity is mainly localized. Thus, the main effects of exposure to AChE activity inhibitors are related to three classes of developmental events:

1. During gamete maturation, activation and interaction (Angelini et al., 2004, 2005).
2. During the early development of invertebrate and vertebrate embryos. In this case cholinergic molecules are located mainly in moving cells and tissues engaged in relevant morphogenetic events, such as gastrulation, and are often co-distributed with special extra-cellular matrix molecules such as fibronectin (Aluigi et al., 2005) and laminin (Johnson et al., 2008).
3. During inductive communications between mesenchyme and other tissues, such as the limb bud development (Falugi and Raineri, 1985), eye and heart formation (Aluigi et al., 2005), neural floorplate induction, etc. By use of different animal models, at different developmental stages, it was demonstrated that neurotoxic insecticides (diazinon, carbaryl, pyrimicarb, and their commercial formulates), may affect calcium dynamics since fertilization events (Pesando et al., 2003) and during embryo and larval development.

By diazinon exposure of sea urchin early embryos, evidence was provided that cytoplasmic dynamics were perturbed in a dose-dependent way, thus affecting the endonuclear delivery of the OTX2 protein. This may be due to altered calcium dynamics, which in turn alter cytoskeleton dynamics: the asters, in fact, appeared strongly positive to the OTX2 immunoreaction (Aluigi et al., 2008). Pesando et al. (2003) also showed that, during sea urchin development, the treatment with organophosphates decreased the rate of early mitotic cycles and affected nuclear and cytoskeleton status as well as DNA synthesis.

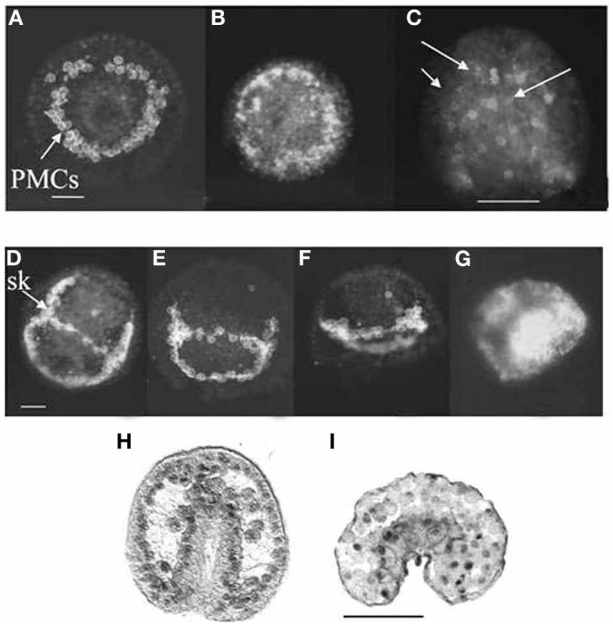

FIGURE 12 | Sea urchin gastrulae exposed to diazinon during fertilization. Arrows point to PMCs. Upper rows: WGA affinity sites for primary mesenchime cells (PMC). **(B,C)** 10^{-4} M diazinon; **(E–G)** 10^{-5} M, 10^{-4}; 10^{-3} M diazinon. **(A,D)** controls (Pesando et al., 2003). **(H,I)** OTX2 immunoreactivity in control **(H)** and exposed to 10^{-5} M diazinon **(I)** larva (Aluigi et al., 2008). Sk, skeletal rod; bar = 10 μm; **(C,H,I)** bar = 50 μm.

EFFECT OF ChE INHIBITORS ON GAMETOGENESIS AND FERTILIZATION

Most of the information about the effects of OPs on reproductive success has been obtained in countries where the socio-economic engine is based on agriculture (Contreras et al., 2006). Exposure to these inhibitors caused a significant decrease of sperm count and increase in teratozoospermia (Bustos-Obregón and González-Hormazabal, 2003). Only a few reports are available in the literature about the effect of cholinesterase activity inhibition on gametogenesis, but recently neurotoxic pesticides have been recognized as emerging endocrine disrupters (Canesi et al., 2011). In porcine *in vitro* models of ovogenesis and fertilization, exposure to two organophosphotionate compounds (diazinon and malathion) caused greater effects than classical endocrine disrupters, such as atrazine and fenoxa-propethyl. The insecticides affected viability and, by a major degree, maturation by blocking the oocytes at the germinal vesicle stage (Casas et al., 2010).

In this case, the activity of the OP compounds seems to be due to the lipofilicity of the molecules that are able to pass through the cell membrane and behave at the same way as steroid hormones, thus addressing the same mechanisms of the classical endocrine disrupters. In addition, OP-induced stress evoked a mutated AChE form, the AChE-R that was demonstrated to be involved in sperm motility regulation (Mor et al., 2008a,b).

In sea urchin gastrulation, the exposure to OPs, CBs, and AChE inhibitors, such as BW284c51, slowed movement of the primary mesenchyme cells (PMCs, **Figures 12A–G**) causing the formation of apparently diblastic gastrulae (Aluigi et al., 2008; **Figure 12I**), more flattened as compared to control embryos (**Figure 12H**). These experiments showed that AChE is also involved in the control of regulatory proteins delivery into the nucleus, thus affecting key genes expression (such as OTX2), which is linked to the cytoskeletal dynamics (in turn regulated by calcium).

Body patterning

Anti-cholinesterase insecticides, widely used for agricultural purposes, mainly affect the cholinergic system by inhibition of cholinesterase activities and muscarinic receptors (Hayes and Laws, 1991; Mineau, 1991). These have been thoroughly studied by a number of researchers using the early development of the Mediterranean sea urchin *Paracentrotus lividus* as a model (Pesando et al., 2003). For the morphological effects on fertilization and first cleavages, the effective concentration of the organophosphate diazinon was found to be 10^{-4} M, while for further stages concentrations between 10^{-5} M and 10^{-7} M were effective. The 10^{-3} M concentration arrested development. Moreover, during embryonic development, the treatment with

organophosphates slowed the rate of early mitotic cycles, affected nuclear and cytoskeleton status as well as DNA synthesis (Pesando et al., 2003).

These results paved the way to new hypotheses: Buznikov et al. (2007) affirmed that sea urchins and generally lower organisms show promise for the screening of neurotoxicants that might target mammalian brain development, based on the use of neurotransmitters as embryonic growth regulatory signals. The Authors compared the effects of the organophosphate insecticide, chlorpyrifos, in sea urchin embryos with those of the monoamine depleter, reserpine. They also stated the fact that chlorpyrifos works simultaneously through actions on acetylcholine, monoamines and other neurotransmitter pathways. Membrane permeable cholinomimetic substances and cannabinoids prevented chlorpyrifos- or reserpine-induced teratogenesis. In this way, the Authors envisaged the possibility to propose both a screening procedure for mammalian neuro teratogenesis and therapeutic approaches to prevent teratogenesis mediated by exposure to chemicals.

Electromagnetic fields

Different experiments showed that electromagnetic fields can affect the speed of cell proliferation, enhancing development of sea urchin early embryos and larvae (Falugi et al., 1987) as well as alter the symmetry of blastomeres (Ravera et al., 2006). These last authors found that exposure of fertilized eggs of the sea urchin *Paracentrotus lividus* to an electromagnetic field of 75-Hz frequency and low amplitudes (from 0.75 to 2.20 mT of magnetic component) caused asynchronous development since the first cell cycle and anomalous embryos showing irregular separation of chromatids during the mitotic events. At the same time, this exposure was also able to impair AChE activity in unicellular organisms (Amaroli et al., 2005) and in particular the one of the PRIMA form, which is linked to the cell membranes. The enzymatic activity was assayed by Ravera et al. (2006) in sea urchin embryo homogenates and decreased by 48% when the homogenates were exposed to the same pulsed field, with a threshold of 0.75 ± 0.01 mT. The same field threshold was able to cause the formation of anomalous embryos of *P. lividus*. Blockers of ACh receptors are able to antagonize those effects. The relationships between the effects of EM fields and the cholinergic system seems to be reliable, but it is still rather puzzling, because also other membrane-bound enzymes, such as phosphatases, are strongly impaired by this kind of electromagnetic fields.

Non-cholinergic stressors

Damage from a number of environmental stressors, causing stress-related electrical potential in cells, was followed by dose-dependent changes in AChE activity. The example reported below (**Figure 13**) shows the effect of 10^{-6} g/L nanoparticles on the coelomocytes of sea urchins exposed for 5 days. PChE was the most affected activity, because in sea urchin coelomocytes it is the preeminent one.

MOLECULAR FORMS OF THE NON-NEUROMUSCULAR ChEs

In the non-neuromuscular fragile cells, such as protists, very early embryos and cnidarians, ChE activity was completely inhibited by the routinely used fixation by alcohol 80, suggested by the protocols running during the 1970s. Following the observation that paraformaldehyde vapors were able to fix protozoans and ascidian embryos preserving the enzyme activity, a modified "gentle" fixation procedure was suggested for the histochemical detection of the "embryonic" AChE activity (Minganti et al., 1981). Using this method, the pre-nervous AChE activity was widely localized in the presumptive differentiating areas, becoming more and more restricted to the differentiated neuromuscular sites, where the activity was strong and resistant to usual fixatives. Minganti and Falugi (1980) advanced the hypothesis that these "embryonic" ChE enzymes were represented by smaller and less active forms than the mature ones, present at synapses. The hypothesis of Minganti et al. (1981) was that the slow cell-to-cell communications taking place during development do not need the high speed and consequently the high differentiation needed at synapses.

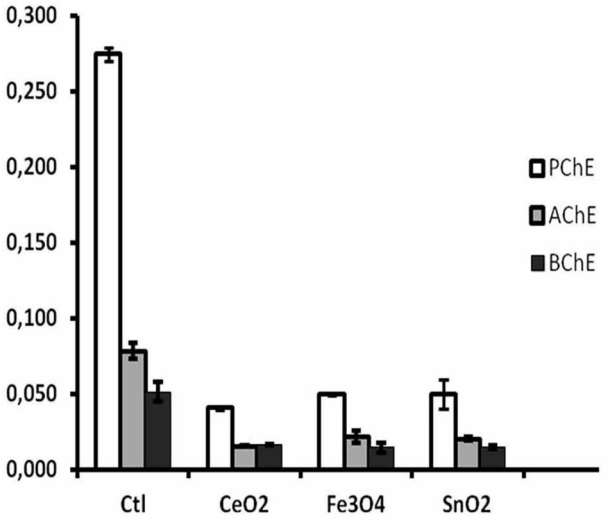

FIGURE 13 | Cholinesterase inhibition by exposure to 10^{-5} mg/L metallic nanopowders (from: Falugi et al., 2012). Ctl, control; CeO2, CeO_2; Fe3O4, Fe_3O_4; SnO2, SnO_2. Y axis, enzyme activity units.

ChEs are present with multiple isoforms formed by monomer and olygomer subunits displaying catalytic activity, with relatively low molecular weight. These are assembled forming more heavy complex polymeres, with either globular or asymmetrical shapes (Massoulié et al., 1993). The most specialized polymeres of AChE are present at synapses, where they exert their mature function, characterized by speed and efficiency. The catalytic activity of the molecular forms seems to depend on the size: according to the parameter of thermolability used by Edwards and Brimijoin (1983) the most thermolabile molecular form in a number of examined tissues was represented by the monomeric 4 S enzyme.

More recently, the group of scientists lead by Hermona Soreq (Mor et al., 2008a,b) reported a further complexity in the possible AChE molecular forms, comprising a mutated type of AChE, the AChE-R, which is expressed in organisms and tissues exposed to different stress kinds. In addition, the scientists

underline the role of microRNAs in post-transcriptional selective splicing of the molecules, which pave the way to understanding the multifunctional role of AChE and BChE both in development and in stress scenarios (Shaked et al., 2009; Meerson et al., 2010; Hanin and Soreq, 2011; Barbash and Soreq, 2012), including inflammation and apoptosis. These findings are very interesting, because alternative splicing is a relevant tool for the control of early development, allowing the creation of stage-dependent alternative proteins from the same gene, and may help to shed light on the evolutive history of AChE molecules and of the cholinergic system.

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Revisiting the role of acetylcholinesterase in Alzheimer's disease: cross-talk with P-tau and β-amyloid

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A common feature in the Alzheimer's disease (AD) brain is the presence of acetylcholinesterase (AChE) which is commonly associated with β-amyloid plaques and neurofibrillary tangles (NFT). Although our understanding of the relationship between AChE and the pathological features of AD is incomplete, increasing evidence suggests that both β-amyloid protein (Aβ) and abnormally hyperphosphorylated tau (P-tau) can influence AChE expression. We also review recent findings which suggest the possible role of AChE in the development of a vicious cycle of Aβ and P-tau dysregulation and discuss the limited and temporary effect of therapeutic intervention with AChE inhibitors.

Keywords: acetylcholinesterase, β-amyloid, presenilin 1, P-tau, Alzheimer's disease

INTRODUCTION

Alzheimer's disease is the most common cause of dementia among the elderly and is characterized by loss of memory and other cognitive functions. The major pathological hallmarks include extensive synaptic and neuronal loss, astrogliosis, and accumulation of proteinaceous deposits. The AD brain is characterized by the presence of β-amyloid plaques and neurofibrillary tangles (NFT), which are the hallmark pathological features (for a review see Blennow et al., 2006). β-amyloid plaques are extracellular deposits of which the major component is the β-amyloid protein (Aβ), a small polypeptide generated by processing of a much larger transmembrane β-amyloid precursor protein (APP; Masters et al., 1985; Kang et al., 1987) through the successive action of proteolytic enzymes known as secretases (for a recent review see Zhang et al., 2011). The intracellular NFT are composed of paired helical filaments of the microtubule-associated protein tau, which is abnormally hyperphosphorylated (P-tau; Grundke-Iqbal et al., 1986). Today, the focus on research has moved away from the proteinaceous deposits toward studies on the role of the triggering effectors, soluble oligomeric Aβ, and P-tau. Accordingly, much research is devoted to understanding how Aβ and P-tau lead to the toxic events associated with AD, how they cause changes in the expression of other key brain proteins and ultimately how they cause neurodegeneration. However, it is also crucial to decipher how both Aβ and P-tau interact in order to reach a better understanding of the mechanism of neurotoxicity and to achieve an effective therapy.

As extensively reviewed in this special issue, acetylcholinesterase (AChE) is a key enzyme in the cholinergic nervous system. During the progression of AD, many different types of neurons deteriorate, although there is a profound loss of forebrain cholinergic neurons, which is accompanied by a progressive decline in acetylcholine (Davies and Maloney, 1976; Perry et al., 1977). Both the acetylcholine-synthesizing enzyme choline acetyltransferase (ChAT), as well in the acetylcholine-hydrolyzing enzyme, AChE are affected. Therapies designed to reverse the cholinergic deficit are in large measure based on the importance of cholinergic function in cognition. In spite the overall decrease in the activity of AChE in the AD brain, current AD therapy is mostly based on inhibitors of AChE (AChE-I), which enhance cholinergic transmission, but which have modest and transient therapeutic effects (Giacobini, 2002; Kaduszkiewicz et al., 2005). As a consequence of its role as a target for AD therapy, AChE is one of the most studied proteins in the Alzheimer's field, with about 1500 manuscripts indexed into the PubMed; the vast majority of reports in the field relate with treatment strategies associated with the use of AChE-I.

It has been well known for almost 50 years that the distribution of AChE molecular forms is particularly affected in the AD brain, but the physiopathological significance and subsequent implications of these intriguing changes in AChE species remain unknown. An increase in AChE levels around amyloid plaques and NFT is a common feature of AD neuropathology, and although the significance of this increase remains to be determined. In another way, up-regulation of AChE activity following long-term AChE-I therapy has been reported in a number of studies during the last decade. All these abnormalities in AChE expression patterns, as well AChE up-regulation in reaction to chronic inhibition, may are related with the limited efficiency and persistence of AChE-I. In summary, after decades of study and hundreds of reports, AChE remains of considerable interest into the AD field. The description of changes in AChE levels and forms in the AD brain has merit extensive revision (see for example Younkin et al., 1986; Mesulam and Geula, 1990; Massoulié et al., 1993; Layer, 1995; Small et al., 1996; Kása et al., 1997; Grisaru et al., 1999; Talesa, 2001; Rees and Brimijoin, 2003; Ballard et al., 2005; Silman and Sussman, 2005;

Greenfield et al., 2008; Inestrosa et al., 2008; and many others). The purpose of this article is to review changes in AChE expression in the AD brain, but with a particular emphasis on the role of these changes in the pathophysiology of AD. In addition, we summarize our recent findings about the cross-talk between AChE and Aβ, and also between AChE and P-tau. The possibility that Aβ and P-tau interact through AChE is considered.

ALTERED AChE MOLECULAR FORM PATTERN IN AD

Acetylcholinesterase can exist in several different molecular forms, which have specific patterns of expression in different cell types (for a review see Massoulié, 2002). Moreover, the specific subcellular distribution of each species of AChE probably reflects different physiological functions for each form. Indeed, a large number of studies suggest that AChE could have novel functions unrelated to cholinergic neurotransmission (for review see Massoulié et al., 1993; Layer, 1995; Small et al., 1996; Soreq and Seidman, 2001). In this regard, it is important to note that AChE is present in both cholinergic and non-cholinergic brain areas, where the functional significance of non-cholinergic AChE remains unknown. We particularly refer to the work of Mesulam (2004) for a detailed view of the distribution of AChE in the non-pathological and AD brain. Even in cholinergic areas, it has been suggested that the reduction of AChE activity in the AD brain is not due to cholinergic depletion alone, as the density of AChE-rich (cholinergic) fibers decreased in cortical areas of the AD patients but was not correlated with the number of AChE-rich neurons (Heckers et al., 1992). Therefore, it is important to note that an alteration in AChE levels may not reflect a change in cholinergic neurotransmission.

Not all molecular forms of AChE are equally affected in the AD brain. Studies using sucrose gradient centrifugation have revealed two major forms of AChE in the mammal brain, tetrameric and monomeric species (**Figure 1**; reviewed in Massoulié et al., 1993). The major forms in the non-AD adult brain are tetramers (G_4) that are anchored in the cell membrane of neurons. These tetramers probably constitute the true cholinergic species. Other minor species are monomers (G_1) and dimers (G_2) that cannot be completely separated from each other by sucrose gradient centrifugation. Regional variations in the AChE molecular form ratio G_4/G_1 usually been studied in relation to neurochemical and neuroanatomical, particularly cholinergic, features of the brain (Atack et al., 1986). However in the AD brain, there is a selective loss in the G_4 form, while the lighter species are preserved (Atack et al., 1983; Fishman et al., 1986) or even increased in severely affected cases of AD (**Figure 1**; see also Arendt et al., 1992; Sáez-Valero et al., 1999). Similarly, changes in AChE molecular forms in cerebrospinal fluid (CSF) reflect changes in the brain (Sáez-Valero et al., 1999, 2000a). Light AChE species, which represent the major forms in plasma, are also increased in the AD plasma (García-Ayllón et al., 2010). In agreement with human studies, AChE monomeric species are also increased in brain of the APPC100 and Tg2576 transgenic mice which overproduce human Aβ (**Figure 2**; see also Sberna et al., 1998; Fodero et al., 2002; Silveyra et al., 2011a) and in rats given intracerebral Aβ (Sáez-Valero et al., 2002). Different reports have corroborated the possibility that Aβ might influence AChE (Sberna et al., 1997; Hu et al., 2003; Melo et al., 2003). So far, the significance of this particular increase in monomeric AChE around plaques and in Aβ models is unclear.

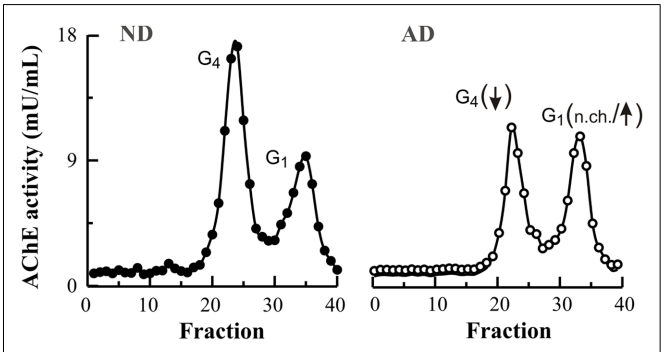

FIGURE 1 | Tetrameric and monomeric AChE molecular forms are differentially affected in the Alzheimer's brain. Human frontal cortex from non-demented individuals (ND; closed circles) and Alzheimer's patients (AD; open circles) were extracted in a Tris–saline buffer containing proteinases inhibitors and 1% (w/v) Triton X-100. Molecular weight forms of AChE were analyzed by ultracentrifugation at 150,000 × *g* in a continuous sucrose gradient (5–20% w/v), containing 0.5% (w/v) Triton X-100, for 18 h at 4°C as previously described (Sáez-Valero et al., 1999). Tetramers (G_4) and monomers (G_1) AChE forms were identified by their coefficient of sedimentation. The proportion of G_4 forms in AD is particularly depleted whereas the minor G_1 species are mostly preserved (no change; n.ch.) or even slightly increased.

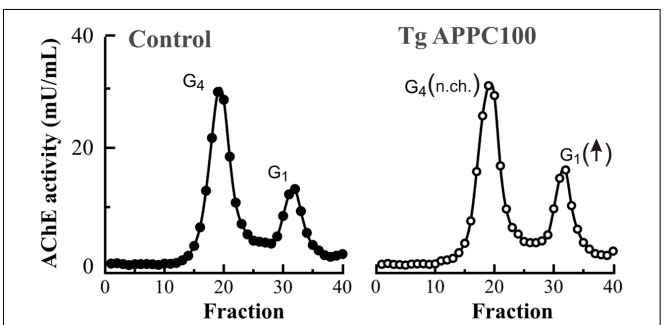

FIGURE 2 | Monomeric AChE molecular forms are increased in the brain of a transgenic mouse model of Aβ over-expression. AChE is expressed as several different species with various molecular weights which can be identified by sucrose density gradient centrifugation (see **Figure 1**). Cerebral cortices from an APPC100 transgenic mice which express human Aβ (Tg APPC100; open circles) display higher levels of monomeric G_1 AChE forms compared to control samples of similar age (closed circles; 3 months old), while levels of tetrameric G_4 AChE species were not different (no change; n.ch.). See also Sberna et al. (1998).

SIGNIFICANCE OF INCREASED MONOMERIC AChE IN AD

Light forms of AChE in the brain have been generally considered as biosynthetic precursors of the G_4 forms due to the fact that oligomeric forms of AChE are assembled from monomeric precursors (Brockman et al., 1986) and that once assembled these forms do not interconvert (Rotundo, 1988). Although it is also possible that a pool of monomers represents a separate pool with a different physiological role than that of the major cholinergic G_4 form (Small et al., 1996; Grisaru et al., 1999). Because G_1 species

are precursors of higher molecular weight species, and as a consequence G_4 is always present in association with the G_1 form, it is difficult to identify a specific functional pool of G_1 species which is distinct from the precursor pool. Interestingly, in this context, the prevalence of lighter AChE forms in AD brain resembles an embryonic pattern of expression (Arendt et al., 1992). In the human embryonic brain, the major form of AChE is a monomeric species (Muller et al., 1985). Indeed, the expression pattern of AChE forms within the embryonic brain depends on the development stage (Zakut et al., 1985; Perry et al., 1986). The G_4 species increase during human brain maturation and become the most abundant before 11 weeks of gestation (Muller et al., 1985). In rodents, the situation is similar with light AChE being the prevalent embryonic forms (Rieger and Vigny, 1976; Sung and Ruff, 1983). However, there is a shift in the molecular forms that occurs post-natally (Muller et al., 1985). The G_1 AChE form is the more abundant during all gestational periods (**Figure 3**). Interestingly, the distribution in molecular form of the structurally related enzyme butyrylcholinesterase (BuChE) does not change significantly either during fetal development or in AD (Atack et al., 1987). The physiological significance of the early and sustained expression of embryonic G_1 AChE, where a role in neurotransmission is not clear, is unknown. However, it has been suggested that AChE has roles in development, such as neuronal differentiation, regulation of cell growth, or cell adhesion. These novel functions may depend on protein–protein interactions rather than the enzyme's catalytic activity (Brimijoin and Koenigsberger, 1999; Paraoanu et al., 2006). On this basis, it has been suggested that the AChE in AD may be similar to embryonic AChE, and that it may reflect the activation of a neuronal repair in the AD brain (Layer, 1995).

Therefore, we can speculate that G_1 AChE has a non-cholinergic role during brain development, and that this role may be unrelated to the enzyme's catalytic properties. In this context, it is assumed that all AChE forms possessed similar catalytic properties, which is probably true when oligomeric and monomeric precursors are compared. Nevertheless, it has been demonstrated subtle differences in sensitivity to inhibitors and in kinetic properties exist between tetrameric and monomeric AChE species (Ogane et al., 1992; Rakonczay, 2003). More interestingly, embryonic G_4 AChE shares similar biochemical and kinetic properties with the adult enzyme, but embryonic G_1 AChE differs in its kinetic properties and in its affinity for several AChE-I from the adult G_1 form (Moreno et al., 1998). In this context, AChE activity present in the AD brain associated with plaques and NFT displays particular enzymatic properties and sensitivity to inhibitors (Geula and Mesulam, 1989; Wright et al., 1993).

Therefore, if we accept the possibility that embryonic AChE may possess a function independent of its catalytic capacities, the large pool of catalytically inactive AChE protein should be considered. The basis for the presence of an embryonic AChE species different from that of the adult, is unknown, but several questions arises regarding the use of AChE-I in AD therapy.

The existence of an unexpectedly large pool of inactive AChE has been demonstrated in brain (Chatel et al., 1993) and other tissues (Stieger et al., 1987; Rotundo, 1988; García-Ayllón et al., 2006), and suggested in CSF (García-Ayllón et al., 2007). This inactive pool may have non-classical functions, as it has been demonstrated that transgenic over-expression of enzymatically inactive AChE can influence neurodevelopment (Dori et al., 2005). The inactive AChE fraction is proportionally more abundant in embryonic than in adult tissues (Massoulié et al., 1993). Because routinely AChE levels are estimated enzymatically using various modifications of the Ellman et al. (1961) method, and are not normally estimated immunochemically, little information is available on this inactive pool of AChE in pathological and non-pathological conditions. A significant decrease of both AChE activity and immunoreactivity has been observed using enzyme-linked immunosorbent assay of AD brain (Hammond and Brimijoin, 1988). However, after decades of studying AChE in the AD brain further research is still necessary in order to determine the AChE protein content and its relationship with altered AChE activity levels. Whether non-catalytic AChE in brain has physiological significance, and how it is affected during pathology and treatment, are issues that warrant further study.

This complex scenario of multiple molecular forms is brought about, at least in part, by the existence of alternative splicing of

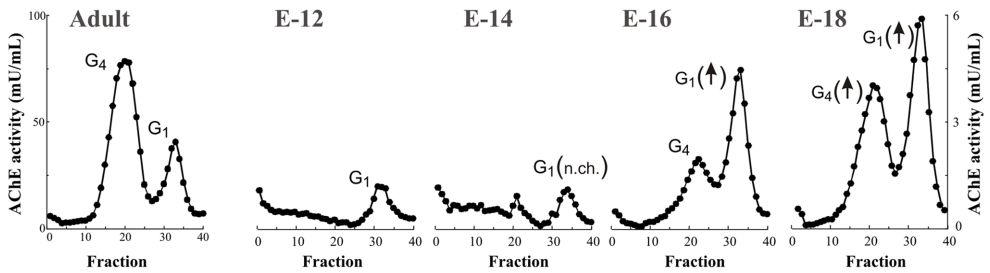

FIGURE 3 | Monomeric AChE molecular forms are the predominant species during embryonic brain development. AChE activity was extracted from rat (Sprague-Dawley) cerebral cortex at embryonic day 12 (E14), 14 (E14), 16 (E16), and 18 (E18) and at 3 months of age (Adult), and AChE forms were separated by sucrose density gradient (see **Figure 1**). At early embryonic stage only light monomeric G_1 AChE is present and levels are maintained (no change; n.ch.) until E15–16, an increase in G_1 AChE parallels emergence of G_4 AChE tetramers, the brain cholinergic species. The G_4 species increase in activity during brain maturation becoming the major molecular forms during post-natal periods (N.B. difference of scales between embryonic and adult stage).

the single ACHE gene, generating different AChE variants, with potential different gene regulation (Grisaru et al., 1999; see also **Figure 4**). Alternative 3′ RNA splicing generates different polypeptide encoding transcripts called "tailed" or T, "hydrophobic" or H, and "readthrough" or R-transcripts (Massoulié et al., 1993; Taylor and Radic, 1994; Grisaru et al., 1999), with the same catalytic domain and distinct C-terminal peptides that determine the ability of the molecule to form oligomers. In the mammalian brain, the T-transcript is the major form and encodes subunits which produce monomers, dimers, and tetrameric forms; whereas the R-transcript, which is normally present at low levels (Kaufer et al., 1998; Perrier et al., 2005), encodes a soluble monomeric form (Sternfeld et al., 2000; **Figure 4**). In addition to the 3′ alternatively spliced species of AChE, the 5′ end is also subject to intricate regulation (Meshorer et al., 2004) generating AChE variants that have extended N-termini; thus, within the brain N-AChE-T and N-AChE-R variants may occur in parallel with AChE-T and AChE-R. The AChE-T and AChE-R monomers or their N-extended variants cannot be distinguished by molecular weight. Whether the increase in monomeric AChE in the AD brain is related to increases in the T or R variants is still an open question. Anyhow, in this context has been suggested that the AChE-R can compete with the main brain AChE-T protein and suppress the formation of insoluble Aβ oligomers (Berson et al., 2008; see below). An increased N-AChE-T expression has been also postulated in the AD brain

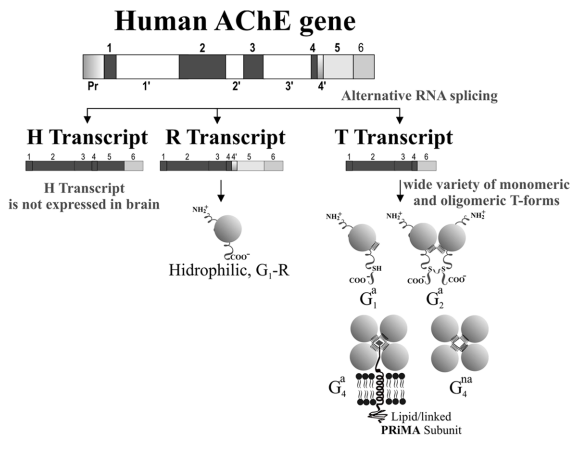

FIGURE 4 | Diagram of the human AChE gene structure, its alternative splicing and generation of variants and molecular forms of AChE in brain. Gene structure showing the splicing pattern of the six major exons. The catalytic core of human AChE is encoded by three exons and additional exons encode the variant-specific carboxy-terminal sequences. In the normal human brain, much more AChE-T than AChE-R mRNA is produced. However, under stress AChE-R expression is increased. To date, no expression of AChE-H has been described in the brain (see Grisaru et al., 1999 for more details). The R-transcript encodes a soluble monomeric form, while the T-transcript encodes a wide variety of monomeric (G_1) and oligomeric (G_2 and G_4) molecular forms (Massoulié et al., 1993). AChE molecular forms can vary in their solubility characteristics (non-amphiphilic: ^{na}; amphiphilic: ^{a}). The main amphiphilic G_4 form in brain is anchored to the membrane by a "proline-rich membrane anchor" PRiMA subunit (Perrier et al., 2002). Additional complexity can be generated by additional 3′ alternatively splicing which can generate AChE variants with extended N-termini (not shown in figure; see Meshorer et al., 2004).

associated with disease progression, including apoptotic cell death (Toiber et al., 2008). In summary, Soreq and Seidman (2001) have presented evidence that N- and C-terminally modified AChE variants, all of which have similar enzymatic activities, can display distinct and in certain cases inverse functions (reviewed in Greenberg et al., 2010). The use of AChE-I which does not distinguish between AChE variants should interfere in all processes indiscriminately.

The central question is whether the changes in the distribution of AChE molecular forms in the AD brain have any physiopathological consequences. As stated previously, gross sedimentation analysis cannot distinguish between monomeric isoforms that are synthesized to be assembled in oligomers, or arise as degradation products, and those specific monomeric species which may have specific functions. Therefore, in previous studies we have further characterized the increase in monomeric AChE associated to Alzheimer's and to Aβ by characterizing its glycosylation pattern by lectin binding analysis, based on the assumption that different functional pools of AChE may have different glycosylation patterns. Correct glycosylation determines the adequate intracellular trafficking, folding, assembly, and final localization of glycoproteins. Thus different forms (glycosylated variants or glycoforms) of the same protein should differ in glycosylation in order to achieve a different oligomerization state, subcellular localization, protein–protein interaction affinity, or a different physiological function. Indeed, for a particular glycoprotein, the abundance of single glycoforms should closely correlate to each other and be regulated within narrow limits. By exploiting the ability of lectins to bind diverse carbohydrate moieties with high specificity (Sharon and Lis, 2004), we have demonstrated that the glycosylation of AChE is altered in the AD brain, postmortem, and lumbar CSF (Sáez-Valero et al., 1997, 1999, 2000a). Tetrameric G_4 and light G_1 have different glycosylation patterns (Sáez-Valero et al., 1999), thus depletion specific loss of the tetrameric form in the AD brain may be responsible, in part, for this change. Changes in AChE glycosylation were also characterized in Aβ transgenic mice models displaying increases in monomeric AChE (Sberna et al., 1998; Fodero et al., 2002). However, we further demonstrated that when the light AChE species from AD and non-demented brain are isolated, G_1 species present in AD brain displayed different affinities for lectins and for conformational anti-AChE antibodies, compared with isoforms from control brains (Sáez-Valero et al., 2000b). These changes indicate that the minor subset of G_1 AChE, whose contribution is increased in AD brain, correspond to isoforms (glycoforms) either not present, or poorly present in adult human brain in non-disease conditions. The physiological relevance of the increase in this minor G_1 form for AD pathogenesis is unclear.

Alterations in the glycosylation state of other glycoproteins have been reported in AD tissue (Guevara et al., 1998; Fodero et al., 2001; Kanninen et al., 2004; Sihlbom et al., 2008). Pathological impairment in the broader protein glycosylation machinery could significantly compromise the processing of many glycoproteins, thereby resulting in loss of physiological function of many of these proteins. Abnormal incorporation of carbohydrate moieties in AChE subunits can compromise its functional role and/or oligomerization. In this context, altered AChE glycosylation has

been also demonstrated in Creutzfeldt-Jakob disease (Silveyra et al., 2006), a neurodegenerative process where impaired glycosylation machinery is suspected (Rudd et al., 1999). Nonetheless, unlike AD, in Creutzfeldt-Jakob disease, altered AChE glycosylation is not caused by changes in the proportion of any particular molecular form (Silveyra et al., 2006). Therefore, we favor the hypothesis that in Creutzfeldt-Jakob disease change in AChE glycosylation is the consequence of perturbed glycosylation machinery, whereas the altered glycosylation pattern of light AChE species in AD reflects an imbalance of protein glycoforms resulting from changes in AChE variants related or not to differentiation state. This is an issue that requires further study.

P-TAU INCREASES AChE, DIFFERENCES FROM Aβ

It has been noted that abnormal AChE expression in the AD brain occurs in association with the two hallmark features of the AD pathology, the amyloid plaques and the NFT (Mesulam and Morán, 1987; Ulrich et al., 1990). As mentioned previously, Aβ peptides influence AChE levels, thus Aβ may be responsible for increased AChE around plaques. However, the increase in AChE associated with NFT has remained largely unexplored. Recently, we showed for the first time that P-tau can trigger an increase in AChE expression (Silveyra et al., 2011a). *In vivo* over-expression of P-tau in transgenic mice (Tg VLW mice) expressing human tau mutations causing frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) led to an increase in the activity of the T-transcript of AChE (Silveyra et al., 2011a). The results suggest that the early increase in AChE expression that occurs around NFT may be a consequence of disturbed tau phosphorylation. In contrast to Aβ transgenic models in which only one specific molecular form of AChE increases, in the P-tau transgenic mice, all major molecular forms of AChE were increased, including the tetrameric species (**Figure 5**). The explanation for differences in the pattern of AChE expression between Aβ and P-tau over-expressing mice remain unclear and will require further research, as will the potential differing physiopathological consequences of increases in tetrameric or monomeric AChE. In this context, it is necessary to consider the different subcellular localization of tetramers and monomers. Light forms probably localized internally whereas G_4 forms are localized extracellularly (Inestrosa et al., 1981). Therefore, the different localization of these forms determines and limits potential interactions with other protein partners. The recent finding that PRiMA (proline-rich membrane anchor) directs a restricted localization of tetrameric AChE not only to synapses but also to membrane microdomains called rafts (Xie et al., 2010), suggests that there may be different functional interactions of AChE species with particular partners.

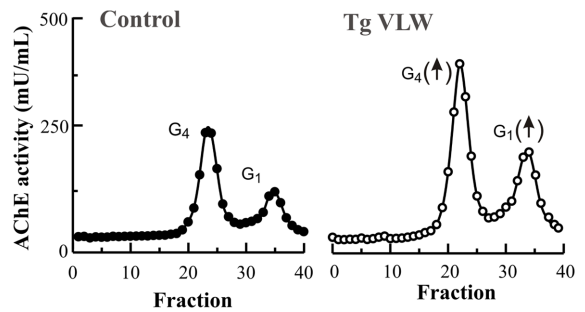

FIGURE 5 | All molecular forms of AChE are increased in the brain of a transgenic mice model of P-tau over-expression. In a transgenic mice (Tg VLW; open circles) expressing human tau mutations causing frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), both the major cholinergic tetramers G_4 AChE and light monomeric forms G_1 AChE activities were higher compared to age-matched control-littermate mice (closed circles; 5 months old).

DOWNSTREAM CONSEQUENCES OF CHANGES IN AChE LEVELS IN AD BRAIN

Notwithstanding the overall loss of total AChE activity in the AD brain, AChE is consistently increased in regions around amyloid plaques and NFT at all stages of the disease, including some of the earliest stages (Perry et al., 1980; Mesulam and Morán, 1987; Ulrich et al., 1990). Extensive studies by Inestrosa et al. (1996) suggest that AChE may directly interact with Aβ in a manner that increases deposition of Aβ to form plaques. Studies using double transgenic mice that over-express AChE and Aβ (Rees et al., 2003) support these observations and they suggest that AChE may play a role in pathogenesis of AD.

Our own recent work also indicate that AChE can modulate APP processing and Aβ production. Aβ is produced through the successive action of two proteolytic enzymes, β-secretase and γ-secretase on APP (Zhang et al., 2011). The active proteolytic component of the γ-secretase complex is presenilin 1 (PS1; Suh and Checler, 2002). Mutations in PS1 cause early-onset AD with an accelerated rate of Aβ deposition (St George-Hyslop, 2000), thus proteins that interact with PS1 are of major functional importance. We have previously demonstrated an interaction between AChE and PS1 by reciprocal co-immunoprecipitation (Silveyra et al., 2008). Recently, we demonstrated that AChE can influence PS1 levels by showing that AChE over-expression increases PS1 levels, while AChE knock-down with siRNA leads decrease PS1 in transfected cells (Silveyra et al., 2011b). Perhaps the most significant conclusion from our recent study is the potential participation of AChE in a degenerative cycle that enhances amyloidogenic APP processing. We can presume that several degenerative cycles, participating as interactive systems within a larger vicious cycle, accelerate the development of AD. Hence, in AD it is possible that Aβ can induce a feedback loop leading to amyloidogenic APP processing (Cribbs et al., 1995; White et al., 2003). A vicious cycle of Aβ generation potentially could involve PS1; recent evidence indicates that Aβ42 can induce an increase in PS1 levels in cultured neurons creating a toxic loop (Matrone et al., 2008). Using different experimental conditions, we have confirmed that both Aβ42 and P-tau trigger an increase in AChE, which can in turn influence PS1 and thereby modulate Aβ production (**Figure 6**). We have found that Aβ42-induced PS1 increase can be prevented by pre-treatment of SH-SY5Y cells with siRNA AChE (Silveyra et al., 2011b). The possibility that different effects may be obtained with different AChE species and variants (tetrameric versus monomeric molecular form, or T-variant versus R-variant or N-extended variant), requires further study. In this context, recent evidence indicates a

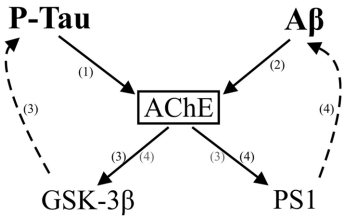

FIGURE 6 | Schematic representation of the multiple relationships between AChE and the Alzheimer's effectors Aβ and P-tau. AChE is proposed to interact with both Aβ and P-tau. P-tau can lead to an increase in the activity of AChE (1) (Silveyra et al., 2011a), whereas over-expression of N-terminally extended T-AChE variant activates the tau kinase GSK-3β inducing tau hyperphosphorylation (3) (Toiber et al., 2008). In addition, Aβ peptides can also increase AChE levels (2) (Sberna et al., 1997). In turn, AChE can affect APP processing and Aβ production by modulating the levels of the γ-secretase catalytic subunit PS1 (4) (Silveyra et al., 2011b). Thus, increased AChE can affect both amyloidogenic and tau hyperphosphorylation pathways.

specific role inducing GSK-3β activation and tau hyperphosphorylation for the alternative N-terminally extended T-AChE variant (Toiber et al., 2008), variant up-regulated by stressors inducing protein misfolding and calcium imbalances, both characteristic of AD.

It appears likely, therefore, that several vicious cycles trigger by Aβ and P-tau involve the potential participation of AChE. The significance of these theoretical considerations to the clinical and neuropathological course of AD remains to be demonstrated.

EFFECTS OF AChE-I

Therapies designed to reverse the cholinergic deficit are in large measure based on the importance of cholinergic function in cognition. Indeed, AChE-Is have proven to be modestly efficacious in treating the cognitive and functional symptoms of AD. In addition disease-modifying effects of AChE inhibition has been also considered (Giacobini, 2002). However, the modest clinical efficacy of AChE-Is has not discouraged the development of new AChE-Is, and particularly the so called dual binding site AChE-Is (Muñoz-Torrero, 2008; Pepeu and Giovannini, 2009), which are both inhibitors of AChE and also of amyloid plaque formation. This latter effect is based on the fact that AChE-binding molecules may, in addition to their effect on enzyme activity, block the effect of AChE on Aβ fibrillogenesis by interaction throughout the peripheral anionic site of the enzyme (Inestrosa et al., 1996). Besides the design of new inhibitors with the capacity to block catalytic and peripheral anionic sites of AChE, there is evidence that inhibitors may also influence APP processing. AChE-Is have been shown to alter APP expression and metabolism in cellular (Lahiri et al., 1994; Pakaski et al., 2001; Peng et al., 2006) and animal models (Mori et al., 1995; Zimmermann et al., 2004; Dong et al., 2009), as well in AD-treated patients (Clarke et al., 2001; Basun et al., 2002; Zimmermann et al., 2005). The modulatory effects of AChE-I on APP metabolism have been attributed to their effect on ADAM10/α-secretase (Zimmermann et al., 2004; Peng et al., 2006), BACE1/β-secretase (Lahiri et al., 2007; Fu et al., 2008; Li et al., 2010), and on PS1/γ-secretase (Silveyra et al., 2011b). The disparity between the effects of AChE-Is and cholinergic agonists on secretase activity would suggest that AChE is not exerting its modulatory action on PS1 via a cholinergic mechanism (Zimmermann et al., 2004; Silveyra et al., 2011b). However, effects on APP processing by cholinergic agonists have been also demonstrated (Nitsch et al., 1992; Rossner et al., 1998; Davis et al., 2010). The mechanisms by which AChE-Is influence APP processing remain unclear, but it is suggested that regulation of APP processing by AChE-Is may involve multiple mechanisms, including cholinergic and non-cholinergic actions, some independent of their anti-cholinesterase (catalytic) activities (Lahiri et al., 1997).

The therapeutic effect of current AChE-Is is both modest and transient. Current AChE-I treatment results are disappointing both because of their poor efficacy and tolerability. Interestingly, these drugs have a limited duration of cognitive benefit. The effects of these drugs on APP processing also fail to be maintained over the long-term in Alzheimer's patients (Basun et al., 2002).

The transience of the response to AChE-I could be associated with AChE up-regulation in reaction to chronic inhibition (Chiappa et al., 1995; Kaufer et al., 1998). Indeed, increases in CSF AChE have been reported after AChE-I treatment (Davidsson et al., 2001; Darreh-Shori et al., 2006; García-Ayllón et al., 2007; Parnetti et al., 2011). The varying responses of different AChE species to AChE-I treatment, were the light AChE species seem not subject to AChE-I induced up-regulation, suggest different modes of regulation and should be also considered (García-Ayllón et al., 2007). Interestingly, we found that the effect of AChE-I treatment on PS1 levels was also not sustained, and that the lack of effect on PS1 was associated with up-regulation of AChE (Silveyra et al., 2011b). Our data suggest that sustained AChE inhibition cannot be effective when the expression of AChE is up-regulated and that this undesired effect needs to be addressed to develop more effective therapies based on AChE-I.

SUMMARY AND CONCLUSION

In summary, AChE species differ in their responses to disease and their interactions with β-amyloid and P-tau. The important question about the nature of the alternative functions of AChE, their association with different AChE species and variants, and their role in AD pathogenesis and therapy needs to be examined further. Recent evidence also suggests the potential participation of AChE in vicious cycles involving Aβ and P-tau. Elucidation of the mechanisms involved in these changes will be useful for understanding the physiological and pathological relevance of altered AChE expression in the AD brain and AChE-I pharmacological intervention. The chronic increases in AChE activity during AChE-I treatment may cause the therapeutic value of AChE-I to be limited and temporary and needs to be addressed in order to improve therapy.

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Stressing hematopoiesis and immunity: an acetylcholinesterase window into nervous and immune system interactions

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Hematopoietic stem cells (HSCs) differentiate and generate all blood cell lineages while maintaining self-renewal ability throughout life. Systemic responses to stressful insults, either psychological or physical exert both stimulating and down-regulating effects on these dynamic members of the immune system. Stress-facilitated division and re-oriented differentiation of progenitor cells modifies hematopoietic cell type composition, while enhancing cytokine production and promoting inflammation. Inversely, stress-induced increases in the neurotransmitter acetylcholine (ACh) act to mitigate inflammatory response and regain homeostasis. This signaling process is terminated when ACh is hydrolyzed by acetylcholinesterase (AChE). Alternative splicing, which is stress-modified, changes the composition of AChE variants, modifying their terminal sequences, susceptibility for microRNA suppression, and sub-cellular localizations. Intriguingly, the effects of stress and AChE variants on hematopoietic development and inflammation in health and disease are both subject to small molecule as well as oligonucleotide-mediated manipulations *in vitro* and *in vivo*. The therapeutic agents can thus be targeted to the enzyme protein, its encoding mRNA transcripts, or the regulator microRNA-132, opening new venues for therapeutic interference with multiple nervous and immune system diseases.

Keywords: acetylcholinesterase, hematopoiesis, stress

INTRODUCTION

Stress can be defined as a psychological, environmental, or physiologic threat on homeostasis (Chrousos, 2009). Psychological stress begins with impulses from high cortical centers via the limbic system and results in the release of chemical mediators. Catecholamine signals, and specifically, nor-adrenaline, are then released from sympathetic nerve fibers in direct proximity to target tissues assisting the body to launch a fight-or-flight reaction, increasing heart rate, and blood flow to skeletal muscles while decreasing immune reactions (Padgett and Glaser, 2003). Another key contributor to these processes is the parasympathetic neurotransmitter acetylcholine (ACh) which is fundamental for nervous system function (Kaufer et al., 1998) and is also produced by peripheral leukocytes (Kawashima and Fujii, 2000). These two pivotal neurotransmitters are both up-regulated during stress and can activate the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic adrenal medulla (Black, 2002). Specifically, sustaining moderate ACh levels is crucial for maintaining homeostasis: abrupt stress-induced elevation of ACh signaling may be lethal whereas its gradual loss, as in Alzheimer's disease, multiple system atrophy, and other neuro-degeneration conditions, is associated with progressive deterioration of cognitive, autonomic, and neuromuscular functions (Soreq and Seidman, 2001). Also, activation of cholinergic receptors is beneficial in pathological states such as atherosclerosis, myocardial infarction, hypertensive vasculopathy and nephropathy, and heart failure, largely due to yet incompletely understood mechanisms (Chrousos, 2009; McEwen and Gianaros, 2011). In our current review, we argue that moderate ACh levels are also crucial for controlling immune and inflammatory functions, both in the brain and in peripheral tissues and that acetylcholinesterase (AChE) is a key contributor toward sustaining these levels.

The dynamic hematopoiesis process of blood cell production and differentiation is notably characterized by a continuous turnover of cells throughout life. Hematopoietic stem cells (HSCs) are characterized by their ability to generate all blood cell lineages while maintaining self-renewal. These properties are modified substantially when the host of these cells is exposed to either psychological or physical stressors (Dudakov et al., 2010), so that cells from the bone marrow and the vascular marginal pool are rapidly mobilized to the circulation (Rafii et al., 1995). Consequently, cytokines and chemokines capable of activating cells to immediately respond to various stimuli regulate the production and longevity and determine the number of circulating blood cells (Cavazzana-Calvo et al., 2011). Cytokines further regulate the division and differentiation of progenitor cells to develop into morphologically recognizable cells with distinctive phenotype features. Myelopoiesis involves the production of

granulocytes, monocytes, red blood cells (RBCs) and the platelet-producing megakaryocytes (Mks). The lymphoid progenitor cells can differentiate into T, B, or natural killer (NK) lymphocytes, depending on the microenvironment. Importantly, mesenchymal stem cells carry both nicotinic and muscarinic ACh receptors (Hoogduijn et al., 2009). Stress-inducible changes in cholinergic signaling and in any of these populations can hence drastically modulate immune functions.

STRESS-AFFECTED HEMATOPOIESIS AND NEUROPOIESIS

There are many interactions between the nervous and hematopoietic systems. These include sequence and structural homology between neuropoietic and hematopoietic factors. Neuropoietic factors such as cholinergic differentiation factor (CDF) and ciliary neurotrophic factor (CNTF) determine the developmental fate of sympathetic neurons from noradrenergic to cholinergic function. These show homologies to the hematopoietic cytokines granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (Bazan, 1991).

Hematopoietic factors may affect differentiation and regeneration of cholinergic neurons. In contrast, scopolamine, a muscarinic ACh receptor blocker, can abolish stress-induced erythropoiesis (Gol'dberg et al., 2000), and deficiencies of neuropeptides. Substance P and calcitonin gene-related peptide (CGRP) lead to a dramatic fall in neutrophil production; these neuropeptides stimulate bone marrow colony formation and affect neutrophils production via both direct and indirect effects on bone marrow colony forming units of granulocyte/monocyte progenitors (CFU-GM). The observed effects do not involve peripheral blood CFU-GM, suggesting a direct neural control over BM hematopoiesis (Broome et al., 2000). Furthermore, erythropoietin was shown to exert neurotrophic and neuroprotective activities in different *in vivo* and *in vitro* models of brain damage as well as an anti-apoptotic effect on microglia, the brain's resident immune cell (Vairano et al., 2002).

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THE PARASYMPATHETIC SYSTEM ACETYLCHOLINE HYDROLYZING ENZYME ACETYLCHOLINESTERASE AND STRESS

The parasympathetic system and the ACh hydrolyzing enzyme AChE together contribute to the delicate balance of hematopoietic and immune events under stress (**Figure 1**). ACh produced by the vagus nerve and/or by peripheral leukocytes (Rosas-Ballina et al., 2011) can potently modulate several classical immune reactions by activating the α7 nicotinic receptor α7nAChR on the leukocytes' membrane, which in turn blocks the NF-kB-mediated production of pro-inflammatory cytokines such as IL-1 and tumor necrosis factor (TNF) α. Compatible with this mechanism of action, vagal stimulation suppresses septic shock-like response after bacterial lipopolysaccharide (LPS) injection (Gol'dberg et al., 2000; Tracey, 2002). Tissue residing mononuclear cells also receive cholinergic signaling via ACh secreted from the vagus and/or synthesized in these nucleated immune cells, which have all the components of the cholinergic signaling system: ACh, the ACh synthesizing enzyme choline acetyl-transferase (ChAT), and the co-regulated vesicular ACh transporter VAChT, transcribed from the same transcription unit, the ACh degrading enzyme AChE and functional muscarinic and nicotinic ACh receptors. Correspondingly, ACh receptors were identified on lymphocytes from the thymus, lymph nodes, spleen, and peripheral blood

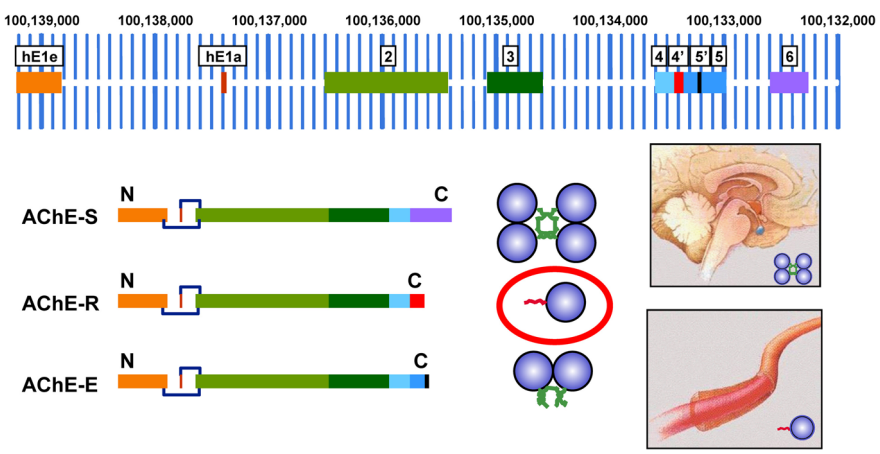

FIGURE 1 | Stress modifies AChE's composition. Shown are the N-terminal and C-terminal changes representing combinatorial differences on AChE splice variants. The soluble monomeric AChE-R variant produced in all tissues expressing this gene accumulates under stress and is found in the circulation, albeit being less stable than AChE-S. Both AChE variants are associated with stress-inducible hematopoiesis. The red-labeled C-terminus of AChE-R represents the hematopoietic peptide ARP [Grisaru et al. (2006)].

(Tracey, 2002). Also, vagus nerve stimulation fails to inhibit TNF α production in splenectomized animals during lethal endotoxemia, demonstrating cholinergic innervation of the spleen (Huston et al., 2006).

In mice, peptidergic nerve fibers entering the bone marrow terminate with synapses on stromal and perivascular cells, interactions that were implicated in local inflammation and regulation of leukocyte trafficking (Gol'dberg et al., 2000). Endothelium, a key regulator of leukocyte trafficking during inflammation, is also a target of anti-inflammatory cholinergic mediators; both vagus nerve stimulation and cholinergic agonists significantly block leukocyte migration *in vivo* (Saeed et al., 2005). Cholinergic neurons are also present in developing thymic rudiments and their presence was associated with increased production of thymic lymphocytes. The cholinergic system also affects HSCs and their niche cells and plays an active role in T cell differentiation. Thus, CD8+ T cells from M1 receptor-deficient mice fail to differentiate into cytolytic T lymphocytes (Zimring et al., 2005). ACh release from spleen T cells can in turn attenuate TNF α production in spleen via α7nAChR expressed on innate immune cells (Rosas-Ballina et al., 2010). Furthermore, T cells show plasticity in their response to cholinergic stimuli, so that nicotine upregulates interferon-γ (IFN-γ) and down-regulates interleukin (IL)-17 secretion, whereas muscarin enhances IL-10 and IL-17 and inhibits INF-γ secretion. As is implicated from the numerous studies stated above, ACh signaling is essential for T-cell activation and its opposite function may be required to synchronize and balance ionic and metabolic events in a single lymphocyte. Within the T-cell, the ACh regulatory axis may provide for a fine tuning of the T cells to changing environment (Qian et al., 2011).

In all peripheral tissues, ACh signals normally remain above a certain threshold sufficient to suppress the production of pro-inflammatory cytokines, yet are transiently reduced following stress due to AChE over-production which lasts several hours (Nance and Sanders, 2007). Within 24 h, elevation of the AChE-targeted microRNA-132 (Shaked et al., 2009; Soreq and Wolf, 2011) reduces AChE levels, retrieving ACh-mediated blockade of pro-inflammatory cytokines production. Therefore, stress responses also facilitate yet more IL-1 production (Gilboa-Geffen et al., 2007). Monocytes, macrophages, dendritic cells, and T lymphocytes which infiltrate peripheral organs and accelerate pathological processes are exposed to the adrenergic and cholinergic transmitters released by the autonomic innervations of these organs, which could dramatically alter cytokine release (Nance and Sanders, 2007). That serum AChE activity continuously increases with age (Sklan et al., 2004) may suggest aging-related deterioration in the efficacy of this regulation process.

Under stress, the elevated glucocorticoid cortisol interacts with two cytosine/guanosine (CpG-rich) sequence motifs in the AChE promoter to induce nuclear transcriptional and post-transcriptional events of over-production of AChE and replace the major stable AChE splice variant AChE-S by the less stable AChE-R variant (**Figure 2**) (Meshorer et al., 2002). This effect associates with release from AChE-R of the cleavable C-terminal peptide ARP. Both AChE-R and ARP can stimulate the proliferation of CD34+ hematopoietic progenitor cells and facilitate the proliferation and differentiation of promegakaryocytes and subsequent platelet production (Pick et al., 2006). When secreted, the extra-cellular AChE-R hydrolyzes ACh as efficiently as AChE-S, further facilitating the production of pro-inflammatory cytokines; and when retained in the intracellular space AChE-R interacts through the C-terminal ARP peptide with several partner signaling proteins, also promoting cell proliferation (Meshorer et al., 2005). *Ex vivo*, ARP was more effective than cortisol and equally effective as stem cell factor in promoting expansion and differentiation of early

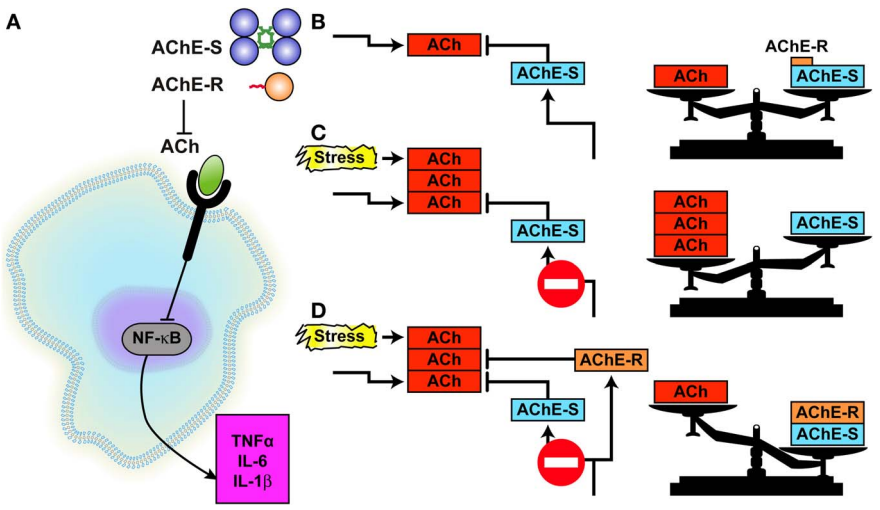

FIGURE 2 | AChE controls the anti-inflammatory reflex. (A) At the extracellular space, both AChE-S and AChE-R hydrolyze ACh, impairing its capacity to block, through the α7 nicotinic ACh receptor, the activation of NFκB which enables pro-inflammatory cytokine secretion (the so-called "anti-inflammatory reflex"). **(B)** Under normal conditions, AChE-S is by far the prominent variant keeping ACh in balance, AChE-R being the soluble, minor component. **(C)** Under stress, excess ACh impairs the cholinergic balance. **(D)** Substitution of AChE-S with excess AChE-R facilitates ACh hydrolysis, shifting the imbalance to AChE excess [Ofek et al. (2007)].

hematopoietic progenitor cells into myeloid and Mk lineages (Pick et al., 2006).

Tissue macrophages are effectively deactivated when exposed to ACh, suppressing the release of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-18 at the post-transcriptional level (Tracey, 2002). Among other effects, immune stimuli induce over-expression of the AChE-targeting microRNA-132 in leukocytes (Soreq and Wolf, 2011). Consequently, microRNA-132 reduces AChE levels, increasing the available ACh and finally inhibiting the secretion of pro-inflammatory cytokines (Wang et al., 2003). Inversely, mice engineered to over-express a 3′-shortened and hence microRNA-132 refractory AChE-R present excessive leukocyte recruitment into the peritoneum, higher levels of inflammatory cytokines than strain-matched controls and impaired cholinergic anti-inflammatory regulation in spite of substantial microRNA-132 up-regulation both in the bone marrow and the hypothalamus. Intravenous injection to wild-type mice of an LNA-modified anti-miR-132 oligonucleotide increases AChE activity in the bone marrow, spleen, and serum, with parallel consequences (Shaked et al., 2009). MiR-132 has been shown to be a regulator of neuronal structure and function. Transgenic mice over-expressing miR-132 in forebrain neurons display increased dendritic spine density and decreased expression of MeCP2, a protein implicated in Rett Syndrome and other disorders of mental retardation. These mice displayed significant deficits in novel object recognition (Hansen et al., 2010). However, shRNA-mediated suppression of the "synaptic" AChE-S variant prevented the increase in miR-132 following footshock stress, suggesting bidirectional regulation (Shaltiel et al., in press).

Furthermore, miR-132 has been shown to be induced during periods of active synaptogenesis and is necessary and sufficient for hippocampal spine formation. Knockdown of the miR-132 target p250GAP increases spine formation while overexpression attenuates this activity (Impey et al., 2010).

AChE AND GRANULOCYTOSIS

AChE-R and ARP both induce granulocytosis (Grisaru et al., 2001). The specificity of ARP is supported by the finding that ASP, the parallel yet distinct C-terminal peptide of AChE-S, fails to induce such effects (**Figure 3**). In the post-partum human serum, AChE-R excess was speculated to lead to reduced ACh concentrations. AChE-R accumulation might hence be perceived as an adaptive response, facilitating the production of pro-inflammatory cytokines to protect the body from post-partum conditions, such as infections. This is compatible with observations of increased production of pro-inflammatory cytokines under stress and further proliferative and cell activation signals (Broome et al., 2000). Protracted post-stress granulocytosis initiated by cortisol would then increase AChE-R production and reduce ACh's anti-inflammatory action.

Subsequent secondary feedback response to the initial excess of ACh and AChE in the periphery is needed to re-balance parasympathetic activities (Erb et al., 2001), suppress the production of pro-inflammatory cytokines in macrophages (Tracey, 2002), and terminate the granulocytosis process. Such responses can potentially involve changes in miR-132 which can suppress AChE activities. In contrast, IL-1β induces ACHE gene expression in phaeochromocytoma cells (Li et al., 2000) (**Figure 4**). The involvement of AChE-R in immune system activities under acute

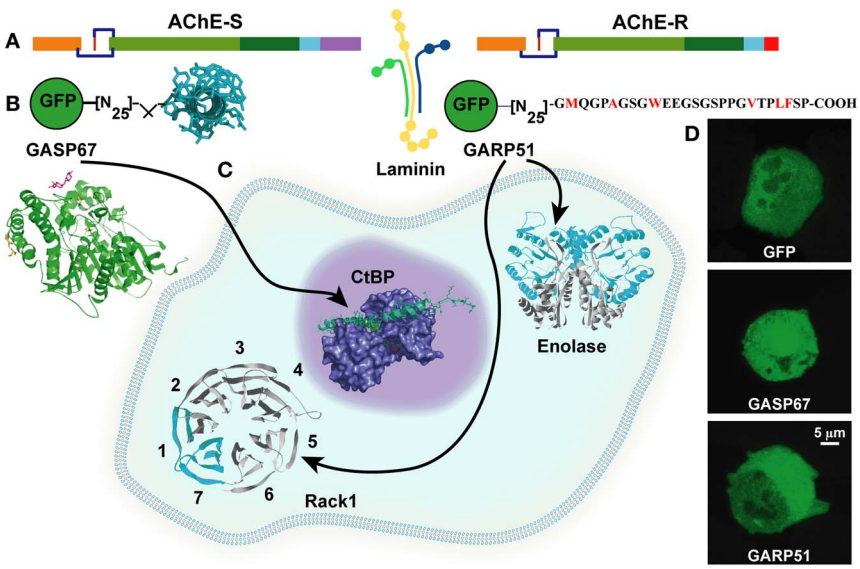

FIGURE 3 | AChE variants exert hematopoietic effects through common and distinct protein partners. (A) The common core domain of AChE interacts with laminin. **(B)** To validate partner interactions, the C-termini of AChE-S and AChE-R were constructed together with GFP. **(C)** The C-terminus of AChE-S interacts with the anti-apoptogenic nuclear CtBp, whereas intracellular AChE-R interacts with the proliferation activating scaffold PKCβ carrier RACK1 and with Enolase. **(D)** Fluorescent cell images: GFP constructs direct the AChE-S and AChE-R C-termini to nucleus and cytoplasm, respectively [Perry et al. (2007)].

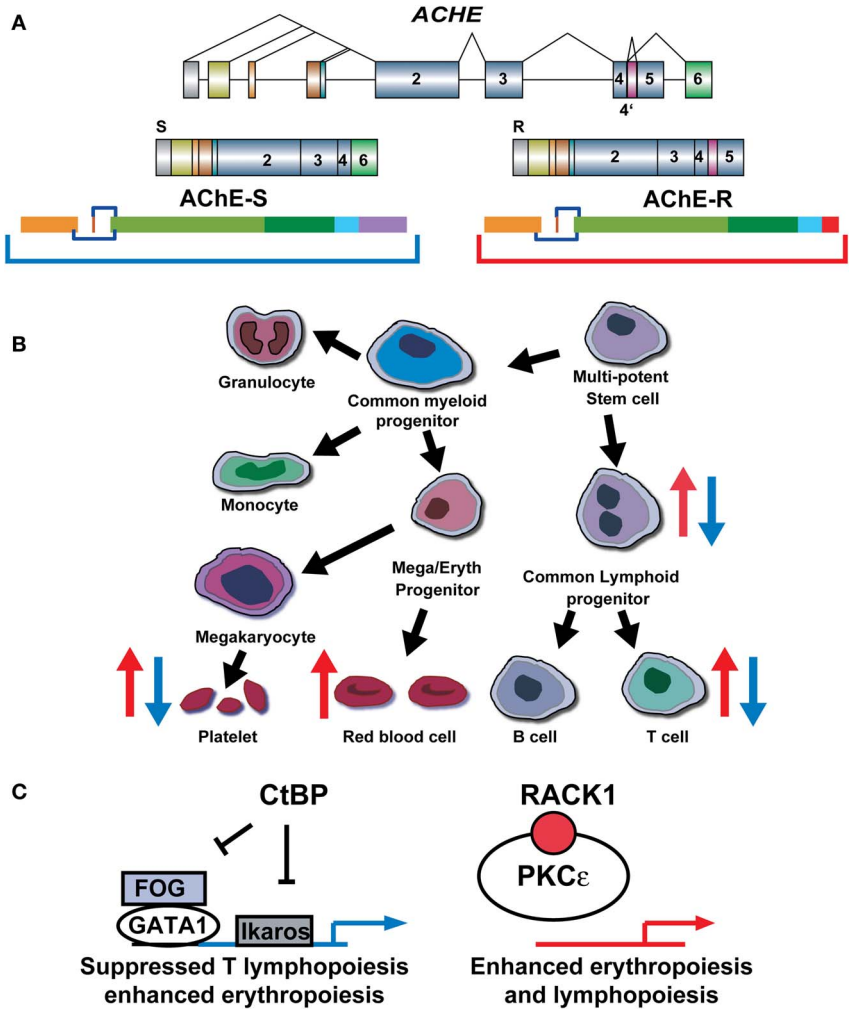

FIGURE 4 | AChE splice variants induce inverse hematopoietic effects. **(A)** The AChE gene and its S and R transcripts. **(B)** The hematopietic pathway (black arrows).Up and down changes in cell compositions are presented as red and blue arrows for transgenic excess in AChE-R (red) and AChE-S (blue). **(C)** Scheme: Inverse outcome and the proteins involved [Gilboa-Geffen et al. (2007)].

stress further prolongs the beneficial (pro-inflammatory) HPA effects mediated through cortisol. While explaining the harmful aspects of stress (chronically high cytokine levels) (Grisaru et al., 2001), increased levels of AChE-R can also maintain the immune system at high alert, avoiding acute suppression of the immune system and providing protection from bacterial infection.

THE AChE-S SPLICE VARIANT CAUSES LEUKOPENIA

AChE gene expression increases during apoptosis (Zhang et al., 2002), and silencing of the AChE gene by small interference (si) RNA prevents apoptosis (Pick et al., 2006). Given that AChE-S is by far the major AChE splice variant in all tissues (Soreq and Seidman, 2001), this suggests that AChE-S induces apoptosis, alternatively or additionally to its known role in the apoptosome formation. One possible mechanism through which this could occur involves the interaction of AChE-S with the anti-apoptotic C-terminal binding protein (CtBP). Supporting this notion, AChE-S excess capable of blocking the anti-apoptotic effect of CtBp reduces T lymphopoiesis and thrombopoiesis, inversely to the effect of AChE-R, which up-regulates hematopoiesis (Gilboa-Geffen et al., 2007). Correspondingly, AChE-S over-expressing mice (TgS) present elevated hemoglobin and total RBC counts accompanied by decreased platelet counts, GR1 + myeloid cells and T lymphocytes [both T helper (CD4+) and T suppressor cells (CD8+)], but unchanged content of monocytes (CD11b+) and B lymphocytes (CD19+) (Perry et al., 2007). This suggests that the CFU-GEMM progenitor cells from AChE-S over-expressing mice preferentially differentiate toward erythropoiesis rather than megakaryocytopoiesis and granulopoiesis, inverse to the stress-induced enhancement of granulopoiesis and thrombocytosis, under AChE-R over-expression (Grisaru et al., 2001). Together, under acute stress (Gilboa-Geffen et al., 2007; Perry et al., 2007), elevated AChE-R and decreased AChE-S levels can, therefore, induce a bimodal myelopoietic and thrombopoietic effect: first, by increasing AChE-R inducing proliferation,

and second by enhancing the CtBP-anti-apoptotic effect due to AChE-S loss which releases CtBP from AChE/CtBP complexes (**Figure 4**).

STRESS-INDUCED THROMBOPOIESIS

Thrombopoiesis increases in response to stress-increased production of AChE-R. This likely involves AChE-R interaction with the scaffold protein RACK1 and its cargo PKCε (Perry et al., 2007), which induces megakaryocytic differentiation in HEL and K562 cells and in primary human HSCs (Lev-Lehman et al., 1997). TPO and platelet counts also increase in the AChE-R overproducing TgR mice as compared to the strain-matched control mice. This affects the non-fatal LPS response: LPS administration (5 ug/kg) induces a rapid fall in platelet counts in both TgR and control mice, but platelet and WBC recovery is considerably faster in TgR mice, likely due to the increased capacity of TgR BM progenitors to proliferate and differentiate into pluripotent CFU-GEMM, CFU-GM, and CFU-Mk (Pick et al., 2006; Perry et al., 2007). Moreover, thrombopoietin (TPO) increases PKCε expression in mouse Mks, whereas blocking PKC activation inhibits platelet formation (Deutsch et al., 2002). Thus, the elevated levels of TPO, AChE-R, and PKCε in plasma and Mks from TgR mice support the notion of a cholinergic promotion of thrombopoietic signal transduction both through the hydrolytic and the non-enzymatic features of AChE-R. The pivotal role played by AChE-R and ARP in hematopoiesis and thrombopoiesis suggests a potential new strategy for improving post-engraftment thrombopoiesis.

STRESS AND THYMOCYTE DEVELOPMENT

In the autoimmune disease Myasthenia gravis (MG) AChE-R translocates to the membrane-associated fraction of thymocytes, reflecting the activation state of the thymic cells. Like thymus from MG patients, the thymus from TgR mice displays an abnormally high number of immature thymocytes compared to control mice. PKCβII which plays a role in B cell receptor survival signaling (Oshevski et al., 1999) is also over-expressed in TgR mice compared to FVB/N controls. AChE-R could thus protect immature thymocytes from apoptosis, maintaining a high level of the anti-apoptotic protein Bcl-2 (Rojnuckarin and Kaushansky, 2001). The high number of thymocytes in TgR mice is compatible with production of AChE-R by cortical stromal cells exerting a protective signal from apoptosis to the neighboring thymocytes. That immature thymocytes located in the cortex require contact signals with the cortical epithelium to survive (Toiber et al., 2008), may therefore, explain why excessive AChE-R production by cortical stromal cells induces an efficient positive selection process in the TgR thymus. Also, AChE-R could be produced by the thymocytes themselves, and thymocytes cultured from TgR mice are more resistant to apoptosis than normal thymocytes, even in the absence of stromal cells. Cholinergic signals and AChE's catalytic activity thus influence thymocytic apoptosis in inverse directions, so that the pro-apoptotic effect of ACh is counterbalanced by a proliferative effect that could be predominant for the AChE-R variant. Supporting this notion, the TgR thymus presents more proliferating cells (expressing the Ki67 antigen) compared to control mice (Gilboa-Geffen et al., 2007) (**Figure 5**).

STRESS, AChE SPLICE VARIANTS AND ANTISENSE MODULATION

Antisense oligonucleotides are short synthetic strands of modified DNA or RNA designed to hybridize within the cell with target-specific mRNA by Watson–Crick base pairing. Binding activates RNAses that degrade them. RNA–antisense complexes block synthesis of the protein encoded by the mRNA sequence and release the antisense agent to bind to a further strand of mRNA, creating a recycling antisense mechanism. An antisense agent which selectively destroys AChE-R, EN101 (currently denoted BL-7040) is a 20-mer oligonucleotide, chemically modified by incorporating 2′oxymethyl groups in the last three nucleotides at its 3′ end. EN101/BL-7040 binds to a coding sequence common to all splice variants of human AChE (Evron et al., 2005). Since the AChE-R mRNA transcript includes less guanosines and cytosines than AChE-S, it is intrinsically less stable and more sensitive to this treatment. BL-7040/EN101 is an FDA approved orphan drug shown to be safe for human use and active both intravenously and orally in the suppression of the progressive muscle fatigue symptoms of rats with experimental myasthenia gravis (MG), an autoimmune neuromuscular disease involved with systemic AChE-R over-production (Brenner et al., 2003). It was also demonstrated to suppress AChE-R in primates (Evron et al., 2005) and in human myasthenic patients (Argov et al., 2007; Sussman et al., 2008).

Other short oligonucleotides, designated aptamers can be selected to tightly and specifically bind a target protein molecule (Que-Gewirth and Sullenger, 2007) and have been approved for the therapeutic use by the US FDA. A 50-mer nuclease-resistant RNA aptamer with 2′-amino-modified pyrimidines can recognize human muscarinic AChR, and cross-react with MG patient autoantibodies (Lee and Sullenger, 1997), efficiently preventing them from down-modulating AChR expression on human cells (Que-Gewirth and Sullenger, 2007). Intriguingly, BL-7040 also operates as an aptamer activator of Toll-like receptor 9 (TLR9) facilitating an alternative route of NFκB, controlling salivary function (Gilboa-Geffen et al., 2011) and avoiding post-traumatic stress in mice exposed to predator scent (Zimmerman et al., 2012).

STRESS AND NEUROIMMUNE SIGNALING

Within the central nervous system, neuronal ACh levels rapidly increase under stress, followed by a feedback response of AChE overproduction which can tilt the balance to reduce ACh levels (Kaufer et al., 1998). Correspondingly, the key pro-inflammatory mediator IL-1 is over-expressed in the brain under stress. That IL-1 can induce neuronal AChE synthesis (Li et al., 2000) would further suppress ACh levels, inducing a feed-forward loop of stress-inducible inflammatory response in the brain. This response is pivotal for those neural systems thought to be involved in stress effects in PTSD in humans (Rauch et al., 2006) and in predator stress effects in animals (Adamec et al., 2005). Systems implicated include the amygdala, hippocampus, and medial prefrontal cortex and brain stem targets of forebrain limbic systems. It is unlikely that within such complex circuitry, changes in one system are responsible for all lasting effects of stress. Nevertheless, neuronal AChE-R has been found to be functionally involved in

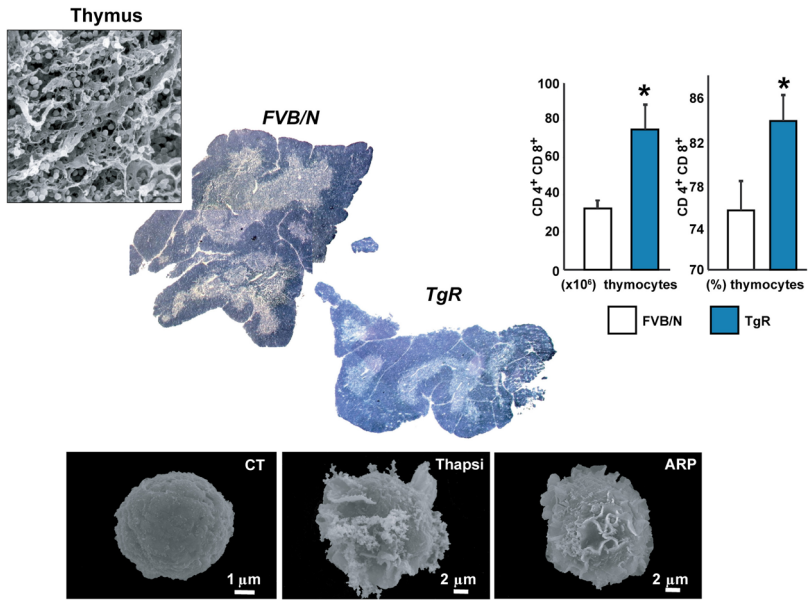

FIGURE 5 | AChE-R potentiates lymphopoiesis and megakaryopoiesis. Excess of the stress-induced AChE-R variant induces thymocytes hyperproliferation, compatible with the clinical phenotype in myasthenic patients, as well as thrombopoiesis. This reflects on myasthenia gravis where antisense suppression of AChE-R was effective in phase I clinical trials [Argov et al. (2007)]. Below: Megakaryoblasts (Meg-01) cells scanned by electron micrographs. Control Meg-01 cells exhibit a smooth surface. Cells treated for 24 h with ARP show initial formation of flat membrane sheets (middle panels) or elongated pseudopodia reflecting proplatelet formation territories (right panels) characteristic of megakaryocytic differentiation [Guimaraes-Sternberg et al. (2006)].

stress-mediated facilitation of fear learning, limbic neural plasticity, and change in affect in predator stress (Adamec and Shallow, 1993; Nijholt et al., 2004; Adamec et al., 2008). Specifically, stress-induced transcription of AChE-R facilitates neuroplasticity in the brain's limbic structures, resulting in long-lasting changes in neural systems (Meshorer et al., 2002; Nijholt et al., 2004).

Severe stress may precipitate affective disorder lasting up to a life time after traumatic stress (Yehuda, 2002). Predator stress (inescapable and unprotected exposure to a cat) likewise lastingly increases rodent anxiety (Adamec and Shallow, 1993). Increased expression of AChE-R following stress has been demonstrated in some of the same areas involved in predator stress-induced limbic neuroplasticity, including the hippocampus and amygdala. Intriguingly, BL-7040/EN101 administration limits these stress reactions. Predator stress abnormally increases the transcription of AChE-R, which in turn alters the neural substrate responsible for the changes in acoustic startle amplitude. BL-7040/EN101 reduces the mRNA for AChE-R, and prevents the occurrence of these changes (Adamec et al., 2008). This effect involves TLR9 activation and is preventable by the TLR9 blocking oligonucleotide ODN 2088 (Zimmerman et al., 2012). It has been proposed that following severely stressful events, AChE-R increases to a level which is no longer adaptive and results in physiological changes which contribute to changes in affect observed in post-traumatic stress disorder (PTSD) (Meshorer et al., 2002; Nijholt et al., 2004). Correspondingly, systemic administration of BL-7040/EN101 selectively lowers the level of AChE-R both peripherally (Argov et al., 2007) and in the brain (Evron et al., 2005; Pollak et al., 2005). In view of the TLR9 involvement, this effect indicates inter-related coordination between the innate immune system and the AChE gene expression. Termination of the granulocytosis response in the periphery thus resembles the termination of stress-inducible neurotransmission in the brain, highlighting the close inter-relationships between the neuronal and immune cell reactions to stress (Kiecolt-Glaser et al., 2003).

NON-CATALYTIC ROLE FOR AChE IN THE CNS

The stress-related role of neural AChE was inferred from experiments in which over-expression of AChE-R mRNA was suppressed in mice subjected to closed head injury by administration of mouse/EN101 (Shohami et al., 2000). This treatment reduced the number of dead neurons and facilitated neurologic recovery, suggesting that AChE-R and/or secondary element(s) induced by overexpression of AChE-R contributed to neuronal death (Metz and Tracey, 2005). This could reflect a direct contribution of the increased hydrolytic capacity of the induced AChE to reduce the tissue level of ACh, promoting inflammation and tissue damage. Alternatively, or in addition, stress-induced AChE could selectively contribute to cellular apoptosis through its non-catalytic properties, inducing cell death in non-cholinergic neurons as well (Zhang et al., 2002; Park et al., 2004).

Photoreceptor loss is the primary cause of blindness in degenerative diseases such as retinitis pigmentosa and age-related macular degeneration. Exposure of albino rats to bright light provides an established model for photoreceptor damage and stress-induced photoreceptor injury (Stone et al., 1999). A variety

of growth hormones, cytokines (LaVail et al., 1992), and antioxidants (Organisciak et al., 1992) were found to partially protect retinal photoreceptors from light damage, suggesting causal involvement of stress-induced processes. The importance of AChE gene expression for retinal maintenance was experimentally validated in a detailed neuro-anatomic survey that demonstrated impaired formation of the inner retina and degeneration of photoreceptors in an AChE knockout mouse where ALL of the AChE variants are missing (Bytyqi et al., 2004). The major excitatory neurotransmitter of the retina is L-glutamate, whereas ACh plays a limited role in visual information processing within the retina and is found mainly in amacrine and ganglion cells in the proximal part of the retina (Hutchins, 1987; Criswell and Brandon, 1993). Either AChE-S or AChE-R can appear either with a cleavable N-terminal signal peptide or with an extended N terminus, potentially making it membrane bound. AChE mRNA expression in human adult photoreceptors (Broide et al., 1999), cells that are not involved in cholinergic synaptic activity further raises the possibility that AChE in photoreceptors exerts stress-related morphogenic function(s). It was shown that the N-AChE protein plays a detrimental role, exacerbating photoreceptor injury after exposure to bright, damaging light. Notably, EN101 treatment limited light-induced damages to the retina (Kehat et al., 2007). Future experiments are needed to find out whether N-AChE-R accumulates in retinal (and other) neurons after other stressful episodes such as increased pressure (glaucoma), metabolic stress (diabetes), and ischemia, where it may play detrimental role(s) promoting neuronal and specifically retinal damage.

CONCLUDING REMARKS

To properly function, the immune system depends on a fine-tuned balance of immune cell activation, proliferation, and differentiation which is perturbed under stress. Consequently, hematopoietic and immune responses are largely modified under stress in an inter-related manner conserved throughout evolution. First, stress inducible changes in hematopoietic processes can substantially modulate both the immune and CNS functions, for example, by over-producing cytokines which can penetrate the brain and affect neuronal activities. Second, the parasympathetic system, largely through AChE modulations, contributes to the delicate balance between hematopoietic and immune events and CNS reactions under stress. The major AChE splice variant AChE-S is replaced by the stress-induced AChE-R variant. Due to modified splicing, consequently impaired AChE-S-CtBp interaction as well as enhanced AChE-R-RACK1 interactions, both enhance granulopoiesis and thrombocytosis. Increased levels of AChE-R can thus trigger beneficial resistance to acute overstimulation under exposure to microbial molecules such as LPS, but at the same time sustaining the harmful effects of chronically high cytokine levels. This balance of stress-inducible positive and negative effects can be maintained by diverse Oligonucleotide treatments, achieving better control over both nervous system and immune functions. While these therapeutic strategies await further studies to discern the corresponding molecular mechanisms, the promising findings can already point at numerous previously unforeseen interactions between the immune and the nervous system.

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Acetylcholinesterase loosens the brain's cholinergic anti-inflammatory response and promotes epileptogenesis

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Recent studies show a key role of brain inflammation in epilepsy. However, the mechanisms controlling brain immune response are only partly understood. In the periphery, acetylcholine (ACh) release by the vagus nerve restrains inflammation by inhibiting the activation of leukocytes. Recent reports suggested a similar anti-inflammatory effect for ACh in the brain. Since brain cholinergic dysfunctions are documented in epileptic animals, we explored changes in brain cholinergic gene expression and associated immune response during pilocarpine-induced epileptogenesis. Levels of acetylcholinesterase (AChE) and inflammatory markers were measured using real-time RT-PCR, *in-situ* hybridization and immunostaining in wild type (WT) and transgenic mice over-expressing the "synaptic" splice variant AChE-S (TgS). One month following pilocarpine, mice were video-monitored for spontaneous seizures. To test directly the effect of ACh on the brain's innate immune response, cytokines expression levels were measured in acute brain slices treated with cholinergic agents. We report a robust up-regulation of AChE as early as 48 h following pilocarpine-induced status epilepticus (SE). AChE was expressed in hippocampal neurons, microglia, and endothelial cells but rarely in astrocytes. TgS mice overexpressing AChE showed constitutive increased microglial activation, elevated levels of pro-inflammatory cytokines 48 h after SE and accelerated epileptogenesis compared to their WT counterparts. Finally we show a direct, muscarine-receptor dependant, nicotine-receptor independent anti-inflammatory effect of ACh in brain slices maintained *ex vivo*. Our work demonstrates for the first time, that ACh directly suppresses brain innate immune response and that AChE up-regulation after SE is associated with enhanced immune response, facilitating the epileptogenic process. Our results highlight the cholinergic system as a potential new target for the prevention of seizures and epilepsy.

Keywords: acetylcholinesterase, epileptogenesis, hippocampus, inflammation, status epilepticus

INTRODUCTION

Accumulating experimental evidence indicates that brain immune response and inflammatory mediators decrease the threshold for individual seizures and contributes to the process of epileptogenesis (for review see: Vezzani and Granata, 2005; Rijkers et al., 2009; Vezzani et al., 2011). Therefore, understanding the mechanisms controlling the extent and duration of the brain's inflammatory response to status epilepticus (SE) and injury may promote the understanding of epileptogenesis and highlight new therapeutic targets for the prevention and treatment of epilepsy.

Peripheral inflammation is controlled, amongst others, by the cholinergic anti-inflammatory pathway which involves inhibition of innate immune responses by ACh released from the vagus nerve. This mechanism is dependent on the alpha7 subunit of the nicotinic acetylcholine receptor (α7nAChR), which inhibits NF-kappaB nuclear translocation and suppresses cytokine release by monocytes and macrophages (Tracey, 2002, 2009). Centrally acting inhibitors of the ACh hydrolyzing enzyme, acetylcholinesterase (AChE), were shown to enhance the peripheral cholinergic anti-inflammatory pathway via central muscarinic receptors (Pavlov et al., 2006, 2009). Recent reports point toward a cholinergic anti-inflammatory mechanism also in the central nervous system (CNS). In cultured microglia, ACh pretreatment inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNFα) release via α7nAChR (Shytle et al., 2004). Accordingly, AChE inhibition reduced microglial production of TNFα in a hypoxia model (Wang et al., 2010). AChE inhibitors have also been shown to reduce glial activation and inflammatory cytokine production in experimental autoimmune encephalomyelitis (Nizri et al., 2008) and in a cerebral hypoperfusion model in the rat (Wang et al., 2010).

We recently reported cholinergic dysfunction and up-regulation of AChE in temporal lobe structures of SE-experienced epileptic rats (Zimmerman et al., 2008). These results raised the

hypothesis that transcriptionally controlled cholinergic imbalance may have a role in brain immune response during epileptogenesis. We thus used the pilocarpine model of epilepsy to study AChE expression and the role of consequent cholinergic imbalance on brain immune response and epileptogenesis.

MATERIALS AND METHODS

All animal experiments were approved by the Committee for Ethics in Animal experimentation of the Faculty of Health Sciences, Ben-Gurion University of the Negev. Wild type (WT) FVB/N (Harlan Laboratories, Jerusalem), and transgenic mice over-expressing human AChE-S (TgS) (Beeri et al., 1995) were maintained on a 12 h light/dark cycle and had access to food and water ad libitum. All chemicals were purchased from Sigma unless otherwise mentioned.

PILOCARPINE TREATMENT

The well-established pilocapine model for temporal lobe epilepsy was induced (Cavalheiro et al., 1996; Shibley and Smith, 2002; Chen et al., 2005). Pilocarpine (290–340 mg/kg i.p.) was injected to adult (8–16 weeks old, 26–34 g BW) male mice 15 min after pre-treatment with methyl-scopolamine (1.5 mg/kg i.p.). Animals were observed for 2 h after pilocarpine injection, and seizures were evaluated according to a modified version of the Racine scale (Racine, 1972; Borges et al., 2003). SE was defined by continuous seizure activity consisting of stages 3.5–5 seizures, five taken as the onset. Forty minutes from onset, SE was interrupted by injection of diazepam (4 mg/kg i.p.). Control mice were treated with the same protocol, except that saline was injected instead of pilocarpine, followed, 1 h later, by diazepam.

Epilepsy was diagnosed in the presence of at least one spontaneous stage five seizure observed during a 48 h period of video-monitoring, 1–3 months after SE (in 11 WT mice). For the comparison of spontaneous seizures frequency between strains, WT (eight mice) and TgS (12 mice) were simultaneously video-monitored 1 month following SE.

PROCESSING OF TISSUE SAMPLES

Forty-eight hours after SE, animals were deeply anesthetized with isofluorane and decapitated. For enzymatic activity and RT-PCR, brains were quickly removed and the hippocampus was dissected and frozen in liquid nitrogen. All tissues were stored fresh at −80°C. For native protein extraction, frozen tissue was thawed and homogenized on ice with 200 µl homogenization buffer (0.01 M sodium phosphate pH 7.4, 1% triton, 4°C). Lysates were pre-cleared by centrifugation at 17,000 rpm at 4°C for 30 min, and the total protein concentration of the supernatant was determined using Bradford solution (Bio-Rad, Israel) (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard. The supernatants were kept on ice until the cholinesterase assay was performed within 2 h after homogenization. For histological staining, hemispheres were either fixed in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 48 h and paraffin-embedded, or horizontally sliced to 400 µm sections, fixed in 4% PFA in phosphate buffer (PB) (0.1 M Na_2HPO_4, 0.02 M NaH_2PO_4, pH 7.4) and kept in 4°C until re-slicing. Slices were immersed in 30% sucrose overnight and then re-sliced to 30 µm sections in a freezing microtome (Leica Microsystems, Wetzlar, Germany).

KARNOVSKY-ROOT STAINING IN NON-DENATURING ELECTROPHORESIS GEL AND BRAIN SECTIONS

Electrophoresis of active AChE was performed in non-denaturing conditions. Fifty milligrams protein from each supernatant was loaded on 7.5% vertical polyacrylamide gel at 4°C for 1 h (Bon et al., 1988). Migration was performed in the presence of 0.25% Triton X-100; AChE activity was revealed as reported by Karnovsky and Roots (Karnovsky and Roots, 1964; Kaufer et al., 1998). Bands intensity was quantified using a homemade MatLab script. AChE activity staining was performed in 200 µm fresh horizontal sections using the same staining solution (Zimmerman et al., 2008). Images were taken with a stereomicroscope (Zeiss, Lumar.V12 and an AxiCam MRc5).

REAL-TIME RT-PCR, *In-situ* HYBRIDIZATION AND IMMUNOSTAINING

Real-time RT-PCR was performed using a standard protocol as previously described (Zimmerman et al., 2008). Total RNA was extracted using Absolutely RNA Miniprep Kit (Stratagene, CA, USA), and reverse transcribed (300 ng) using Verso™ cDNA Kit (Thermo, MA, USA). RT-PCR was performed using ABsolute™ QPCR SYBR® Green ROX (ABgene, UK) in a 7900 HT Sequence Detection System (Applied Biosystems, CA, USA). Quantification was assessed at the logarithmic phase of the PCR reaction. The PCR annealing temperature was 60°C for all primer pairs (**Table 1**). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene.

For double labeling histological experiments we combined *in-situ* hybridization (FISH) with immunofluorescence (IF). Stainings were done on seven µm-thick paraffin-embedded sagittal sections or 30 µm-thick freezing microtome horizontal sections. Prior to staining, 30 µm sections were washed three times in PB and mounted on SuperFrost slides (Thermo, MA, USA). FISH was performed as previously described (Berson et al., 2008) with slight modifications: for antigen retrieval, slides were soaked in 0.01 M citrate buffer pH six, heated in the micro-wave for 15 min, washed in double-distilled water and with PBT. Hybridization mix [including 50-mer 5′-biotinylated, 2-*O*-methylprotected complementary RNA probes (**Table 2**) 10 µg/ml] was applied for 10 min in 60°C and then for 90 min in 52°C (90 min and overnight respectively for 30 µm sections). After washing with TBST buffer (25 mM Tris–HCl pH 7.5, 136 mM NaCl, 2.7 mM KCl, 0.05% Tween-20), slides were incubated in blocking solution for 30 min. Primary antibodies (**Table 3**) diluted in the blocking solution were incubated (90 min) at room temperature (30 µm sections—overnight in 4°C). After three rinses in TBST, sections were incubated in a mix of streptavidin-conjugated Cy2 and Cy3 or Cy5 conjugated goat IgG (Jackson Immunoresearch, 1:100 dilution) in TBST for 1 h at room temperature. Slides were then washed with TBST, and mounted with ImmunoMount (Thermo, MA, USA). Slides stained for Iba1 were incubated in PBS with 0.5% Triton X-100 for 1 h, before blocking.

Table 1 | Primer pairs.

Gene	Accession #	Primers
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM017008	F(1242): GGGTGTGAACCACGAG R(1417): AGGGGCCATCCACAGTCT
Acetylcholinesterase (AChE)S-variant	AF002993	F(7855): CTTCTGGAACCGCTTCCTCCCCAAATT R(6916): GTAGTGGTCGAACTGGTTCTTCCAGTGCAC
Acetylcholinesterase (AChE)R-variant	AF002993	F(7855): CTTCTGGAACCGCTTCCTCCCCAAATT R(7779): GGTTACACCTGGCGGGCTCC
Acetylcholinesterase (AChE)N-variant	AY389979	F(188): AATGCTAGGCCTGGTGATGT R(285): GGCAGTGGAAACTTCTGGA
Interleukin 1 beta (IL-1β)	NM008361.3	F(368): GCCCATCCTCTGTGACTCAT R(597): AGGCCACAGGTATTTTGTCG
Interleukin 12 (IL-12)	M86672.1	F(600): CATCGATGAGCTGATGCAGT R(762): CAGATAGCCCATCACCCTGT
Tumor necrosis factor alpha(TNF-α)	NM013693	F(946): AGCCCCCAGTCTGTATCCTT R(1157): CTCCCTTTGCAGAACTCAGG
High-mobility group box 1 (HMGB1)	NM010439.3	F(4): ACAGAGCGGAGAGAGTGAGG R(247): GGGTGCTTCTTCTTGTGCTC

Table 2 | Complementary RNA probes.

Probe	Beginning point in the mouse AChE gene	Sequence
mAChE-S	Exon E6 position 1,392	5′-CCCCUAGUGGGAGGAAGUCGGGGAGGAGUGGACAGGGCCUGGGGGCUCGG-3′
mAChE-R	Intron I4 position 74	5′-AACCCUUGCCGCCUUGUGCAUUCCCUGCUCCCCCCACUCCAUGCGCCUAC-3′

Table 3 | Dilution, blocking, and designated sections for all primary antibodies.

Primary antibody	Source	Dilution	Blocking	Designated sections
Mouse anti-GFAP	Sigma	1:400	4% goat serum (Sigma) +	7 μm-thick paraffin-
Rabbit anti-GAD65/67	Sigma	1:400	2% BSA (Sigma)	embedded sections
Mouse anti-NeuN	Chemicon	1:100		30 μm-thick freezing
Rabbit anti-Iba1	Wako	1:1000 (+0.3% Triton X-100)	Dyna Antibody Diluant (Dyna Scientific) (+0.5% Triton X-100)	microtome sections

Abbreviations: GFAP, glial fibrillary acidic protein; GAD, glutamic acid decarboxylase; NeuN, neuronal nuclei; Iba1, ionized calcium binding adaptor molecule 1.

ACUTE SLICES PREPARATION AND DRUG APPLICATION

Preparation and maintenance of brain slices *ex-vivo* was performed using standard procedures as previously reported (Zimmerman et al., 2008). In short, mice (2–4 month old) were deeply anesthetized; brains quickly removed and 400 μm horizontal slices were obtained (NVSLM1—motorized advance vibroslice, WPI, USA). Slices were maintained in a standard interface chamber at 36 ± 1°C and were superfused with artificial cerebrospinal fluid (ACSF) contained (in mM): NaCl, 129; $NaHCO_3$, 21; NaH_2PO_4, 1.25; $MgSO_4$, 1.8; $CaCl_2$, 1.6; KCl, 3; glucose, 10; pH 7.4. Treated slices were incubated simultaneously with matched controls (in separate chambers). Drugs were added to the bathing solution after 30 min incubation with ACSF for 4 h. Slices were then transferred to liquid nitrogen for RT-PCR. Drugs included carbamylcholine chloride (CCh, 50 μM), acetylcholine (ACh 10 or 50 μM), physostigmine (1 μM), Atropine (1 μM), Mecamylamine (50 μM), α-bungarotoxin (100 μM) and tetrodotoxine (TTX, 1 μM).

MICROSCOPY AND IMAGE ACQUISITION

All fluorescent images were obtained using XYZ scanning with an Olympus FluoView FV1000 confocal microscope (Olympus, Hamburg, Germany) or with a Nikon inverted TI microscope (Nikon, Japan) and a cooled 14 bit CCD camera (Coolsnap HQ2, Photometrics, Tucson AZ). For quantification, Six to eight images (×20) from non-overlapping hippocampal fields were taken from each slice (three slices per animal, two to three animals per group) using the same acquisition parameters. Analysis was done using a homemade MatLab script: intensity values for each image were normalized to the mean. A constant cut-off value was set to establish a threshold defining background ("black") and signal ("white") pixels. The total number of white pixels (stained area)

for each binary image was summed and that value was later used for statistical analysis.

STATISTICAL ANALYSIS

The non-parametric Mann–Whitney test, One-Way ANOVA, Chi-square (χ^2), and two-tailed independent student *t*-test were applied (SPSS; SPSS Inc., Chicago, USA) as mentioned in the text. $P < 0.05$ was considered significant. Data is expressed as the mean ± SEM.

RESULTS

SE was achieved in 75.7% of mice injected with pilocarpine ($n =$ 53 out of 70) with a mortality rate of 22.6%. In WT mice, 48 h video-monitoring at 3 months after SE revealed a high percentage ($n = 9$ out of 11, 82%) of epileptic mice (≥1 spontaneous unprovoked stage 5 seizure).

AChE IS UP-REGULATED DURING EPILEPTOGENESIS

As previous studies showed AChE upregulation in chronic epileptic animals, we studied AChE expression 48 h after SE to challenge the hypothesis that cholinergic imbalance occurs early during the epileptogenesis process. All known alternatively spliced AChE mRNA transcripts, including AChE-S, AChE-R, and N-AChE were found to be significantly up-regulated in the hippocampus of treated mice compared to saline-injected controls (**Figure 1A**). Karnovsky–Root staining confirmed increased AChE activity in hippocampal homogenates (**Figure 1B**) as well as in sections obtained from SE-experienced mice (**Figure 1C**). Within the hippocampus, AChE activity was most prominent in the stratum molecular of the dentate gyrus, the stratum oriens of the CA1 and CA3 regions, the subiculum and the entorhinal cortex (**Figure 1C**).

To identify specific cell populations expressing AChE mRNA following SE, we combined AChE FISH with immunostaining against specific cell-type markers. FISH showed intensified AChE expression in neurons within all regions of the hippocampus, which was most prominent in the pyramidal cell layer of all CA sub-regions (**Figures 2A,B,G**). In addition, ACHE mRNA was co-localized with GAD positive inhibitory interneurons in all hippocampal sub-regions (**Figure 2H**). We further searched for AChE expression in glia cells, known to be activated early following SE and suggested to play a role in epileptogenesis (Borges et al., 2003; Turrin and Rivest, 2004; Ivens et al., 2007; Pitkanen et al., 2007; David et al., 2009). GFAP immunostaning indeed indicated a strong astroglial activation after SE (**Figures 2C,D**); However, while AChE mRNA was observed along blood vessels, probably in endothelial cells, co-labeling experiments failed to reveal significant ACHE mRNA expression within GFAP-positive astrocytes (**Figures 2I,J**). Conversely, in SE-exposed mice we found a robust increase in immunolabeling for the microglia marker, Iba1 (**Figures 2E,F**; $p < 0.001$; control: $n = 47$, treated: $n = 70$ fields from three sections per animal, 2/3 animals per group; independent *t*-test), co-labeled with a strong FISH signal for AChE mRNA (**Figures 2K,L**). In addition, Iba1 positive cells following SE presented perikaryal hypertrophy and processes retraction (**Figure 2L**), a clear morphological pattern for their activation (Rappold et al., 2006).

BRAIN AChE LEVELS AND LOCAL IMMUNE RESPONSE

The rapid up-regulation and expression pattern of AChE after SE together with the known cholinergic control of immune response in the periphery led us to challenge the hypothesis that the observed cholinergic imbalance is sufficient to induce a local innate immune response within the brain. We thus tested the immune response in transgenic mice with a constitutive overexpression of the human synaptic variant of acetylcholinesterase (AChE-S) (TgS) before and 48 h following SE. In TgS mice, showing 50% more AChE activity than WT animals (Beeri et al., 1995; and **Figure 3A**), immunolabeling against Iba1 was significantly

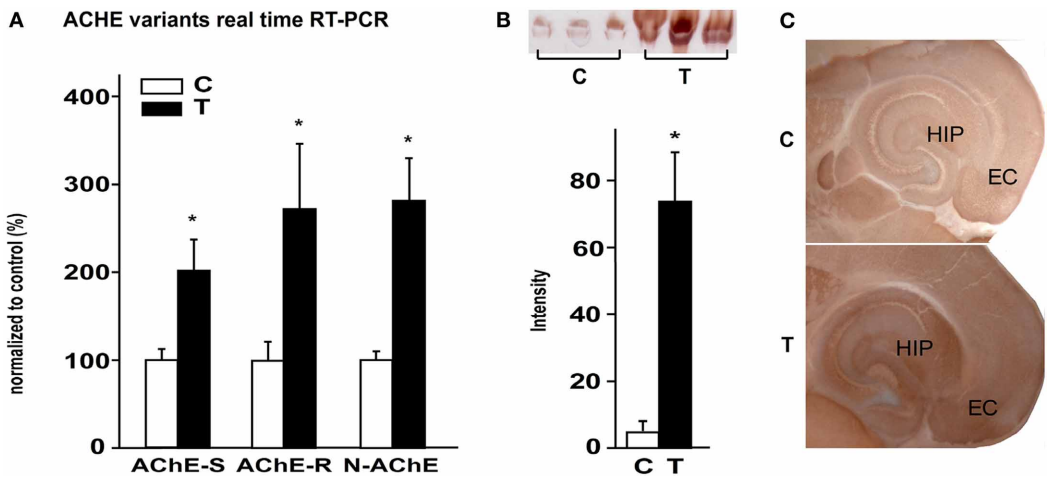

FIGURE 1 | AChE up-regulation 48 h post SE. (A) expression levels of AChE-S, AChE-R, and N-AChE 48 h after SE, as revealed by real-time RT-PCR ($p = 0.05$, 0.053, and 0.025 respectively; $n = 5$ controls, $n = 3$ or 4 treated; mean ± SEM; Mann–Whitney test). **(B)** Top: Karnovsky–Roots staining on non-denatured activity gel of control and 48 h after SE samples. Bottom: gel quantification ($p = 0.05$; $n = 3$; mean ± SEM; Mann–Whitney test). **(C)** Karnovsky–Roots staining of 200 μm horizontal brain sections from control and 48 h post SE mice. C, control; T, treated.

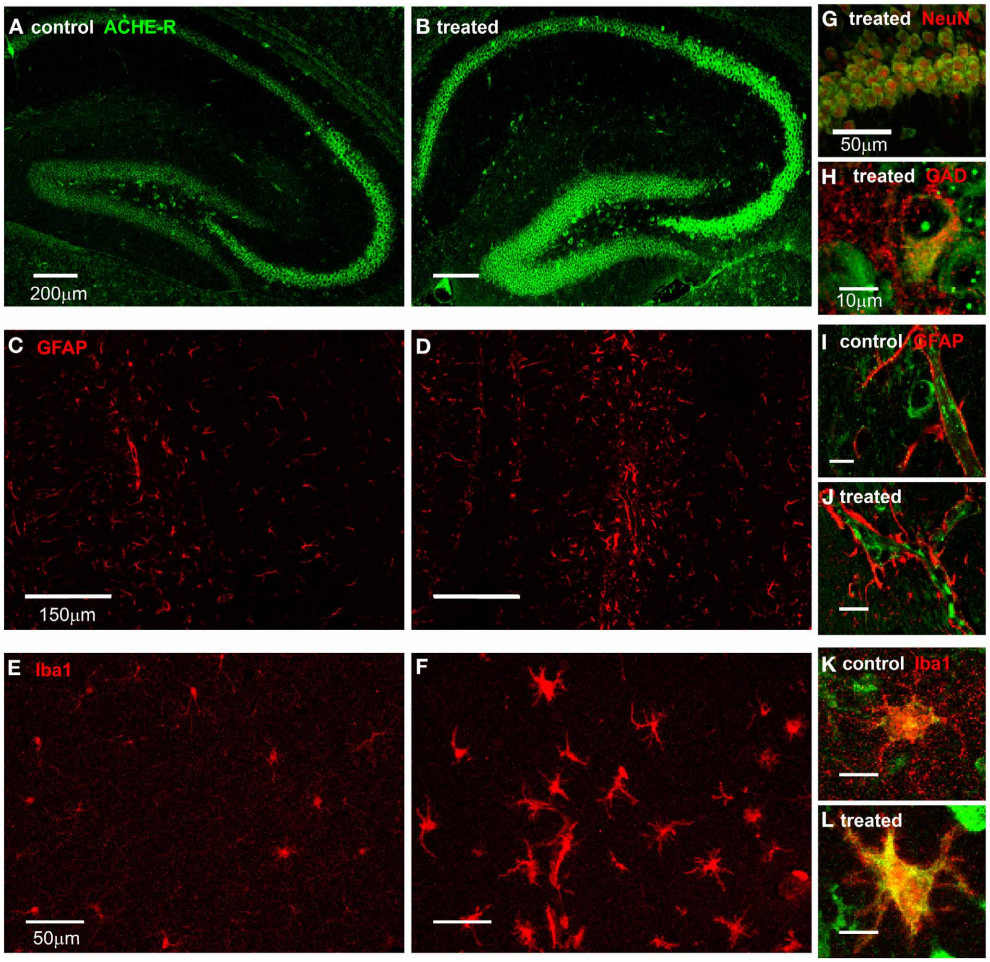

FIGURE 2 | AChE expression in the hippocampus. (A,B) FISH for AChE-R in the hippocampus of control **(A)** and 48 h after SE **(B)** mice. **(C,D)** GFAP staining in the hippocampus of control **(C)** and 48 h post SE **(D)** mice. **(E,F)** Iba1 staining in the hippocampus of control **(E)** and 48 h post SE **(F)** mice. **(G)** Double labeling of AChE (green) and NeuN (red) in CA1 area of a treated animal. **(H)** Double labeling of AChE (green) and GAD65/67 (red) in the hilus of a treated animal. **(I,J)** Double labeling of AChE (green) and GFAP (red) in hippocampus of control **(I)** and treated **(J)** mice (scale 10 μm). Note the clear positive AChE staining in the blood vessel and the endothelial cells but not in the end fit of GFAP-positive astrocytes surrounding it. **(K,L)** Double labeling of AChE (green) and Iba1 (red) in hippocampus of control **(K)** and treated **(L)** mice (scale 10 μm).

increased compared to WT ($p = 0.046$; WT: $n = 47$, TgS: $n = 70$ fields from three sections per animal, 2/3 animals per group; independent t-test). This increase was associated with upregulation of mRNA for high mobility group box 1 (HMGB1)—a pro-inflammatory cytokine-like mediator expressed in activated microglia (Lotze and Tracey, 2005; Kim et al., 2006; Hayakawa et al., 2008) (**Figure 3F**; $p = 0.014$). Forty-eight hours following SE both WT and TgS mice showed higher labeling of Iba1 compared to their matched controls (**Figure 3B**); However, treated TgS mice showed significantly higher (30%) labeling compared to that observed in treated WTs (17%) ($p = 0.043$; control: $n = 47$, treated: $n = 71$ fields from three sections per animal, 2/3 animals per group; independent t-test). In addition, up-regulation of IL-1β and IL-12 was significantly higher in TgS mice compared to WT (**Figures 3C,D**). SE-induced up-regulation of TNFα mRNA was similar in both WT and TgS mice (**Figure 3E**). HMGB1 was also upregulated ($p = 0.027$) in WT exposed to SE and matched the already high levels in non-treated TgS (**Figure 3F**).

TgS MICE OVER-EXPRESSING AChE SHOW ACCELERATED EPILEPTOGENESIS

To test whether the observed increase in immune response in treated TgS mice is associated with altered epileptogenesis (Vezzani et al., 2008), we video-monitored WT and TgS mice for 48 h one month after SE. Importantly, no differences in the acute response to pilocarpine (rate of SE, delay to SE, seizure phenotypes, response to diazepam or survival) were observed between WT and TgS mice (**Figures 4A–C**). In contrast, video-monitoring at 1 month uncovered a significantly higher rate of TgS mice presenting spontaneous seizures compared to WT mice (**Figure 4D**). These results support the notion that AChE over-expression is associated with enhanced immune response and facilitated epileptogenesis.

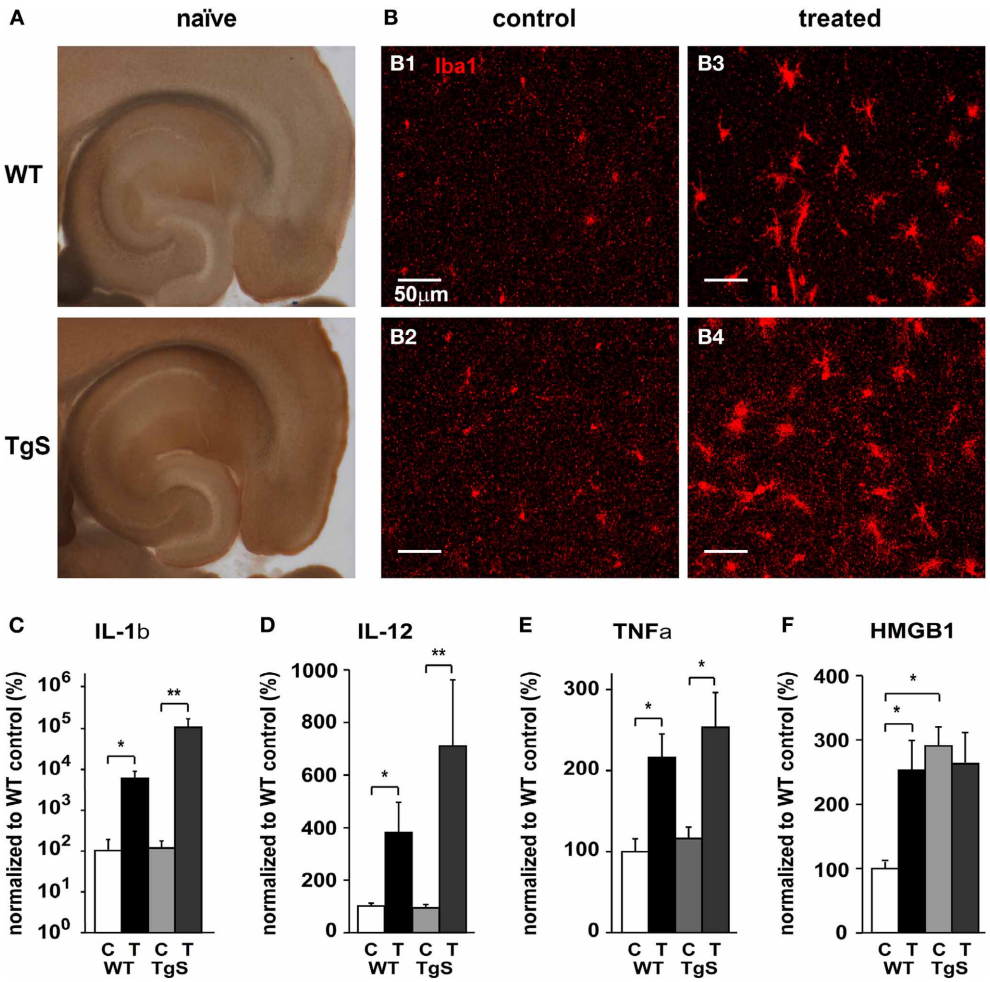

FIGURE 3 | Compared microglia status in WT and TgS mice. **(A)** Karnovsky–Roots staining of 200 μm horizontal brain sections from naïve WT and TgS mice. **(B)** Iba1 staining in the hippocampus of control WT (B1), control TgS (B2), treated WT (B3), and treated TgS (B4) mice. **(C–F)** Real-time RT-PCR for key inflammatory cytokines. **(C)** IL-1β mRNA relative levels (*$p = 0.014$; **$p = 0.004$). **(D)** IL-12 mRNA relative levels (*$p = 0.014$; **$p = 0.004$). **(E)** TNFα mRNA relative levels. WT: $p = 0.027$, TgS: $p = 0.054$. **(F)** HMGB1 mRNA relative levels. $p < 0.05$ (see text). (WT: $n = 5$ controls, $n = 4$ treated, TgS: $n = 6$ controls/treated. In F; TgS control: $n = 4$; C, control; T, treated; Statistical analysis was done using the non-parametric Mann–Whitney test, mean ± SEM.)

CHOLINERGIC ACTIVATION IS ANTI-INFLAMMATORY IN HIPPOCAMPAL SLICES

Our results suggested that the rapid up-regulation of AChE following SE is associated with enhanced local immune response. To explore to what extent this effect is associated with reduced cholinergic control over the brain innate immune response, we studied the immune response in acute brain slices maintained *ex-vitro* 4 h following the slicing procedure. We hypothesized that the injury associated with the slicing procedure would activate a significant inflammatory response (Lucas et al., 2006) which could be suppressed by cholinergic stimuli. Indeed, cholinergic activation by 50 μM CCh reduced mRNA levels of IL1β and TNFα (**Figure 5A**). Similar results were found with low levels of the physiological agonist, ACh (10 μM), in the presence of the AChE inhibitor physostigmine (1 μM, **Figure 5A**). The addition of TTX did not prevent CCh action on the immune response, suggesting it is not dependent on neuronal firing (**Figure 5B**). Importantly, while the non-specific muscarinic receptor antagonist atropine blocked the cholinergic-suppression of inflammatory response, the non-specific nicotinic receptor antagonist, mecamylamine (50 μM) or the α7 nicotinic receptor antagonist α-bungarotoxin (100 nM) did not (**Figure 5B** and data not shown).

DISCUSSION

In this study we offer experimental evidence indicating that (1) cholinergic imbalance due to upregulation of AChE within the hippocampus is associated with a local immune response; (2) such imbalance and the associated inflammation following SE is associated with epileptogenesis; and (3) ACh acts directly as a modulator of brain innate immune response and thereby affect epileptogenesis.

AChE is the fastest enzyme in mammalian cells and as the enzyme in charge of executing ACh breakdown it is a key component of cholinergic signaling (Meshorer and Soreq, 2006). AChE

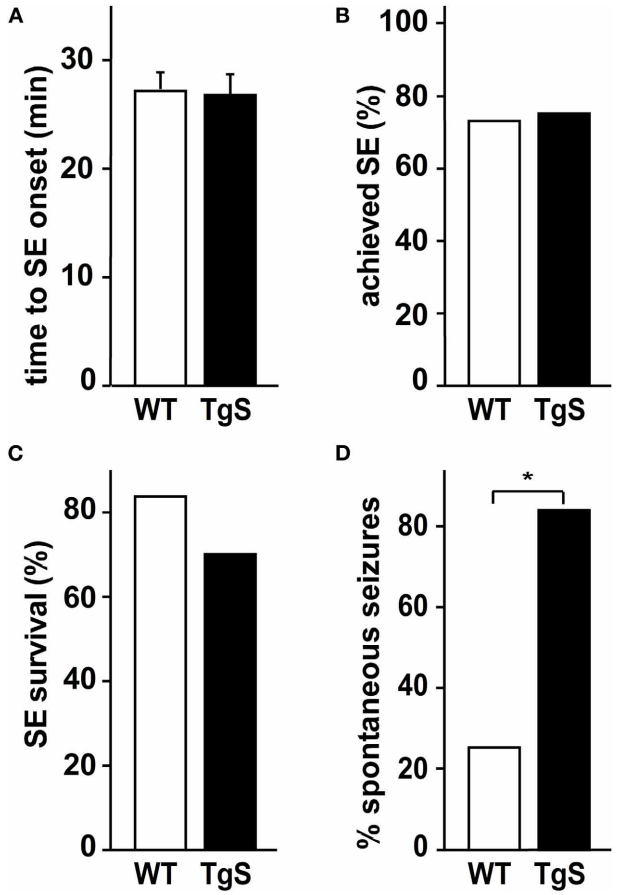

FIGURE 4 | SE and spontaneous seizures in TgS and WT mice. (A) Time (in min) was measured from pilocarpine injection to onset of SE. **(B)** Percentage of mice achieving SE. **(C)** Percentage of mice surviving 40 min of SE (total number of injected animals: WT: $n = 33$, TgS: $n = 44$). **(D)** Percentage of mice presenting spontaneous seizures 1 month after SE (*$p = 0.019$, Chi-square test; number of monitored animals: WT: $n = 8$, TgS: $n = 12$).

levels are controlled via transcriptional and post-transcriptional mechanisms and are dramatically increased under toxicological, physical and psychological stress (Kaufer et al., 1998; Shohami et al., 2000; Meshorer et al., 2002). This fast and robust increase in AChE levels in response to neuronal activation has been suggested to act as an homeostatic control to reduce neuronal excitability (Meshorer et al., 2005b) and on the long-run this response was reported to be associated with enhanced network excitability (Meshorer et al., 2002; Zimmerman et al., 2008). These previous findings raised the hypothesis that AChE will be similarly up-regulated following SE in the pilocarpine model of epilepsy. Indeed, 48 h following SE we recorded increased AChE mRNA and protein levels in SE-experienced mice. Unlike milder and more specific stress manipulations (e.g., psychological) where upregulation was reported to include the readthrough alternatively spliced AChE-R variant (Kaufer et al., 1998; Meshorer et al., 2002), SE was associated with a significant increase in mRNA levels of all three alternatively spliced transcripts (**Figure 1**). In this study we used the muscarinic agonist, pilocarpine, to induce SE and thus it is possible that AChE upregulation represents a specific response to the activation of muscarinic receptors. While we cannot entirely exclude this possibility, it seems unlikely in light of a similar up-regulation observed in the brains from chronic epileptic rats following kainic acid-induced SE (Zimmerman et al., 2008). The presence of c-fos binding sites in the promoter of the gene encoding AChE (Meshorer et al., 2004) further suggest that its upregulation is part of the transcriptional response to excess neuronal activation. AChE transcripts were found in both principle and inhibitory interneurons, supporting its role in controlling the hyperexcitable network. In addition, following SE high expression levels of AChE mRNA were also found in morphologically identified endothelial cells, suggesting it may have a role in vascular changes observed following SE, specifically the robust increase in blood-brain barrier (BBB) permeability (van Vliet et al., 2007; Weissberg et al., 2011). This notion is supported by reports on compromised BBB in transgenic mice over-expressing AChE (Meshorer et al., 2005a). The activation of the immune system, and specifically HMGB1 upregulation in these transgenic animals (**Figure 3**) may also underlie BBB opening (Zhang et al., 2011). AChE transcripts were not found in GFAP positive astrocytes, despite their robust activation during SE. These results are consistent with a previous report showing AChE activity in different lines of neuronal and non-neuronal cells except for astrocytes (Thullbery et al., 2005), although mixed astroglia cells were reported to upregulate the readthrough ACHE transcript following oxidative stress in culture (Bond et al., 2006). High expression levels of ACHE transcripts were found in Iba1 positive microglia following SE and to much less extent in control animals. These results together with the well-described brain immune activation following SE (review by Vezzani et al., 2011) raised the hypothesis that AChE upregulation in microglia may have a role in modulating the innate immune response. This hypothesis is supported by previous studies showing that in microglial cell cultures AChE induces changes in cell morphology as well as iNOS upregulation (von Bernhardi et al., 2003). That the AChE effect is likely to be associated with cholinergic signaling is suggested by the observation that ACh inhibits LPS-induced TNFα release in microglia cultures, an effect attenuated by α-bungarotoxin (Shytle et al., 2004). To test directly the role of AChE overexpression on microglia functions we studied transgenic mice with CNS limited, constitutive up-regulation of synaptic AChE-S (Sternfeld et al., 2000). Indeed, the increase in AChE activity led to increased immunostaining of the microglial marker Iba1 and significant upregulation of mRNA levels for HMGB1 (**Figure 3**), a newly defined cytokine that can be passively released from necrotic cells or actively released from immune activated cells (Wang et al., 1999; Abraham et al., 2000). Our finding showing that AChE over expression in transgenic animals facilitated epileptogenesis are consistent with recent reports showing a proconvulsant effect of HMGB1 in kainic acid treated mice (Maroso et al., 2010). It is worth noticing that TgS mice showed a robust increase in IL-1β expression (18-fold higher than WT mice) 48 h following SE, consistent with the well-described epileptogenic effects of IL-1β (Vezzani et al., 1999; Friedman and Dingledine, 2011). Facilitated epileptogenesis in

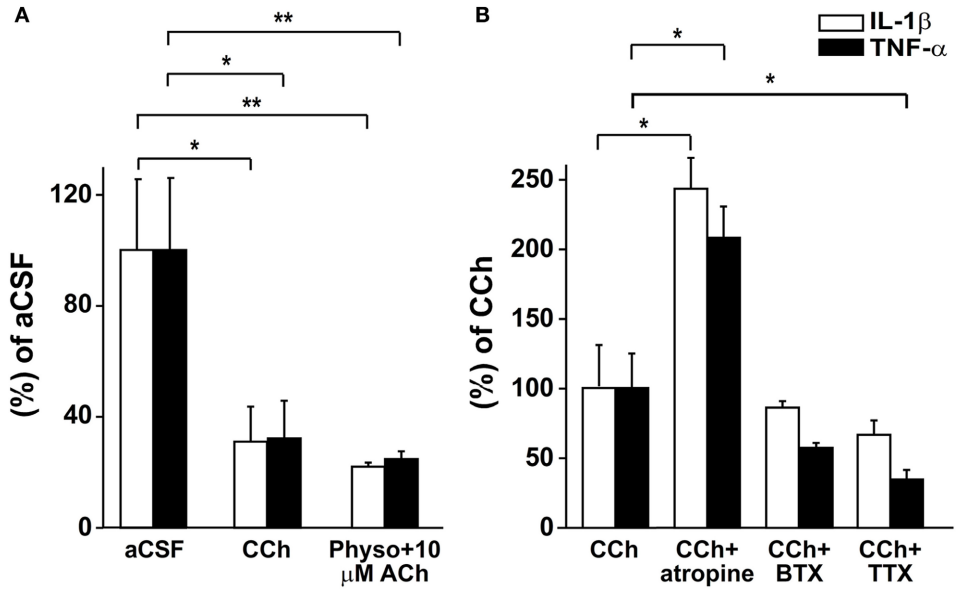

FIGURE 5 | Real-time RT-PCR for IL-1β and TNF-α in acute slices. **(A)** mRNA relative levels in slices treated with aCSF, 50 μM CCh (*$p = 0.025$; $n = 6$) or 1 μM physostigmine with 10 μM ACh (**$p = 0.006$; $n = 5$–6). **(B)** mRNA relative levels in slices treated with 50 μM CCh ($n = 5$) and 50 μM CCh with 1 μM atropine (*$p = 0.028$; $n = 5$), 100 nM BTX ($n = 5$) or 1 μM TTX (*$p = 0.027$; $n = 4$). Statistical analysis was done using the non-parametric Mann–Whitney test, mean ± SEM. Physo, physostigmine; BTX, bungarotoxine.

TgS mice could not be solely attributed to enhanced seizure activity due to increased network sensitivity to ACh (Meshorer et al., 2002) since the latency and duration of SE in response to pilocarpine were similar in WT mice. This strengthens the notion that AChE upregulation following SE is a response to excess neuronal activation rather than a direct effect of pilocarpine. We cannot rule out at this stage the contribution of other epileptogenic mechanisms including vascular changes (i.e., enhanced BBB permeability) (Marchi et al., 2007; Friedman et al., 2009) or changes in peripheral immune response (Fabene et al., 2008; Marchi et al., 2009). When monitoring WTs for 1–3 month, 82% showed spontaneous seizures, while TgS were not monitored at such a late time point. Therefore, it is feasible that the rate of epileptogenesis and not the end result are different between the strains.

The most direct evidence for cholinergic control over the innate immune response is given in the acute slices experiments demonstrating that adding ACh or CCh to the bathing solution suppressed mRNA levels of IL-1β and TNF-α. In accordance with these results, previous studies demonstrated anti-inflammatory effect for ACh and AChE inhibitors in glial cultures (Shytle et al., 2004; Wang et al., 2010) and for AChE inhibitors in animals (Nizri et al., 2008; Wang et al., 2008, 2010). It is important to note that exposure to centrally acting potent AChE inhibitors induces SE and neuroinflammation that may promote epileptogenesis (Dhote et al., 2007, 2012; De Araujo Furtado et al., 2010). Thus, it seems clear that AChE inhibition *per se*, perhaps due to enhanced neuronal excitability is usually associated with increased brain inflammation and the transient increase in ACh levels are not sufficient to block completely the brain innate immune processes associated with SE and chronic recurrent seizures (Pernot et al., 2009). Unlike previous studies (mostly in the peripheral nervous system) (Tracey, 2002, 2007; Shytle et al., 2004; Wang et al., 2008), our findings point to muscarinic dependant activation as the underlying pathway. Although muscarinic receptors in the CNS were shown to control the peripheral nicotinic pathway, hinting to complex interactions between the involved pathways (Pavlov et al., 2006, 2009), these interactions could not explain the observations in the *ex-vivo* slice preparation. Since recent studies did show that cytokines act as pro-convulsants (Vezzani et al., 2011), their upregulation may also be attributed to a positive feedback enhancement mechanism. However, it is noteworthy that the sodium channel blocker, TTX had no effect on cholinergic-suppression of cytokine production suggesting that this cholinergic effect is independent of neuronal firing.

In summary, our study suggests for the first time, a role for activity-dependant cholinergic imbalance in brain immune response, epileptogenesis and seizures and may offer a new basis for understanding the role of AChE-inhibitors in the treatment of Alzheimer's disease and similar neurodegenerative disorders. Future studies are warranted to delineate the contribution of cholinergic signaling to brain immune response in neurological and neurodegenerative disorders where immune response has been reported (Amor et al., 2010) as well as its potential as a novel target in the treatment of uncontrolled brain immune response.

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Cholinesterase-targeting microRNAs identified *in silico* affect specific biological processes

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MicroRNAs (miRs) have emerged as important gene silencers affecting many target mRNAs. Here, we report the identification of 244 miRs that target the 3′-untranslated regions of different cholinesterase transcripts: 116 for butyrylcholinesterase (BChE), 47 for the synaptic acetylcholinesterase (AChE-S) splice variant, and 81 for the normally rare splice variant AChE-R. Of these, 11 and 6 miRs target both AChE-S and AChE-R, and AChE-R and BChE transcripts, respectively. BChE and AChE-S showed no overlapping miRs, attesting to their distinct modes of miR regulation. Generally, miRs can suppress a number of targets; thereby controlling an entire battery of functions. To evaluate the importance of the cholinesterase-targeted miRs in other specific biological processes we searched for their other experimentally validated target transcripts and analyzed the *gene ontology* enriched biological processes these transcripts are involved in. Interestingly, a number of the resulting categories are also related to cholinesterases. They include, for BChE, *response to glucocorticoid stimulus*, and for AChE, *response to wounding* and two child terms of *neuron development: regulation of axonogenesis* and *regulation of dendrite morphogenesis*. Importantly, all of the AChE-targeting miRs found to be related to these selected processes were directed against the normally rare AChE-R splice variant, with three of them, including the neurogenesis regulator miR-132, also directed against AChE-S. Our findings point at the AChE-R splice variant as particularly susceptible to miR regulation, highlight those biological functions of cholinesterases that are likely to be subject to miR post-transcriptional control, demonstrate the selectivity of miRs in regulating specific biological processes, and open new venues for targeted interference with these specific processes.

Keywords: AChE, BChE, microRNA

INTRODUCTION

MicroRNAs (miRs) are small RNA molecules which target many mRNA transcripts, leading to their post-transcriptional silencing (Bartel, 2009). Many mRNAs can be silenced by multiple miRs and miRs often target more than one mRNA participating in a particular biological function (Bartel, 2009). Together, this suggests that the miR networks affecting specific mRNA transcripts may provide useful information on the biological roles in which these transcripts are involved. Cholinesterases are involved in many biological functions (Massoulie, 2002). However, miR-132 is the only miR so far that has been experimentally validated as targeting AChE, with consequences on inflammatory responses (Shaked et al., 2009). To delineate additional miRs which might regulate cholinesterase functions, we explored the 3′-untranslated regions (3′-UTR) of human cholinesterase transcripts (acetyl- and butyrylcholinesterase, AChE, BChE; Soreq and Seidman, 2001).

Given that several of the proteins involved in a specific function are often repressed by the same miR (Girardot et al., 2010), changes in a particular miR might down-regulate the entire process. Hence, we surmised that those functions that are shared by cholinesterases and the other targets of the cholinesterase-complementary miRs would be more susceptible for being affected by miR control than other processes. That concept is schematically presented as a workflow in **Figure 1**.

MATERIALS AND METHODS

MicroRNA candidates were identified on each of the 3′-UTR sequences of AChE and BChE, which are 235, 1030, and 478 nucleotides long for BChE, the major "synaptic" AChE-S variant and the stress-inducible AChE-R variant, respectively (**Figure 2A**). We used the PicTar[1], miRanda[2], miRbase[3], and microCosm[4] algorithms to identify these transcript-specific miRs. All predictions ensured a threshold P-value < 0.05, and analysis specifications allowed both evolutionarily conserved and non-conserved miRs, which enabled us to include primate-targeting miRs as well.

Validation of miR-target interactions generally involved a 3′UTR luciferase assay. In some cases, it was complemented by protein blots, real-time RT-qPCR, microarrays, transgenic technology, β-galactosidase, or GFP-tagged targets. See, for example

[1] www.pictar.mdc-berlin.de
[2] www.microRNA.org
[3] www.mirbase.org
[4] http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/

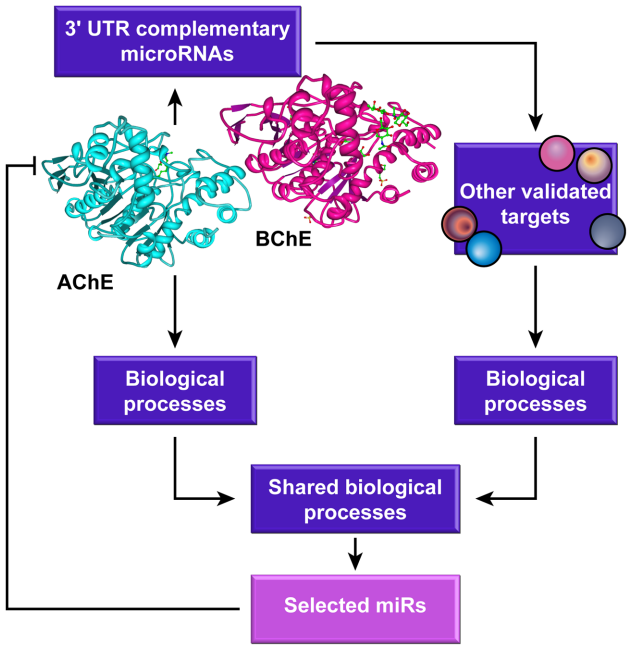

FIGURE 1 | The study's flow chart. MicroRNAs complementary to the 3′-UTR domains of AChE and BChE transcripts were identified using several algorithms and other validated targets for those miRs were searched for and analyzed for common biological processes in which both these miR targets and cholinesterases are involved.

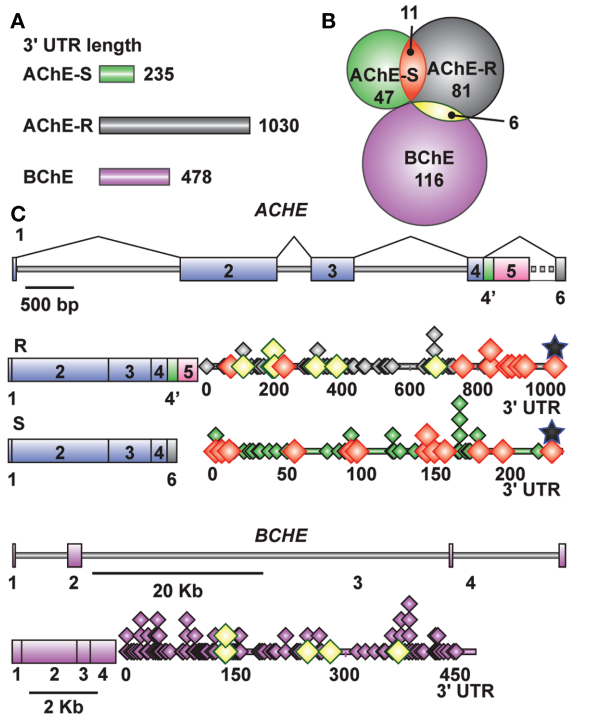

FIGURE 2 | Cholinesterase-targeted miRs show distinct 3′UTR distributions and partial overlaps. (A) The length of the studied 3′UTR domains. **(B)** Each 3′-UTR is targeted by many different miRs, part of which shared by BChE/AChE-R and AChE-R/AChE-S. **(C)** Gene and transcript compositions (exons shown as boxes, introns – as lines) and miR distribution patterns on the 3′-UTR domain (not to scale). Overlaps are color coded. MiR (diamonds) localizations are marked. Black stars show miR-132 position.

the Shaked et al. (2009) report for several of the latter technologies used to explore the miR-132 target AChE, and (Hansen et al., 2010) for the "classical" 3′-UTR and transgenic approaches, in exploring p250GAP which is also a miR-132 target.

To search for gene ontology (GO) categories which are also relevant for the other mRNA targets of cholinesterase-related miRs, we used the DAVID functional annotation clustering tool[5]. For each of the miRs identified as targeting one of the cholinesterases we searched for other experimentally validated targets; and we then used the lists of the other validated targets as gene lists for the DAVID search. Each list was normalized to the entire human genome, which served as a background.

RESULTS

We identified 116, 81, and 47 miRs (24, 8, and 20 miRs/100 nucleotides) that are complementary to the 3′-UTR domains of the BChE, AChE-R, and AChE-S transcripts, respectively. Of these, 6 miRs target both BChE and AChE-R whereas 11 miRs are common to both AChE-R and AChE-S, but BChE and AChE-S do not share any miR (**Figure 2B**). Positions of the identified miRs are presented in **Figure 2C**, with miR-132 targeting a similar seed domain localized at the very 3′-end of the 3′-UTR in both the AChE-S and AChE-R transcripts. Of the cholinesterase-targeting miRs, seven had multiple binding sites to the target AChE-S, nine to AChE-R, and seven to the BChE transcript, suggesting that they have a higher prospect for being functional (John et al., 2004).

[5]http://david.abcc.ncifcrf.gov/

Compatible with the different conceptual principles on which each of the algorithms employed is based, only 8.6, 17, and 13.7% (7/81), (8/47), (16/116) of the miRs identified as targeting AChE-R, AChE-S, and BChE, respectively, were predicted by more than one of the algorithms. For AChE-R, these are hsa-miR-28-5p, -423-3p, -484, -483-5p, -663, -582-3p, -380*. For AChE-S, hsa-miR-194, -939, -658, -608,-615-5p, -423-5p -920, and let-7f-2* and for BChE, hsa-miR-203, -218, -221, -222, -181a, -181b, -181c, -181d, -494, -200b, -200c, -576-3p, -16-2*, -625, -195*, -889.

These cholinesterase-targeting miRs and their other validated non-cholinesterase targets are listed in **Tables 1–3** with the corresponding functions attributed to these other targets. The relevant citations appear in **Tables A1–A4** in Appendix. Of note, numerous cholinesterase-targeting miRs have no experimentally validated targets at this time, yet others have more than one validated target and associate with more than one biological function. Examples include miR-124 which targets both the AChE-S and IQGAP1-(Furuta et al., 2010), a GTPase activating protein which promotes neurite outgrowth (**Table 1**). Additionally miR-152 and miR-148a, which target AChE-R, also target the calmodulin regulating kinase CaMKIIα (Liu et al., 2010; **Table 2**). Lastly, the BChE-targeting cluster of miRs-222 and -221 also target the neuronal early immediate protein c-fos (Ichimura et al., 2010; **Table 3**).

Table 1 | Additional targets of AChE-S targeting microRNAs.

miR ID	Validated targets		
hsa-miR-491-5p	Bcl-X(L; cell death)		
hsa-miR-605	Mdm2 (ubiquitination)		
hsa-miR-608	CD44 (cell–cell/cell–matrix interaction)	CDC42 (cell division)	
hsa-miR-124	Glucocorticoid receptor	LAMC1 (laminin γ1)	IQGAP1(neurite outgrowth; Furuta et al., 2010)
	NeuroD1 (neurogenic differentiation 1)	BAF53a (chromatin remodeling)	C14orf24 (chromosome 14 ORF 24)
	Mtpn (myotrophin)	PTBP1 (splicing)	CDK6 (cyclin-dependent kinase 6)
	Mapk14 (mitogen activated protein kinase 14)	PTBP2 (splicing)	SOX9 (glial cell specification)
	CDK2 (cyclin-dependent kinase 2)	C/EBPα (transcription)	Lhx2 (transcription)
	MCP1 (monocyte chemoattractant protein 1)	FOXA2 (transcription)	EfnB1(projecting axons)
	Itgb1 (integrin 1)	VIM (cytoskeleton; Furuta et al., 2010)	NR3C2 (Mineralocorticoid and glucocorticoid receptor)
	SCP1 (synaptonemal filaments)	SMYD3 (transcription; Furuta et al., 2010)	
hsa-let-7g	C-Myc (transcription)	Collagen alpha2 (COL1A2)	Bcl-xL (cell death)
hsa-miR-196a	HOX-B7 (transcription)	SPRR2C (small proline-rich protein 2C)	Annexin A1 (exocytosis)
	S100A9 (calcium-binding protein A9)	KRT5 (keratin 5)	HOXC8 (transcription)
hsa-miR-542-3p			Survivin
hsa-miR-525-5p	VPAC1 (vasoactive intestinal peptide receptor 1)		

Table 2 | Additional targets of microRNAs targeting AChE-R.

miR ID	Validated targets		
Hsa-miR-708	MPL (thrombopoietin receptor; Girardot et al., 2010)		
Hsa-miR-28-5p	MPL (thrombopoietin receptor; Girardot et al., 2010)	OTUB1 (immune system transcription; Girardot et al., 2010)	
	N4BP1 (NEDD4 binding protein 1; Girardot et al., 2010)	TEX261 (apoptosis; Girardot et al., 2010)	MAPK1 (megakaryocyte differentiation; Girardot et al., 2010)
hsa-miR-503	ANLN (actin-binding protein anillin)	ATF6 (activating transcription factor 6)	CHEK1 (cell cycle)
	EIF2C1 (argonaute1)	KIF23 (mitotic kinesin-like protein 1)	WEE1 (mitosis regulator)
	CCNE1 (cyclin E1)	CDC25A (cell cycle)	
	CCND1 (cyclin D1)	CDC14A (CDC14 cell division cycle 14 homolog A)	
hsa-miR-148a	CaMKIIα (CNS kinase; Liu et al., 2010)	MLC1 (megalencephalic leukoencephalopathy with subcortical cysts 1)	MSK1 (histone phosphorylase)
	DNMT1 (DNA methyltransferase 1)	DNMT3B (CpG island methylation)	MITF (microphthalmia-associated transcription factor)
	CCKBR (modulates anxiety and neuroleptic activity)	EPAS1 (endothelial PAS domain-containing protein 1)	HLA-G (asthma susceptibility)
	POMC (pro-opiomelanocortin)	CAND1 (ubiquitin ligase regulation)	PXR (pregnane X receptor)
hsa-miR-152	CaMKIIα (CNS kinase; Liu et al., 2010)	DNMT1 (DNA methyltransferase 1)	
hsa-miR-125b	TNFα (tumor necrosis factor α)	ERBB2 (erythroblastic leukemia viral oncogene homolog 2)	BMPR1B (bone morphogenic receptor type 1B)
	IRF4 (interferon regulatory factor 4)	ERBB3 (erythroblastic leukemia viral oncogene homolog 3)	E2F3 (cell cycle)
	Blimp1 (zinc finger protein)	TEF (thyrotroph embryonic factor)	Bcl2 modifying factor (apoptosis)
	Vdr (vitamin D receptor)	MUC1 (adhesion)	Bak1 (pro-apoptotic Bcl2 antagonist killer 1)
	CYP24A1 (cytochrome P450 family 24A)	p53 (tumor suppressor)	SMO (smoothened receptors)
	IGF2 (insulin-like growth factor 2)	Suv39h1 (histone methyltransferase)	Stat3 (Transcription factor, binds to IL-6)
	LIN28 (translational enhancer)	NMDA receptor subunit NR2A	ATM (ataxia telangiectasia mutated)

(Continued)

Table 2 | Continued

miR ID	Validated targets		
hsa-miR-125a-5p	LIN28 (translational enhancer)	T-TrkC (neurotrophic tyrosine kinase receptor 3)	HuR (cell growth)
	p53 (tumor suppressor)	KLF13 (transcription factor)	AT-rich interactive domain 3B (transcription)
	PDPN 9 (actin organization)	Bak1 (pro-apoptotic Bcl2 antagonist killer 1)	
	N-ras (oncogene)	MEK3 (phosphorylation of MAP kinase)	
hsa-miR-214	SrGAP1(neuronal migration)	Ezh2 (stem cell identity)	N-ras (oncogene)
	JNK1 (MAPK8)	PTEN (tumor suppressor)	MEK3 (phosphorylation)
hsa-miR-199a-5p	Hif-1α (Hypoxia-inducible factor 1)	IKKβ (NFκB activation)	DDR1 (discoidin domain receptor 1)
	Sirt1 (apoptosis)		
hsa-miR-31	ICAM-1 (leukocyte adhesion protein)	Fgf13 (fibroblast growth factor 13)	Dkk-1 (canonical Wnt signaling)
	DACT-3 (epigenetic regulator of Wnt)	E-selectin (inflammation)	p16Ink4a (cell cycle)
	LATS2 (tumor suppression)	PPP2R2A (signal transduction)	Krt16 (keratin 16)
	Krt17 (keratin 17)	Dlx3 (development of ventral forebrain)	E2F6 (cell cycle)
	TIAM1 (T-cell lymphoma invasion and metastasis 1)	Fzd3 (accumulation of β-catenin)	Integrin α (fibronectin)
	M-RIP (regulation of actin)	MMP16 (blood vessels matrix remodeling)	RDX (actin filaments binding to plasma membrane)
	RhoA (signal transduction)	SATB2 (upper-layer neurons initiation)	PROX1 (CNS development)
	WAVE3 (signal transmission)		
hsa-miR-185	Six1 (limb development)		
hsa-miR-193b	Estrogen receptor α Mcl-1 (myeloid cell leukemia sequence 1)	ETS-1 (oncogene) uPA (urokinase-type plasminogen activator)	CCND1 (cyclin D1)
hsa-miR-7	Alpha-synuclein (SNCA)	SFRS1 (splicing)	ERF (cell proliferation)
	LSH (lymphoid-specific helicase)	DAP (cell death-associated protein)	MRP1 (human multidrug resistance-associated protein 1)
	Associated cdc42 kinase 1	Yan (cell differentiation)	EGFR (epidermal growth factor receptor)
	CD98 (sodium transport)	Pak1 (p21-activated kinase 1)	IGF1R (insulin-like growth factor 1 receptor)
hsa-miR-483-5p	Socs-3 (cytokine signaling)	BBC3/PUMA (apoptosis)	
hsa-miR-663	TGFβ1 (proliferation)	JunB (jun B proto-oncogene)	JunD (jun D proto-oncogene)
hsa-miR-765	TRK3 (neurotrophic tyrosine kinase)		
hsa-miR-146b-3p	IRAK1 (IL1 receptor-associated kinase 1)	EGFR (epidermal growth factor receptor)	MMP16 (degrades extracellular matrix)

Table 3 | Additional targets of BChE-targeting microRNAs.

miR ID	Validated target		
hsa-miR-203	SOCS-3 (cytokine signaling)	Lef1 (lymphoid enhancer-binding factor)	p63 (transcription)
	ABL1 (cell growth)	Barx1 (transcription)	CKAP2 (cytoskeleton associated protein 2)
	LASP1 (cytoskeletal activities)	BIRC5 (regulator of mitosis)	WASF1 (signal transmission)
	ASAP1 (membrane trafficking)	RUNX2 (runt-related transcription factor 2)	
hsa-miR-340	MITF (microphthalmia-associated transcription factor)		
hsa-miR-218	IKK-β (NFκB activation)	ROBO1 (roundabout, axon guidance receptor, homolog 1)	BIRC5 (mitosis)
	GJA1 (gap junction protein, α1)	ROBO2 (roundabout, axon guidance receptor homolog 2)	GLCE (glucuronic acid epimerase)
	PXN (paxillin, cytoskeletal protein)		

(Continued)

Table 3 | Continued

miR ID	Validated target		
hsa-miR-221	ERα (estrogen receptor α)	ICAM-1(leukocyte adhesion protein)	p27 (cell cycle)
	p57 (cyclin-dependent kinase inhibitor 1C)	DNA damage-inducible transcript 4 (DDIT4)	TIMP3 (TIMP metallopeptidase inhibitor 3)
	PTEN (tumor suppressor)	PUMA (apoptosis)	C-fos (cell proliferation; Ichimura et al., 2010)
	Bmf (apoptosis)	Mdm2 (ubiquitination)	CDKN1B (cyclin-dependent kinase inhibitor 1B)
hsa-miR-222	ERα (estrogen receptor α)	p27 (cell cycle)	PTEN (tumor suppressor)
	STAT5A (transcription)	p57 (cyclin-dependent kinase inhibitor 1C)	TIMP3 (TIMP metallopeptidase inhibitor 3)
	Bim (apoptosis)	ETS-1 (transcription)	PUMA (apoptosis)
	PPP2R2A (protein phosphatase 2A subunit B)	C-fos (cell proliferation; Ichimura et al., 2010)	ICAM-1(leukocyte adhesion protein)
	MMP1 (cleaves collagens)	SOD2 (superoxide dismutase 2)	
hsa-miR-181a	SIRT1 (apoptosis)	Ataxia telangiectasia mutated (ATM; cell cycle)	Hox-A11 (transcription)
	p27(cell cycle)	PLAG1 (transcription)	BCL2 (B-cell CLL/lymphoma 2; apoptosis)
	Bim (apoptosis)	Tcl1 (cell proliferation)	OPN (osteopontin)
hsa-miR-181b	AID (RNA-editing)	PLAG1 (transcription)	BCL2 (B-cell CLL/lymphoma 2; apoptosis)
	TIMP3 (TIMP metallopeptidase inhibitor 3)	Ataxia telangiectasia mutated (ATM; cell cycle)	SIRT1 (apoptosis)
	ZNF37A (transcriptional regulation)	ZNF83 (transcriptional regulation)	ZNF182 (transcriptional regulation)
	Mcl-1 (myeloid cell leukemia-1; apoptosis)		
hsa-miR-181c	IL2 (immune response)	BCL2 (B-cell CLL/lymphoma 2; apoptosis)	NOTCH4 (transcriptional activator)
	KRAS (GTPase activity)		
hsa-miR-181d	BCL2 (B-cell CLL/lymphoma 2; apoptosis)		
hsa-miR-494	CaMKIIδ (CNS kinase)	ROCK-1 (apoptosis)	LIF [leukemia inhibitory factor (cholinergic differentiation factor)]
	PTEN (phosphatase and tensin homolog)	TEL-AML1 (hematopoiesis)	FGFR2 (fibroblast growth factor receptor 2)
hsa-miR-129-5p	CAMTA1 (calmodulin binding transcription activator 1)	EIF2C3 (eukaryotic translation initiation factor 2C, 3)	GALNT1 (oligosaccharide biosynthesis)
	SOX4 (transcriptional activator)		
hsa-miR-30d	Galphai2 (G protein, α inhibiting activity polypeptide 2)		
hsa-miR-30c	Runx1 (runt-related transcription factor 1)	CTGF (connective tissue growth factor)	
hsa-miR-30a	SOD2 (superoxide dismutase 2)	BDNF (brain-derived neurotrophic factor)	Beclin 1 (autophagy)
	Xlim1/Lhx1 (transcription factor)		
hsa-miR-30e	Ubc9 (ubiquitin-conjugating enzyme E2I)	B-Myb (transcription factor)	
hsa-miR-320a	(Hsp20 heat-shock protein 20)	AQP1 (aquaporin 1)	AQP4 (aquaporin 4)
	TfR-1; CD71 (development of erythrocytes and the nervous system)	Mcl-1 (myeloid cell leukemia sequence 1; apoptosis)	
hsa-miR-140-5p	Smad3 (transcription)	HDAC4 (histone deacetylase 4)	
hsa-miR-519c-3p	HIF-1α (hypoxia-inducible factor 1α)	ABCG2 (exclusion of xenobiotics from the brain)	
hsa-miR-489	PTPN11 (signal transduction)		
hsa-miR-584	NXA1 (exocytosis)	ROCK-1 (actin assembly)	

We focused our survey on those functions of those miRs for which experimental validation is available. **Table 4** presents these miRs which are shared for AChE-R and AChE-S or AChE-R and BChE and some of their additional targets, highlighting the multitude of miR targets with predicted regulatory functions (e.g., the chromatin modulator zinc finger proteins ZEB1 and ZEB2 targeted by miR-200b, miR-200c, and miR-429 that are also directed to both AChE-R and AChE-S; Gregory et al., 2008). Likewise, the AChE-S-targeted miR-132 (Shaked et al., 2009; Soreq and Wolf, 2011) also targets the GTPase regulator p250GAP

Table 4 | Additional targets of ChE-targeting miRs (common to more than one ChE).

miR ID	Validated target common to ACHE-R and AChE-S		
hsa-miR-186	Pro-apoptotic P2 × 7 purinergic receptor	AKAP12 (tumor suppressor)	
hsa-miR-199b-5p	Dyrk1a (brain development)	HES1 (transcriptional repressor)	SET (apoptosis)
hsa-miR-429	ZEB1 (transcriptional repression of IL2; Gregory et al., 2008) RERE (apoptosis)	ZEB2 (SIP1; zinc finger protein; Gregory et al., 2008)	PLCgamma1(apoptosis)
hsa-miR-200b	ZEB1 (transcriptional repression of IL2; Gregory et al., 2008)	ZEB2 (SIP1; zinc finger protein; Gregory et al., 2008)	PLCgamma1(apoptosis)
	Serca2 (sarco/endoplasmic reticulum Ca2+ ATPase)	Suz12 (chromatin silencing)	Ets-1 (transcriptions factor)
	OREBP (osmotic response element)	Cyclin D1	
hsa-miR-200c	ZEB1 (transcriptional repression of IL2; Gregory et al., 2008)	ZEB2 (SIP1; zinc finger protein; Gregory et al., 2008)	PLCgamma1(apoptosis)
	VEGF (angiogenesis)	TUBB3 (neurogenesis and axon guidance)	TRPS1 (transcription factor)
	KLF13 (transcription factor)	MBNL2 (muscleblind-like protein 2)	FAP1 (apoptosis)
miR ID	**Validated targets common to ACHE-R and BChE**		
hsa-miR-24	SOD1 (superoxide dismutase 1)	ALK4 (transducer of activin)	Notch1 (Bergmann glia differentiation)
	MKK4 (survival signal in T cells)	E2F2 (cell cycle)	H2AX (histone-formation)
	FAF1 (apoptosis)	HNF4α (cell proliferation)	FURIN (processing of TGFβ1)
	DHFR (dihydrofolate reductase)	DND1(miRNA-mediated gene suppression)	
hsa-miR-212	MeCP2 (interaction with histone deacetylase) PED (apoptosis)	MYC (transcription)	Rb1(tumor suppressor)
hsa-miR-132	AChE-S (Shaked et al., 2009)	P250GAP (neuron-associated GTPase; Vo et al., 2005)	Per1 (circadian clock)
	SirT1 (apoptosis)	MeCP2 (modification of eukaryotic genomes)	p300 (chromatin remodeling)
	Jarid1a (histone demethylase) p120RasGAP (angiogenesis)	Btg2 (cell cycle)	Paip2a (translation regulation)
hsa-miR-198	Cyclin T1		
hsa-miR-194	Rac1 (GTP-binding protein) MDM2 (p53 negative regulator)	Per family (circadian)	EP300 (transcriptional co-activator)

involved in neurite extension (Vo et al., 2005; Hansen et al., 2010; **Table 4**).

The process-regulation hypothesis of miR function predicts the existence of biological functions in which both cholinesterases, and those other targets which share miRs with cholinesterases, would be involved. To challenge this hypothesis, we first identified the GO categories in which AChE and BChE are involved, and found 24 and 11 biological processes for these two proteins, respectively. Twenty-three, 13, and 18 enriched biological processes emerged as shared processes for the other validated targets of AChE-R, AChE-S, and BChE-targeting miRs, respectively (P-value threshold < 0.05).

Out of over 20 ontology categories attributed to AChE, only two are shared with the categories attributed to the other validated targets of the cholinesterase-targeting miRs. These are: *Response to wounding* (GO: 0009611; 68 transcripts) and *Neuron development* (GO: 0048666), and specifically its AChE-relevant child terms *Regulation of axonogenesis* (GO: 0050770; 78 transcripts) and *regulation of dendrite morphogenesis* (GO: 0048814; 27 transcripts). Surprisingly, all 10 miRs that regulate *Response to wounding* and *Neuron development* selectively target the normally rare, stress-responsive AChE-R transcript, (miR-186, -125b, -200c, -199a-5p, -199b-5p, -125a, -214, -7, -663, -31, and -148a) whereas only three of these miRs also target the prevalent AChE-S mRNA (miR-194, -24, and -132). For BChE, we found only one shared category out of 11 relevant ontology groups: *Response to glucocorticoid stimulus* (GO: 0051384; 119 transcripts), and no overlap with the AChE-relevant categories (**Figures 3A,B**).

DISCUSSION

Using a variety of available algorithms, we found a plethora of cholinesterase-targeted miRs. Some of these were already validated as functionally capable of silencing other mRNA transcripts. A study of the functionally relevant biological processes in which these other targets are involved revealed a highly focused overlap with only few of the biological processes in which cholinesterases participate. Given that miRs regulate targets which share biological

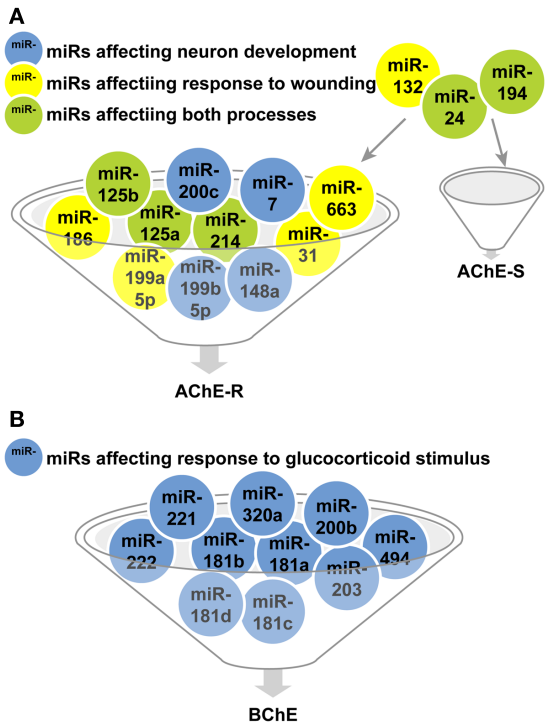

FIGURE 3 | MiR regulators of biological processes shared by cholinesterases and validated targets of these miRs. (A) miRs targeting transcripts participating in the AChE-S and AChE-R relevant *response to wounding* (yellow)and *neuron development* processes(blue) or both categories(green). **(B)** miRs targeting transcripts participating in the BChE-relevant *response to glucocorticoid stimulus* category.

processes, cholinesterases appear to be primarily subject to miR regulation when involved in neuronal development, response to wounding, and glucocorticoid stimulus; and specific cholinergic processes are regulated by miRs targeting both AChE and other targets participating in the same biological process.

Several limitations should be considered in the context of this study. First, the currently available search algorithms for miR candidates appear to differ substantially, which casts a shadow on the veracity of such identification. Second, research bias has focused much of the efforts in the miR field toward cancer research, whereas neuroscience-focused miRs were relatively neglected. Therefore, we might have overlooked important miRs simply because they have not yet been validated experimentally. This being said, that many of the biological functions in which cholinesterases are involved show no relevant cholinesterase-targeting miR sequences suggests other modes of regulation of cholinesterase levels for most of these functions [e.g., transcriptional (Hill and Treisman, 1995), epigenetic (Allshire and Karpen, 2008), or post-translational processes (Fukushima et al., 2009)]. Alternatively, or in addition, miRs might exist which control these functions, but have no role in cancer biology and are therefore not yet characterized. MiR regulation of cholinesterase functions will therefore need to be re-inspected in the near future.

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APPENDIX

Table A1 | Additional targets of AChE-S targeting microRNAs.

miR ID	Validated targets		
hsa-miR-491-5p	Bcl-X (L; cell death; Nakano et al., 2010)		
hsa-miR-605	Mdm2 (ubiquitination; Xiao et al., 2011)		
hsa-miR-608	CD44 (cell–cell/cell–matrix interaction; Jeyapalan et al., 2011)	CDC42 (cell division; Jeyapalan et al., 2011)	
hsa-miR-124	Glucocorticoid receptor (Vreugdenhil et al., 2009)	LAMC1 (laminin γ1; Cao et al., 2007)	IQGAP1(neurite outgrowth; Furuta et al., 2010)
	NeuroD1 (neurogenic differentiation 1; Liu et al., 2011)	BAF53a (chromatin remodeling; Yoo et al., 2009)	C14orf24 (chromosome 14 open reading frame 24; Ko et al., 2009)
	Mtpn (myotrophin; Krek et al., 2005)	PTBP1 (splicing; Makeyev et al., 2007)	CDK6 (cyclin-dependent kinase 6; Pierson et al., 2008)
	Mapk14 (mitogen activated protein kinase 14; Krek et al., 2005)	PTBP2 (splicing; Makeyev et al., 2007)	SOX9 (glial cell specification; Cheng et al., 2009)
	CDK2 (cyclin-dependent kinase 2; Nakamachi et al., 2009)	C/EBPα (transcription factor; Hackanson et al., 2008)	Lhx2 (transcription; Qiu et al., 2009)
	MCP- (1 monocyte chemoattractant protein 1; Nakamachi et al., 2009)	FOXA2 (transcription factor; Baroukh et al., 2007)	EfnB1(projecting axons; Arvanitis et al., 2010)
	Itgb1 (integrin 1; Cao et al., 2007)	VIM (cytoskeleton; Furuta et al., 2010)	NR3C2 (mineralocorticoid and glucocorticoid receptor; Sober et al., 2010)
	SCP1 (synaptonemal filaments; Cao et al., 2007)	SMYD3 (transcription; Furuta et al., 2010)	
hsa-let-7g	C-Myc (transcription factor; Lan et al., 2011)	Collagen alpha2 (COL1A2; Ji et al., 2010)	Bcl-xL (cell death; Shimizu et al., 2010)
hsa-miR-196a	HOX-B7 (transcription factor; Braig et al., 2010)	SPRR2C (small proline-rich protein 2C; Maru et al., 2009)	Annexin A1 (exocytosis; Luthra et al., 2008)
	S100A9 (calcium-binding protein A9; Maru et al., 2009)	KRT5 (keratin 5; Maru et al., 2009)	HOXC8 (transcription factor; Kim et al., 2009a)
hsa-miR-542-3p	Survivin (Yoon et al., 2010)		
hsa-miR-525-5p	VPAC1 (vasoactive intestinal peptide receptor 1; Cocco et al., 2010)		

miRs without validated targets: hsa-miR-920, -506, -27b, -541, -92a-2*, -658, -423-5p, -615-5p, -25*, -4688, -4776-3p, -668, -3613-5p, -4700-5p, -718, let-7f-2*, -455-3p, -633, -554, -524-3p, -638, -525-3p, -611, let-7e*, -4283, -4329, -4278, -4300, -3184, -149*.*

Table A2 | Additional targets of microRNAs targeting AChE-R.

miR ID	Validated targets		
hsa-miR-708	MPL (thrombopoietin receptor; Girardot et al., 2010)		
hsa-miR-28-5p	MPL (thrombopoietin receptor; Girardot et al., 2010)	OTUB1 (immune system transcription; Girardot et al., 2010)	
	N4BP1 (NEDD4 binding protein 1; Girardot et al., 2010)	TEX261 (apoptosis; Girardot et al., 2010)	MAPK1 (megakaryocyte differentiation; Girardot et al., 2010)
hsa-miR-503	ANLN (actin-binding protein anillin; Forrest et al., 2010)	ATF6 (activating transcription factor 6; Forrest et al., 2010)	CHEK1 (checkpoint mediated cell cycle arrest; Forrest et al., 2010)
	EIF2C1 (argonaute1; Forrest et al., 2010)	KIF23 (mitotic kinesin-like protein 1; Forrest et al., 2010)	WEE1 (mitosis regulator; Forrest et al., 2010)
	CCNE1 (cyclin E1; Forrest et al., 2010)	CDC25A (cell cycle progression; Forrest et al., 2010)	
	CCND1 (cyclin D1; Forrest et al., 2010)	CDC14A (CDC14 cell division cycle 14 homolog A; Forrest et al., 2010)	
hsa-miR-148a	CaMKIIα (CNS kinase; Liu et al., 2010e)	MLC1 (megalencephalic leukoencephalopathy with subcortical cysts 1; Geisler et al., 2011)	MSK1 (histone phosphorylase; Fujita et al., 2010)
	DNMT1 (DNA methyltransferase 1; Pan et al., 2010)	DNMT3B (CpG island methylation; Duursma et al., 2008)	MITF (microphthalmia-associated transcription factor; Haflidadottir et al., 2010)
	CCKBR (modulates anxiety, analgesia, arousal, and neuroleptic activity; Muinos-Gimeno et al., 2011)	EPAS1 (endothelial PAS domain-containing protein 1; Giraud-Triboult et al., 2011)	HLA-G (asthma susceptibility; Tan et al., 2007)
	POMC (pro-opiomelanocortin; Muinos-Gimeno et al., 2011)	CAND1 (ubiquitin ligase regulation; Murata et al., 2010)	PXR (pregnane X receptor; Takagi et al., 2008)
hsa-miR-152	CaMKIIα (CNS kinase; Liu et al., 2010e)	DNMT1 (DNA methyltransferase 1; Braconi et al., 2010a)	
hsa-miR-125b	TNFα (tumor necrosis factor α; Tili et al., 2007)	ERBB2 (erythroblastic leukemia viral oncogene homolog 2; Scott et al., 2007)	BMPR1B (bone morphogenic receptor type 1B; Saetrom et al., 2009)
	IRF4 (interferon regulatory factor 4; Malumbres et al., 2009)	ERBB3 erythroblastic leukemia viral oncogene homolog 3; Scott et al., 2007)	E2F3 (cell cycle control; Huang et al., 2011a)
	Blimp1 (zinc finger protein; Zhang et al., 2011b)	TEF (thyrotroph embryonic factor; Gutierrez et al., 2011)	Bcl2 modifying factor (apoptosis; Xia et al., 2009b)
	Vdr (vitamin D receptor; Zhang et al., 2011b)	MUC1 (adhesion; Rajabi et al., 2010)	Bak1 (pro-apoptotic Bcl2 antagonist killer 1; Zhou et al., 2010)
	CYP24A1 (cytochrome P450, family 24A, polypeptide 1; Komagata et al., 2009)	p53 (tumor suppressor; Le et al., 2009)	SMO (smoothened receptors; Ferretti et al., 2008)
	IGF2 (insulin-like growth factor 2; Ge et al., 2011)	Suv39h1 (histone methyltransferase; Villeneuve et al., 2010)	Stat3 (transcription factor binds to IL-6; Surdziel et al., 2011)
	LIN28 (translational enhancer; Zhong et al., 2010)	NMDA receptor subunit NR2A (Edbauer et al., 2010)	ATM (ataxia telangiectasia mutated; Smirnov and Cheung, 2008)
hsa-miR-125a-5p	LIN28 (translational enhancer; Wu and Belasco, 2005)	T-TrkC (neurotrophic tyrosine kinase receptor 3; Ferretti et al., 2009)	HuR (cell growth; Guo et al., 2009)
	p53 (tumor suppressor; Zhang et al., 2009)	KLF13 (transcription factor; Zhao et al., 2010)	AT-rich interactive domain 3B (transcription factor; Cowden Dahl et al., 2009)
	PDPN 9 (actin organization; Cortez et al., 2010)	Bak1 (pro-apoptotic Bcl2 antagonist killer 1; Guo et al., 2010)	
	N-ras (oncogene; Juan et al., 2009)	MEK3 (phosphorylation of MAP kinase; Li et al., 2011)	

(Continued)

Table A2 | Continued

miR ID	Validated targets		
hsa-miR-214	SrGAP1(neuronal migration; Zhang et al., 2011a)	Ezh2 (stem cell identity; Juan et al., 2009)	N-ras (oncogene; Liu et al., 2010b)
	JNK1 (MAPK8; Yang et al., 2009)	PTEN (tumor suppressor; Yang et al., 2009)	MEK3 (phosphorylation; Yang et al., 2009)
hsa-miR-199a-5p	Hif-1α (hypoxia-inducible factor 1; Rane et al., 2009)	Sirt1 (apoptosis; Rane et al., 2009)	DDR1 (discoidin domain receptor 1; Shen et al., 2010)
	IKKβ (NFκB activation; Chen et al., 2008)		
hsa-miR-31	ICAM-1 (leukocyte adhesion protein; Suarez et al., 2010)	Fgf13 (fibroblast growth factor 13; Mardaryev et al., 2010)	Dkk-1 (canonical Wnt signaling; Xi et al., 2010)
	DACT-3 (epigenetic regulator of Wnt; Xi et al., 2010)	E-selectin (inflammation; Suarez et al., 2010)	p16Ink4a (cell cycle; Malhas et al., 2010)
	LATS2 (tumor suppression; Liu et al., 2010c)	PPP2R2A (signal transduction; Liu et al., 2010c)	Krt16 (keratin 16; Mardaryev et al., 2010)
	Krt17 (keratin 17; Mardaryev et al., 2010)	Dlx3 (development of ventral forebrain; Mardaryev et al., 2010)	E2F6 (cell cycle; Bhatnagar et al., 2010)
	TIAM1 (T-cell lymphoma invasion and metastasis 1; Cottonham et al., 2010)	Fzd3 (accumulation of β-catenin; Valastyan et al., 2009)	Integrin α (fibronectin; Valastyan et al., 2009)
	M-RIP (regulation of actin; Valastyan et al., 2009)	MMP16 (blood vessels matrix remodeling; Valastyan et al., 2009)	RDX (actin filaments binding to plasma membrane; Valastyan et al., 2009)
	RhoA (signal transduction; Valastyan et al., 2009)	SATB2 (upper-layer neurons initiation; Aprelikova et al., 2010)	PROX1 (CNS development; Pedrioli et al., 2010)
	WAVE3 (signal transmission; Sossey-Alaoui et al., 2010)		
hsa-miR-185	Six1 (limb development; Imam et al., 2010)		
hsa-miR-193b	Estrogen receptor α (Leivonen et al., 2009)	ETS-1 (oncogene; Xu et al., 2010a)	CCND1 (cyclin D1; Xu et al., 2010a)
	Mcl-1 (myeloid cell leukemia sequence 1; Braconi et al., 2010b)	uPA (urokinase-type plasminogen activator; Li et al., 2009b)	
hsa-miR-7	Alpha-synuclein (SNCA; Junn et al., 2009)	SFRS1 (Wu et al., 2010b)	ERF (cell proliferation; Chou et al., 2010)
	LSH (lymphoid-specific helicase; Ilnytskyy et al., 2008)	DAP (cell death-associated protein; Yu et al., 2009)	MRP1 (human multidrug resistance-associated protein 1; Pogribny et al., 2010)
	Associated cdc42 kinase 1 (Saydam et al., 2011)	Yan (cell differentiation; Li and Carthew, 2005)	EGFR (epidermal growth factor receptor; Kefas et al., 2008)
	CD98 (sodium transport; Nguyen et al., 2010)	Pak1 (p21-activated kinase 1; Reddy et al., 2008)	IGF1R (insulin-like growth factor 1 receptor; Jiang et al., 2010)
hsa-miR-483-5p	Socs-3 (cytokine signaling; Ma et al., 2011)	BBC3/PUMA (apoptosis; Veronese et al., 2010)	
hsa-miR-663	TGFβ1 (proliferation; Tili et al., 2010b)	JunB (jun B proto-oncogene; Tili et al., 2010a)	JunD (jun D proto-oncogene; Tili et al., 2010a)
hsa-miR- 765	TRK3 (neurotrophic tyrosine kinase; Guidi et al., 2010)		
hsa-miR- 146b-3p	IRAK1 (interleukin-1 receptor-associated kinase 1; Taganov et al., 2006)	EGFR (epidermal growth factor receptor; Shao et al., 2011)	MMP16 (degrades extracellular matrix; Xia et al., 2009a)

miRs without validated targets that are predicted to target AChE-R: hsa-miR-590-3p, -148b, -193a-3p, -182, -4298, -4644, -4739, -1224-3p, -4769-5p, –582-3p, -380*, -1825, -892b, -1275, -3155, -765, -3119, -3139, -563, -92b*, -1321, -4283, -1228*, -4323, -4319, -761, -767-5p, -224*, -522, -4271, -1226*, -3179, -92a-1*, -3202, -20b*, -4303, -4306, -3065-5p, -4297, -4329, -3148, -3163, -22*, -4302, -513a-5p, -542-5p, -377*, -1908, -92a-2*, -608, -625.*

Table A3 | Additional targets of BChE-targeting microRNAs.

miR ID	Validated target		
hsa-miR-203	SOCS-3 (cytokine signaling; Wei et al., 2010)	Lef1 (lymphoid enhancer-binding factor; Thatcher et al., 2008)	p63 (transcriptional activator or repressor; Yi et al., 2008)
	ABL1 (cell growth; Bueno et al., 2008)	Barx1 (transcription factor; Kim et al., 2011)	CKAP2 (cytoskeleton associated protein 2; Viticchie et al., 2011)
	LASP1 (cytoskeletal activities; Viticchie et al., 2011)	BIRC5 (regulator of mitosis; Viticchie et al., 2011)	WASF1 (signal transmission; Viticchie et al., 2011)
	ASAP1 (membrane trafficking; Viticchie et al., 2011)	RUNX2 (runt-related transcription factor 2; Viticchie et al., 2011)	
hsa-miR-340	MITF (microphthalmia-associated transcription factor; Goswami et al., 2010)		
hsa-miR-218	IKK-β (cytokine-activated intracellular signaling pathway; Song et al., 2010)	ROBO1 (roundabout, axon guidance receptor, homolog 1; Alajez et al., 2011)	BIRC5 (regulator of mitosis; Alajez et al., 2011)
	GJA1 (gap junction protein, α1; Alajez et al., 2011)	ROBO2 (roundabout, axon guidance receptor homolog 2; Alajez et al., 2011)	GLCE (glucuronic acid epimerase; Small et al., 2010)
	PXN (paxillin, cytoskeletal protein; Wu et al., 2010a)		
hsa-miR-221	ERα (estrogen receptor α; Zhao et al., 2008)	ICAM-1 (leukocyte adhesion protein; Hu et al., 2010)	p27 (cell cycle; Garofalo et al., 2008)
	p57 (cyclin-dependent kinase inhibitor 1C; Kim et al., 2009b)	DNA damage-inducible transcript 4 (DDIT4; Pineau et al., 2010)	TIMP3 (TIMP metallopeptidase inhibitor 3; Garofalo et al., 2009)
	PTEN (tumor suppressor; Garofalo et al., 2009)	PUMA (apoptosis; Zhang et al., 2010)	C-fos (cell proliferation; Ichimura et al., 2010)
	Bmf (apoptosis; Gramantieri et al., 2009)	Mdm2 (ubiquitination; Kim et al., 2010)	CDKN1B (cyclin-dependent kinase inhibitor 1B; Kotani et al., 2009)
hsa-miR-222	ERα (estrogen receptor α; Zhao et al., 2008)	p27 (cell cycle; Garofalo et al., 2008)	PTEN (tumor suppressor; Garofalo et al., 2009)
	STAT5A (signal transducer and activator of transcription 5; Dentelli et al., 2010)	p57 (cyclin-dependent kinase inhibitor 1C; Kim et al., 2009b)	TIMP3 (TIMP metallopeptidase inhibitor 3; Garofalo et al., 2009)
	Bim (apoptosis; Terasawa et al., 2009)	ETS-1 (transcription factor; Zhu et al., 2011)	PUMA (apoptosis; Zhang et al., 2010)
	PPP2R2A (protein phosphatase 2A subunit B; Wong et al., 2010)	C-fos (cell proliferation; Ichimura et al., 2010)	ICAM-1 (Ueda et al., 2009)
	MMP1 (cleaves collagens; Liu et al., 2009)	SOD2 (superoxide dismutase 2; Liu et al., 2009)	
hsa-miR-181a	SIRT1 (apoptosis, muscle differentiation; Saunders et al., 2010)	Ataxia telangiectasia mutated (ATM; cell cycle; Wang et al., 2011)	Hox-A11 (transcription factor; Naguibneva et al., 2006)
	p27(cell cycle; Cuesta et al., 2009)	PLAG1 (transcription factor; Pallasch et al., 2009)	BCL2 (B-cell CLL/lymphoma 2; apoptosis; Zhu et al., 2010)
	Bim (apoptosis; Lwin et al., 2010)	Tcl1 (cell proliferation; Pekarsky et al., 2006)	OPN (osteopontin; Bhattacharya et al., 2010)
hsa-miR-181b	AID (activation-induced cytidine deaminase; RNA-editing; De Yebenes et al., 2008)	PLAG1 (transcription factor; Pallasch et al., 2009)	BCL2 (B-cell CLL/lymphoma 2; apoptosis; Zhu et al., 2010)
	TIMP3 (TIMP metallopeptidase inhibitor 3; Wang et al., 2010a)	Ataxia telangiectasia mutated (ATM; cell cycle; Wang et al., 2011)	SIRT1 (apoptosis, muscle differentiation; Saunders et al., 2010)
	ZNF37A (transcriptional regulation; Huang et al., 2010)	ZNF83 (transcriptional regulation; Huang et al., 2010)	ZNF182 (transcriptional regulation; Huang et al., 2010)
	Mcl-1 (myeloid cell leukemia-1; apoptosis; Zimmerman et al., 2010)		
hsa-miR-181c	IL2 (immune response; Xue et al., 2011)	BCL2 (B-cell CLL/lymphoma 2; apoptosis; Zhu et al., 2010)	NOTCH4 (transcriptional activator complex; Hashimoto et al., 2010)

(Continued)

Table A3 | Continued

miR ID	Validated target		
	KRAS (GTPase activity; Hashimoto et al., 2010)		
hsa-miR-181d	BCL2 (B-cell CLL/lymphoma 2; Zhu et al., 2010)		
hsa-miR-494	CaMKIIδ (CNS kinase; Wang et al., 2010b)	ROCK-1 (apoptosis; Wang et al., 2010b)	LIF (leukemia inhibitory factor (cholinergic differentiation factor); Wang et al., 2010b)
	PTEN (phosphatase and tensin homolog; Wang et al., 2010b)	TEL-AML1 (hematopoiesis; Diakos et al., 2010)	FGFR2 (fibroblast growth factor receptor 2; Wang et al., 2010b)
hsa-miR-129-5p	CAMTA1 (calmodulin binding transcription activator 1; Liao et al., 2008) SOX4 (transcriptional activator; Dyrskjot et al., 2009)	EIF2C3 (eukaryotic translation initiation factor 2C, 3; Liao et al., 2008)	GALNT1 (oligosaccharide biosynthesis; Dyrskjot et al., 2009)
hsa-miR-30d	Galphai2 (G protein, α inhibiting activity polypeptide 2; Yao et al., 2010)		
hsa-miR-30c	Runx1 (runt-related transcription factor 1; Ben-Ami et al., 2009)	CTGF (connective tissue growth factor; Duisters et al., 2009)	
hsa-miR-30a	SOD2 (superoxide dismutase 2; Xia et al., 2006) Xlim1/Lhx1 (transcription factor; Agrawal et al., 2009)	BDNF (brain-derived neurotrophic factor; Mellios et al., 2008)	Beclin 1 (autophagy; Zhu et al., 2009)
hsa-miR-30e	Ubc9 (ubiquitin-conjugating enzyme E2I; Wu et al., 2009)	B-Myb (transcription factor; Martinez et al., 2011)	
hsa-miR-320a	(Hsp20 heat-shock protein 20; Ren et al., 2009)	AQP1 (aquaporin 1; Sepramaniam et al., 2010)	AQP4 (aquaporin 4; Sepramaniam et al., 2010)
	The transferrin receptor 1(TfR-1; CD71; development of erythrocytes and the nervous system; Schaar et al., 2009)	Mcl-1 (myeloid cell leukemia sequence 1; apoptosis; Chen et al., 2009)	
hsa-miR-140-5p	Smad3 (transcriptional modulator; Pais et al., 2010)	HDAC4 (histone deacetylase 4; Tuddenham et al., 2006)	
hsa-miR-519c-3p	HIF-1α (hypoxia-inducible factor 1α; Cha et al., 2010)	ABCG2 (exclusion of xenobiotics from the brain; To et al., 2008)	
hsa-miR-489	PTPN11 (signal transduction; Kikkawa et al., 2010)		
hsa-miR-584	NXA1 (exocytosis; Luthra et al., 2008)	ROCK-1 (actin assembly; Ueno et al., 2011)	

miRs without validated targets: hsa-miR-147b, -532-5p, -508-3p, -889, -325, -573, -195, -567, -193b*, -625, -16-2*, -576-3p, -190b, -518e*, -518f*, -518d-5p, -147, -320d, -320c, -320b, -875-5p, -758, -30b, -1279, -3145, -1183, -664, -4261, -4262, -1237, -1972, -3146, let-7a-2*, let-7g*, -1911*, -2052, -15a*, -3148, -555, -656, -636, -3182, -513a-3p, -501-3p, -502-3p, -579, -4316, -4312, -1294, -142-5p, -3128, -30a*, -30d*, -30e*, -4268, -3137, -20b*, -651, -32*, -362-5p, -500b, -501-5p, -1976, -449c*, -1224-5p, -302a*, -1248, -99b*, -99a*, -369-3p, -1256, -629, -187*, -514b-3p, -378*, -1305, -331-5p, -1200, -4272, -4260, -493*, -582-5p, -4255, -3133, -4273, -19a*, -19b-1*, -19b-2*, -4271, -15b*, -1826.*

Table A4 | Additional targets of ChE-targeting miRs (common to more than one ChE).

miR ID	Validated target common to ACHE-R and AChE-S		
Hsa-miR-186	Pro-apoptotic P2 × 7 purinergic receptor(Zhou et al., 2008)	AKAP12 (tumor suppressor; Goeppert et al., 2010)	
Hsa-miR-199b-5p	Dyrk1a (brain development; Da Costa Martins et al., 2010)	HES1 (transcriptional repressor; Garzia et al., 2009)	SET (apoptosis; Chao et al., 2010)
Hsa-miR-429	ZEB1 (transcriptional repression of IL2; Gregory et al., 2008) RERE (apoptosis; Karres et al., 2007)	ZEB2 (SIP1; zinc finger protein; Gregory et al., 2008)	PLCgamma1(apoptosis; Uhlmann et al., 2010)
Hsa-miR-200b	ZEB1 (transcriptional repression of IL2; Gregory et al., 2008) Serca2 (sarco/endoplasmic reticulum Ca2+-ATPase; Salomonis et al., 2010) OREBP (osmotic response element; Huang et al., 2011b)	ZEB2 (SIP1; zinc finger protein; Gregory et al., 2008) Suz12 (chromatin silencing; Iliopoulos et al., 2010) Cyclin D1 (Xia et al., 2010)	PLCgamma1(apoptosis; Uhlmann et al., 2010) Ets-1 (transcriptions factor; Chan et al., 2011)
Hsa-miR-200c	ZEB1 (transcriptional repression of IL2; Gregory et al., 2008) VEGF (angiogenesis; Liu et al., 2010a) KLF13 (transcription factor; Li et al., 2009a)	ZEB2 (SIP1; zinc finger protein; Gregory et al., 2008) TUBB3 (neurogenesis and axon guidance; Cochrane et al., 2009) MBNL2 (muscleblind-like protein 2; Li et al., 2009a)	PLCgamma1(apoptosis; Uhlmann et al., 2010) TRPS1 (transcription factor; Li et al., 2009a) FAP1 (apoptosis; Schickel et al., 2010)
miR ID	**Validated targets common to ACHE-R and BChE**		
Hsa-miR-24	SOD1 (superoxide dismutase 1; Papaioannou et al., 2011) MKK4 (survival signal in T cells; Marasa et al., 2009) FAF1 (apoptosis; Qin et al., 2010) DHFR (dihydrofolate reductase; Mishra et al., 2009)	ALK4 (transducer of activin; Wang et al., 2008) E2F2 (cell cycle; Lal et al., 2009a) HNF4α (cell proliferation; Takagi et al., 2010) DND1(miRNA-mediated gene suppression; Liu et al., 2010d)	Notch1 (Bergmann glia differentiation; Fukuda et al., 2005) H2AX (histone-formation; Lal et al., 2009b) FURIN (processing of TGFβ1; Luna et al., 2011)
Hsa-miR-212	MeCP2 (interaction with histone deacetylase; Im et al., 2010) PED (apoptosis; Incoronato et al., 2010)	MYC (transcription; Xu et al., 2010b)	Rb1(tumor suppressor; Park et al., 2011)
Hsa-miR-132	AChE-S (Shaked et al., 2009) SirT1 (apoptosis; Strum et al., 2009) Jarid1a (histone demethylase; Alvarez-Saavedra et al., 2011) p120RasGAP (angiogenesis; Anand et al., 2010)	P250GAP (neuron-associated GTPase; Vo et al., 2005) MeCP2 (modification of eukaryotic genomes; Klein et al., 2007) Btg2 (cell cycle; Alvarez-Saavedra et al., 2011)	Per1 (circadian clock; Cheng et al., 2007) p300 (chromatin remodeling; Lagos et al., 2010) Paip2a (translation regulation; Alvarez-Saavedra et al., 2011)
Hsa-miR-198	Cyclin T1(Xu et al., 2010b)		
Hsa-miR-194	Rac1 (GTP-binding protein; Venugopal et al., 2010) MDM2 (p53 negative regulator; Pichiorri et al., 2010)	Per family (circadian; Nagel et al., 2009)	EP300 (transcriptional co-activator; Mees et al., 2010)

miRs without validated targets: hsa-miR-423-3p, -484, -4728-3p, -939, -484, -4728-3p.

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Acetylcholinesterase (AChE) is an important link in the apoptotic pathway induced by hyperglycemia in Y79 retinoblastoma cell line

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Acetylcholinesterase (AChE) expression was found to be induced in the mammalian CNS, including the retina, by different types of stress leading to cellular apoptosis. Here, we tested possible involvement of AChE in hyperglycemia-induced apoptosis in a retinal cell line. Y79 retinoblastoma cells were incubated in starvation media (1% FBS and 1 mg/ml glucose) for 16–24 h, and then exposed to hyperglycemic environment by raising extracellular glucose concentrations to a final level of 3.5 mg/ml or 6 mg/ml. Similar levels of mannitol were used as control for hyperosmolarity. Cells were harvested at different time intervals for analysis of apoptosis and AChE protein expression. Apoptosis was detected by the cleavage of Poly ADP-ribose polymerase (PARP) using western blot, and by Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) assay. AChE protein expression and activity was detected by western blot and by the Karnovsky and Roots method, respectively. *Mission*^{TM} shRNA for AChE was used to inhibit AChE protein expression. Treating Y79 cells with 3.5 mg/ml of glucose, but not with 3.5 mg/ml mannitol, induced apoptosis which was confirmed by TUNEL assay and by cleavage of PARP. A part of the signaling pathway accompanying the apoptotic process involved up-regulation of the AChE-R variant and an N-extended AChE variant as verified at the mRNA and protein level. Inhibition of AChE protein expression by shRNA protected Y79 cell from entering the apoptotic pathway. Our data suggest that expression of an N-extended AChE variant, most probably an R isoform, is involved in the apoptotic pathway caused by hyperglycemia in Y79 cells.

Keywords: acetylcholinesterase, apoptosis, hyperglycemia, retinoblastoma

INTRODUCTION

Acetylcholinesterase (AChE) is an essential enzyme in cholinergic synapses since it hydrolyses acetylcholine, thereby terminating cholinergic excitation. However, AChE was also found in tissues devoid of cholinergic innervations (Small et al., 1996), indicating potential non-cholinergic functions for this enzyme including response to stress (Grisaru et al., 2001) and neurogenesis (Robitzki et al., 1998; Bytyqi et al., 2004). There are three different isoforms of AChE resulting from alternative splicing of a single gene: synaptic (S), erythrocytic (E), and read-through (R) (Soreq and Seidman, 2001). Each of these three C-terminal variants can appear in one of two forms depending upon the length of its N-terminus; a normal N-terminus and an extended one. Thus, in principal AChE can appear in six different isoforms. Different AChE variants are believed to be involved in different physiological and/or pathological processes. AChE-S is the variant located in cholinergic synapses and responsible for termination of cholinergic excitation (reviewed in Meshorer and Soreq, 2006). AChE-R was found to play a role in the response to stress in laboratory animals (Kaufer et al., 1998; Shohami et al., 2000). Forced swimming (physiological stress) induced AChE-R mRNA expression in FVB/N mice (Kaufer et al., 1998). 2′-O-methyl RNA-capped antisense oligonucleotide (AS-ODN) against AChE mRNA blocked over-expression of AChE-R in head-injured mice, reducing the number of dead CA3 hippocampal neurons and facilitating neurological recovery (Shohami et al., 2000). A novel AChE variant with an extended N-terminus was found to exacerbate light-induced photoreceptor degeneration in albino rats. Treatment with AS-ODNs significantly reduced the expression of this protein, and lowered the degree of light-induced functional damage as expressed by electroretinogram recordings (Kehat et al., 2007). AChE was found to be expressed during apoptosis in different cell types in which the apoptotic pathway could be prevented by AS-ODNs of AChE (Soreq et al., 1994; Zhang et al., 2002). It was later reported that the AChE variant involved in apoptosis was the N-extended AChE-S one (Toiber et al., 2008, 2009). There is evidence regarding the correlation between stress-activated protein kinase family and apoptosis-associated AChE expression. Phosphorylation of c-Jun N-terminal kinase (JNK) and its downstream transcription factor, c-Jun, was enhanced during apoptosis induced by the DNA topoisomerase inhibitors etoposide or excisanin A, in colon cancer cell line SW620. A corresponding

increase in AChE expression in the apoptotic cells was observed. This up-regulation in AChE was eliminated by administering a JNK inhibitor, silencing JNK with siRNA or antagonizing c-Jun with a dominant-negative c-Jun mutant (Deng et al., 2006).

Apoptosis—programmed cell death, is an active process that has well-documented biochemical and morphological characteristics, including nuclei break into several pycnotic granules as a result of DNA fragmentation and cell shrinkage (Majno and Joris, 1995). In the retina, apoptosis plays a major role in the loss of visual cells in degenerative disorders such as Retinitis Pigmentosa and Age-Related Macular Degeneration (reviewed in Wenzel et al., 2005), and also occurs in Diabetic Detinopathy (Barber et al., 1998). Biochemical, physiological, and morphological alterations have been observed in non-vascular retinal cells (Barber et al., 1998; Li et al., 2002; Mohr et al., 2002; Kusner et al., 2004; Xi et al., 2005) during early stages of diabetes in animal models, raising the possibility that hyperglycemia is detrimental to retinal neurons prior to any vascular involvement. Rats with streptozotocin-induced-diabetes exhibited reduced electroretinogram responses as early as two weeks after the onset of the diabetes. The rats also expressed glial fibrilary acidic protein (GFAP) in Muller cells before any vascular abnormality was detected in the retina, a sign of early retinal damage (Li et al., 2002). Several studies have shown that Muller cells incubated in elevated glucose concentrations undergo apoptosis as determined in part by annexin-V staining and activation of caspase 3 (Lorenzi and Gerhardinger, 2001; Mohr et al., 2002; Kusner et al., 2004; Xi et al., 2005). Our goals were to test the hypothesis that AChE is involved in hyperglycemia-induced apoptosis of retinal cell line and to identify the AChE variant included in this process.

MATERIALS AND METHODS

CELL CULTURE

Y79 (HTB18) retinoblastoma cell line was obtained from the American Type Culture Collection (ATCC, The global Bioresource Center, USA), and cultured in DMEM containing 10% fetal bovine serum (FBS), 4.5 mg/ml glucose, 1% glutamine, 1% penicillin, and streptomycin (HG-medium) at 37°C in a humidified atmosphere with 5% CO2/95% air. Culture medium was changed twice weekly. Twenty-four hours before experiments, the cells were transferred to a starvation medium containing 1 mg/ml glucose, mimicking physiologic levels of blood glucose concentrations and 1% FBS, 1% glutamine, 1% penicillin, and streptomycin (LG-medium). Then, glucose was added to the Y79 cells to reach a final concentration of 3.5 mg/ml or 6 mg/ml mimicking hyperglycemia (final concentrations in media: 3.5 mg/ml, 19.4 mM, 350 mg/dl and 6 mg/ml, 33.3 mM, 600 mg/dl, respectively). Identical mannitol concentrations were added to the media instead of glucose, to isolate the effect of hyperglycemia from hyperosmolarity.

Cells were harvested at different time points (1, 2, and 24 h after the addition of glucose/mannitol) and analyzed for apoptosis and AChE expression and activity. It is important to note that since serum is known to contain a complex mixture of growth factors, cytokines, and other signaling molecules that stimulate the expression of several genes like *c-fos*, *c-myc*, *cyclin D1*, and *VEGF* in cultured cells (Harris et al., 2001), it was important to reduce the percentage of the FBS to the minimum (1%) needed for the Y79 retinoblastoma cells to maintain the cells in a quiescent un-stimulated phase.

DETECTION OF APOPTOSIS BY TUNEL

Apoptosis was detected by Terminal Deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) using the fluorescein-dUTP-*in situ* cell death detection kit (Roche Diagnostic GmbH, Mannheim, Germany). For TUNEL assay, cells were seeded on microscope cover glasses pre-coated with 0.1% poly-D-lysine [prepared in 100 mM $Na_2B_4O_7.10(H_2O)$ buffer at pH 8.2], and cultured over-night in the HG-medium. After confirming attachment of cells to the cover slips, medium was changed to LG-medium for over-night starvation. Glucose was added to the starved cells at different concentrations and for different time periods, after which TUNEL was performed according to manufacturer's instructions. Briefly, cells were fixed in 4% paraformaldehyde for 1 h, and then washed in PBS. Cells were permeabilized in a solution containing 0.1% Triton X-100 in 0.1% sodium citrate on ice, washed twice with PBS and incubated with TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere in the dark. Slides were washed twice with PBS, stained with DAPI for nuclei detection, and washed again with PBS. The cover glasses with cells were flapped on glass slides covered with a few drops of mounting media (DakoCytomation), photographed using an upright fluorescence microscope (Axioscop 2, ZEISS), and processed by the Image Pro software.

WESTERN BLOT ANALYSIS

Apoptosis was also detected by PARP cleavage from 116 kDa into 89 kDa fragments using western blot analysis. Since PARP is a nuclear protein, we decided to test its cleavage in nuclear extracts and not in whole-cell extracts to prevent dilution. Nuclear extracts were prepared and collected as described previously (Schreiber et al., 1989). Proteins were separated by 10% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were incubated in 5% non-fat dry milk in PBS for 1 h, and subsequently probed with a primary monoclonal antibody: anti-PARP (SeroTec, UK) that detects the full length as well as the cleaved PARP. HRP-conjugated Donkey-anti-mouse (Jackson ImmunoResearch Laboratories, Inc., USA) was used as secondary antibody. Bound antibodies were detected using enhanced chemiluminescence reagent-ECL (prepared locally) and visualized by autoradiography. AChE expression was also detected using western blot analysis. Cytoplasmic extracts were prepared (Schreiber et al., 1989), and separated on 12.5% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane as described above. A rabbit anti-human polyclonal AChE antibody, which detects all isoforms of AChE, was used (sc-11409, diluted 1:500 from Santa Cruz Biotechnology, Inc., USA). A mouse monoclonal antibody anti-α-Tubulin (T6199, diluted 1:4000 from Sigma-aldrich, USA) was used for normalizing cytoplasmic protein loading. HRP-conjugated Donkey-anti-rabbit or anti mouse (1:5000 diluted in 50% glycerol, Jackson ImmunoResearch Laboratories, Inc., USA) was used as secondary antibody. Densitometry analysis was done by TotalLab Quant software.

AChE ACTIVITY

The method of AChE cytochemical staining is based on the ability of AChE to hydrolyze acetylthiocholine, which contains a sulfur atom substituted for oxygen in the ester linkage. Enzymatic cleavage of acetylthiocholine by AChE yields acetate and thiocholine, whose sulphydryl group reduces ferricyanide to ferrocyanide. Brown copper ferrocyanide (Hatchett's brown), is formed at the site of enzymatic activity and immediately precipitates thus marking the site of AChE activity. The procedure for detecting AChE activity was originally described by Karnovsky and Roots (Karnovsky and Roots, 1964), and modified by Hanker et al. (Hanker et al., 1973) and Kobayashi et al. (Kobayashi et al., 1994). Briefly, cells were seeded on microscope cover glasses while cultured in the DMEM culturing medium. Upon being dried at room temperature, the cells were fixed in 4% paraformaldehyde for 1 h, and then washed in PBS. The cover glasses were then covered with 1 ml per slide of a mixture containing: 65 mM sodium phosphate pH 6.0 containing 0.5 mg acetylthiocholine iodide, 5 mM sodium citrate solution, 10 mM copper sulphate solution, 0.5 mM potassium ferricyanide, 0.1 ml DDW, and 10^{-2} mM of Iso-OMPA (Tetra-isopropyl pyrophosphoramide for inhibition of Butyrylcholinesterase) at room temperature for overnight incubation. The cover glasses were flapped on glass slides covered with few drops of immuno-mount (Thermo scientific, USA). Cells preparations were viewed and photographed under light microscopy.

RNA EXTRACTION AND REAL-TIME RT-PCR

Total RNA was extracted by TRI-REAGENT™ (Sigma Aldrich, St. Louis, USA), according to Sigma's product information. RNA samples (0.2 μg) were used for cDNA synthesis prepared by the Verso™ cDNA Kit (Thermo SCIENTIFIC, UK). Real-time RT-PCR was performed using the Rotor-Gene Q (Qiagen, Corbett life science, Germany) and SYBR green master mix ABsolute™ Blue (Thermo SCIENTIFIC, UK). Different primer sequences for human AChE variants were used according to previous description; Primer sequences (listed below), for AChE-S, AChE-R, 18S rRNA (house-keeping reference transcript) (Berson et al., 2008) and for N-extended AChE (Ofek et al., 2007). Serial dilutions of samples served to evaluate primers efficiency and the appropriate cDNA concentration that yields linear changes. Annealing temperature was 60°C for all primers. Melting curve analysis verified the identity of end products.

PCR primers were employed as follows:

hAChE-S (Accession no NM000665);
(Forward primer position 1789-1807) – 5′-cttcctccccaaattgctc-3′
(Reverse primer position 1901-1920) – 5′-tcctgcttgctgtagtggtc-3′
hAChE-R (Accession no AY750146);
(Forward primer position 7092-7110) – 5′cttcctccccaaattgctc-3′
(Reverse primer position 7177-7196) – 5′-ggggagaagagaggggt tac-3′

h N-extended-AChE (Accession no AY389977);
(Forward primer position 189-207) – 5′-atgctaggcctggtgatgt-3′
(Reverse primer position 285-303) – 5′-ggcagtggaaacttctgga-3′

h18S rRNA (Accession no M10098);
(Forward primer position 1049-1068) – 5′-cgccgctagaggtgaaa ttc-3′
(Reverse primer position 1092-1110) – 5′-ttggcaaatgctttcgctc-3′

MISSION™ shRNA FOR AChE

shRNA packed in Lenti viral particles were prepared using Lenti compatible vectors; VSVG and delta-NRF which were kindly provided by Prof. Gera Neufeld (Faculty of Medicine, Technion), and five pLKO.1—puro vectors each cloned with a potential translational—blocker-complimentary sequence (21 bp long) to AChE exons (Sigma Aldrich, St. Louis, USA). Each pLKO.1 vector together with the two vectors mentioned above were co-transfected in 293-T cell line (using TransIT®-LT-1 Transfection reagent, Mirus Corporation, USA). Forty-eight hours post-transfection, medium containing viruses was collected and filtered through a 0.45 μm filter, and was used to infect Y79 cells for 48 h. Puromycin (2.5 μg/ml) was added to culture media for an additional 48 h for selecting positive-Lenti-infected-cells. Prior to the addition of 3.5 mg/ml glucose, infected-Y79 cells were serum starved under the same conditions as mentioned above. At different time intervals following exposure to glucose, cells were harvested for testing by Western Blot and TUNEL assays as mentioned above.

STATISTICS

Statistically significant differences were calculated by *t-test* (2 tails).

RESULTS

APOPTOSIS INDUCTION BY HYPERGLYCEMIA

Keeping Y79 cells for 1 or 24 h in 1 mg/ml glucose (5.5 mM), mimicking physiologic conditions did not induce apoptosis (**Figure 1A**). Raising the level of glucose to 3.5 mg/ml (19.4 mM) or to 6 mg/ml (33.3 mM), for 1 h also did not result in apoptosis (**Figure 1A**). However, after 24 h in high glucose (3.5 mg/ml or 6 mg/ml—mimicking hyperglycemia in diabetes), apoptosis was stimulated as evident by abundance of TUNEL positive cells (**Figure 1A**).

Counting TUNEL positive cells, indicated that incubating cells in 3.5 mg/ml or 6 mg/ml glucose for 24 h increased apoptosis significantly ($p = 0.05$), by 7- and 10-fold, respectively, as compared to cells exposed to the same concentrations of glucose for 1 h (**Figure 1B**).

Full-length PARP is an 116 kDa nuclear protein involved in the repair of DNA, in differentiation and in chromatin structure formation. During apoptosis this protein is cleaved by caspase-3, and possibly by other caspases, into an 89 kDa fragment (Lazebnik et al., 1994). Thus, demonstration of PARP cleavage is accepted as a molecular indicator for the induction of an apoptotic process. Since cleavage of PARP is an early stage (between 1 and 3 h) in apoptosis, we used western blot to measure its cleavage in nuclear extracts of cells prepared after 1 h incubation in different glycemic conditions as shown in **Figure 2A**. PARP cleavage was induced in cells exposed to 3.5 mg/ml or 6 mg/ml of glucose for 1 h, as compared to 1 mg/ml of glucose. To test a possible osmotic effect induced by hyperglycemia and leading

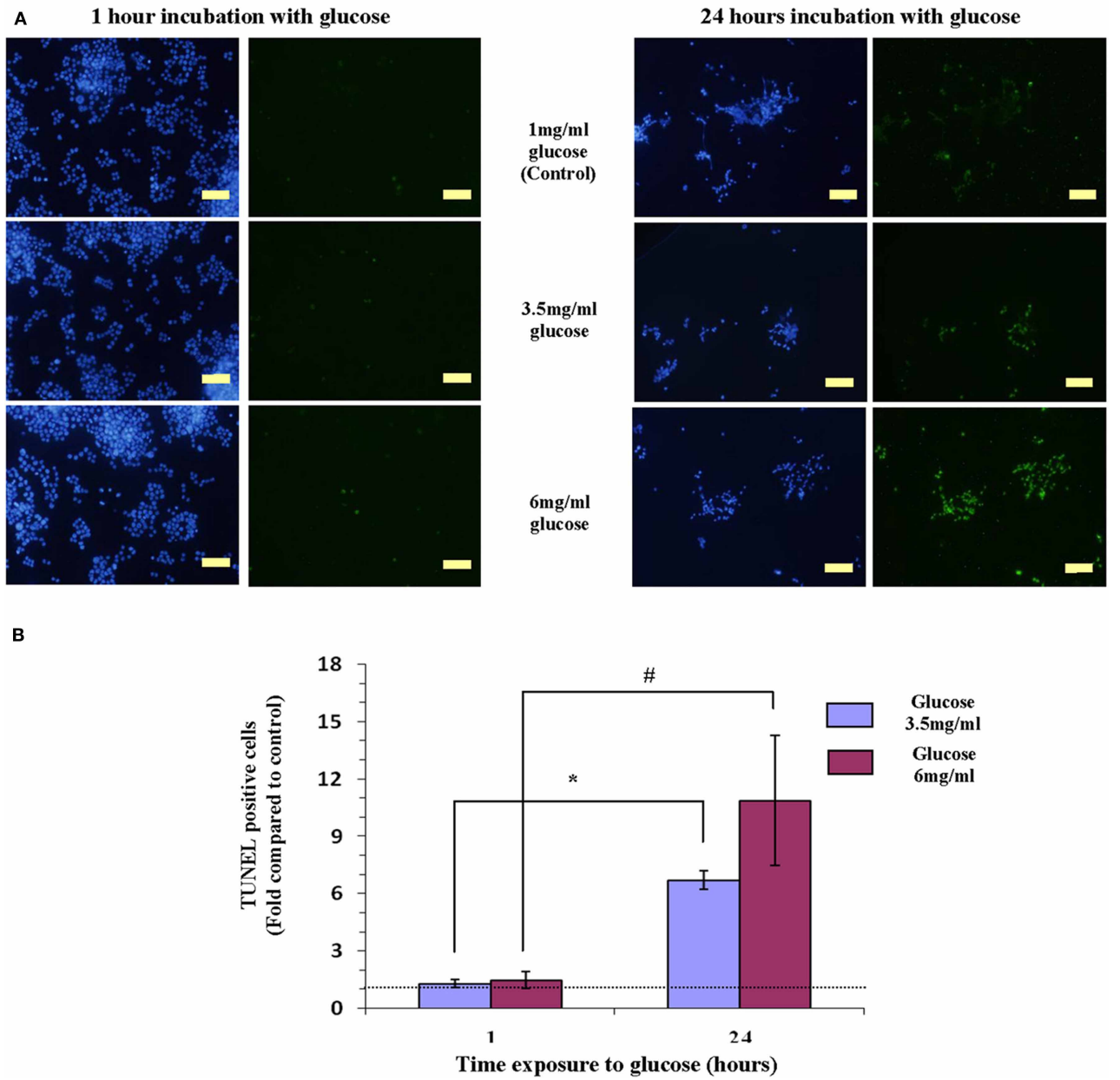

FIGURE 1 | Hyperglicemia (3.5 mg/ml or 6 mg/ml of glucose)-induced apoptosis induction in Y79 cells. (A) Cells tested by TUNEL to detect apoptosis (green) and stained with DAPI for detecting nuclei (blue). Y79 cells were pre-treated for 16–24 h in starvation medium (1% FBS and 1 mg/ml glucose), and then incubated for 1 h with 3.5 mg/ml glucose did not show signs of apoptosis (left); while cells incubated for 24 h in 3.5 mg/ml or 6 mg/ml glucose are TUNEL positive. Scale bar is 50 μm. **(B)** TUNEL positive cells were counted and calculated as fold of control (cells cultured in starvation media containing 1% FBS and 1 mg/ml glucose) and plotted in histograms. Values are means ± SEM, ($N = 3$). *$p < 0.05$, #$p = 0.05$.

to apoptosis, identical mannitol concentrations were added to starved-Y79 cells and PARP cleavage was examined after 1 h. We assessed quantitatively the magnitude of PARP cleavage and its significance from the cleaved/non-cleaved PARP ratio, as suggested by others (Biggs et al., 2001; Grader-Beck et al., 2007). We scanned the western blots and calculate the ratio of cleaved/non-cleaved PARP as shown in **Figure 2B**. PARP cleavage was similar in cells treated with 6 mg/ml of glucose or mannitol, but was more prominent, and statistically significant ($p < 0.05$), in cells treated with 3.5 mg/ml of glucose which showed 2.2 ± 0.16-fold of control, as compared to the same concentration of mannitol which showed 1.3 ± 0.22-fold of control. These results indicate that hyperglycemia caused by 6 mg/ml of glucose, induced apoptosis primarily as a result of hyperosmolarity, while at a lower concentration of 3.5 mg/ml of glucose, apoptosis was induced mainly as a result of hyperglycemia and to a lesser extent by hyperosmolarity.

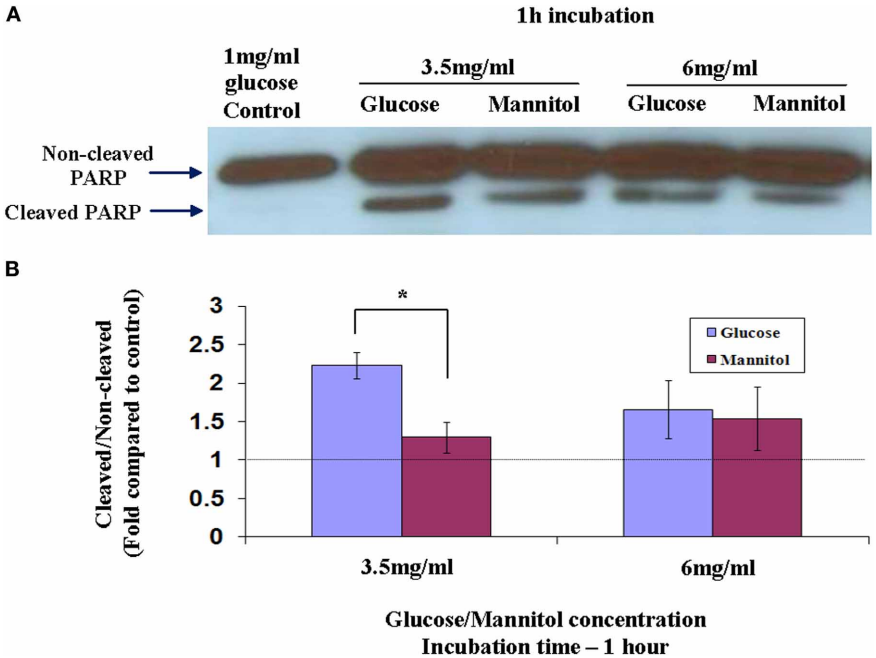

FIGURE 2 | Cleavage of PARP in nuclear extracts of Y79 cells treated by adding glucose or mannitol. Y79 cells were incubated for 16–24 h in starvation medium (1% FBS and 1 mg/ml glucose), and then glucose or mannitol was added (except in control) to reach a concentration of 3.5 mg/ml or 6 mg/ml and incubated for 1 or 2 h. **(A)** Nuclear extracts were prepared and run on SDS-PAGE, blotted, and probed with anti-PARP as described under Materials and Methods. **(B)** Three independent experiments were conducted and densitometry values were calculated for cleaved to non-cleaved ratios, and plotted in histograms as fold of control (cells cultured in starvation media containing 1% FBS and 1 mg/ml glucose). Values are means ± SEM, ($N = 3$). $^*p < 0.05$.

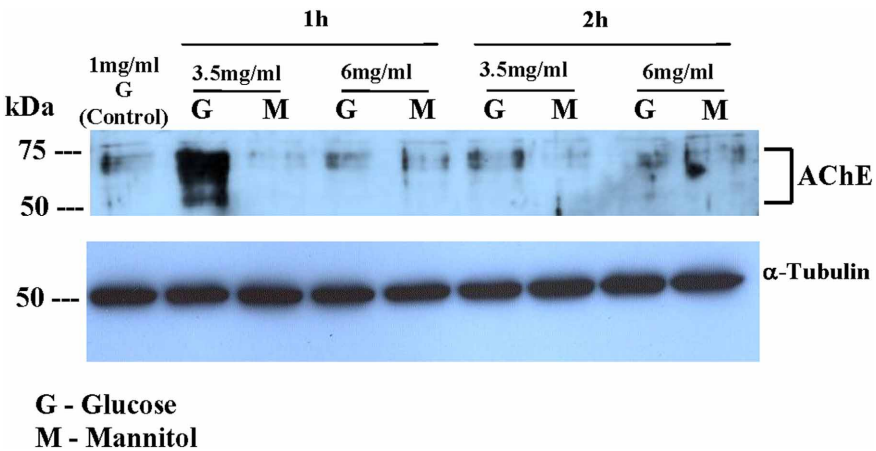

FIGURE 3 | AChE expression in Y79 cells treated by adding glucose or mannitol. Y79 cells were treated first for 16–24 h in starvation medium (1% FBS and 1 mg/ml glucose), and then incubated with 3.5 mg/ml or 6 mg/ml of glucose or mannitol for 1 or 2 h. Following 1 or 2 h incubation cytoplasmic extracts were prepared and run on SDS-PAGE, blotted, and probed with anti- AChE or anti-α-Tubulin (for loading control) as described under Materials and Methods. The western blot shown is one of two independent experiments.

AChE EXPRESSION INDUCED BY HYPERGLYCEMIA

Figure 3 shows western blot analysis for AChE expression in Y79 cells following 1 h or 2 h incubation in different media. AChE expression was increased in Y79 cells treated with 3.5 mg/ml glucose (G) for 1 h and decreased after 2 h to control levels. AChE expression was not increased in cells treated with 6 mg/ml glucose or in cells treated with either 3.5 mg/ml or 6 mg/ml mannitol (M). The dispersed bands seen in **Figure 3** in the range of 50–75 kDa probably reflect the formation of covalent PRiMA-AChE complexes or differences in the degree of glycosylation of AChE subunits (Darreh-Shori et al., 2004).

To test whether the hyperglycemia-induced expression of AChE is also accompanied by increased AChE activity, we tested AChE activity in Y79 cells exposed to glucose concentrations that induced apoptosis. AChE activity, as demonstrated by Karnovsky and Roots staining (Karnovsky and Roots, 1964) (**Figure 4A**), was calculated relative to control conditions (1 mg/ml glucose), and was found to increase by 7.0 ± 1.9-fold in cells treated with 3.5 mg/ml glucose for 1 h. In agreement with AChE expression pattern, AChE activity decreased with time after exposure to high glucose (3.5 mg/ml) and after 24 h was only 1.3 ± 0.34-fold relative to control (**Figure 4B**). This decrease within 24 h was statistically significant ($p < 0.05$). AChE activity in cells exposed to 3.5 mg/ml glucose for 1 h was significantly ($p < 0.05$) higher compared to cells exposed for the same time period to 6 mg/ml glucose. AChE activity was not increased in cells treated with 6 mg/ml glucose for the same time intervals (**Figure 4B**).

Since AChE was detected in the cytoplasmic (**Figure 3**) fraction, and in part of the experiments also in the membranous fraction (data not shown), we hypothesized that the isoforms detected by the western blot assays might be the short cytoplasmic R monomeric soluble isoform, and the membranous one might be the longer N-extended AChE (R or S variants) and/or the AChE-S variant. To test this hypothesis, Reverse Transcription (RT) real-time PCR was performed. As seen in **Figure 5**, mRNA expression levels corresponding to the AChE-S isoform were low and did not change significantly after 15-, 30- or 60- min incubation with 3.5 mg/ml of glucose. However, mRNA expression transcripts tested for the AChE-R or the N-extended isoforms, were significantly ($p < 0.05$) up-regulated by 1.5 ± 0.15 and by 1.4 ± 0.14-fold respectively, compared to control Y79 cells.

AChE EXPRESSION AND APOPTOSIS

In order to examine whether AChE expression plays a role in the hyperglycemia-induced apoptosis of Y79 cells, we tested five AChE shRNA sequences each cloned in pLKO.1- Lenti compatible vector (Sigma-Aldrich, St. Louis, USA). An empty pLKO.1 vector (Sigma-Aldrich, St. Louis, USA), devoid of a relevant shRNA sequence was used as control. As shown in **Figure 6A**, the expression of AChE tested under glucose exposure in the presence of the first sequences sh-1 (lane 3), was high and similar to that expressed in the presence of the sh-control-sequence (lane 2). The expression of AChE in these samples was considerably higher compared to sh-control Lenti-infected Y79 cells, which remained under 1 mg/ml glucose (lane1). These data indicate that sh-1 was not an efficient sequence for blocking AChE translation.

A partial inhibition of AChE protein expression was observed with sh-2, sh-3, and sh-5 (lanes 4, 5, and 7, respectively), as indicated by the weaker band of AChE protein. The most efficient

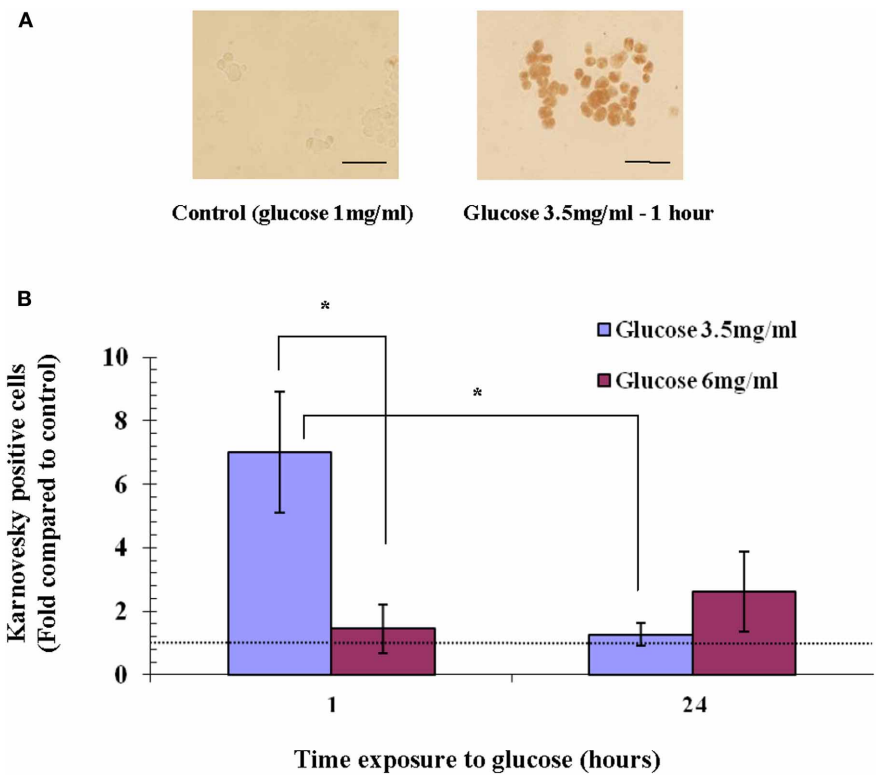

FIGURE 4 | AChE activity in Y79 cells treated with glucose. Y79 cells were pre-treated for 16–24 h in starvation medium (1% FBS and 1 mg/ml of glucose), and then with 3.5 mg/ml or 6 mg/ml glucose for 1 h or 24 h, after which Karnovsky and Roots assay was performed. Positive Karnovsky and Roots staining is seen in **(A)** on the right side compared to the negative control at the left. Scale bar is 50 μm. **(B)** Three independent experiments at which about 20 fields of cells (from each experiment) were counted for each treatment, calculated, and plotted as fold of control (cells cultured in starvation media containing 1% FBS and 1 mg/ml glucose) in histograms. Values are mean ± SEM, ($N = 3$). *$p < 0.05$.

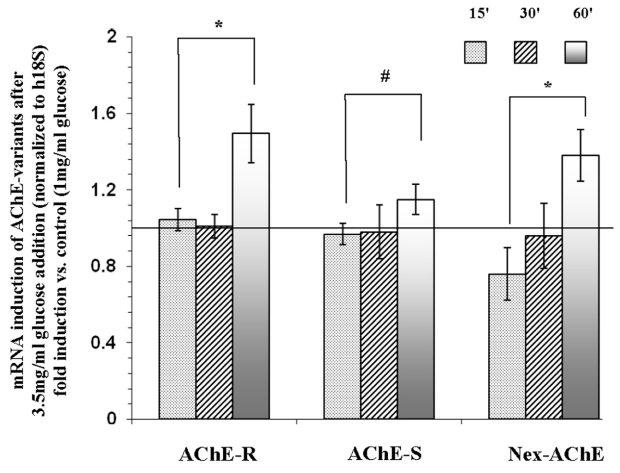

FIGURE 5 | mRNA expression of N-extended AChE, AChE-S, and AChE-R isoforms following treatment of Y79 cells with glucose for 1 h. Y79 cells were pre-treated for 16–24 h in starvation medium (1% FBS and 1 mg/ml of glucose) and then with 3.5 mg/ml glucose for different time intervals. Total RNA was extracted and cDNA was prepared for real-time PCR procedure as described under Materials and Methods. Results are presented as fold of control cells cultured in starvation media. Values are means ± SEM, ($N = 4$). $^*p < 0.05$, $\#p = 0.12$.

blockage of translation was found with sh-4 (lane 6) in which the AChE band is barely visible. Equal loading of total protein is confirmed by the α-Tubulin band, shown in **Figure 6A**.

We chose Y79 cells, expressing either AChE mRNA sh-4 or AChE mRNA sh-5 to test the effectiveness of sh-4 and sh-5 in blocking hyperglycemia-induced apoptosis. Starved Lenti-infected Y79 cells with sh-4, sh-5, or sh-control were incubated for 24 h in 3.5 mg/ml or left in 1 mg/ml glucose media and TUNEL assay was performed. **Figure 6B** shows representative pictures of cells undergoing TUNEL assay, and **Figure 6C** shows the fraction (percentage) of TUNEL positive cells of total number of cells. Y79 cells expressing sh-control underwent a significant apoptotic effect 24 h after incubation in 3.5 mg/ml glucose (**Figure 6B**, 2nd row), compared to sh-control Lenti-infected Y79 cells which remained under 1 mg/ml glucose (**Figure 6B**, 1st row); 75 ± 7.1% and 14.9 ± 3.0%, respectively ($p < 0.05$) (**Figure 6C**). A significant ($p < 0.05$) inhibition of apoptosis was seen in sh-4 Lenti-infected Y79 cells following 24 h incubation in 3.5 mg/ml of glucose addition (**Figure 6B**, 4th row), compared to sh-control Lenti-infected Y79 treated under the same conditions (**Figure 6B**, 2nd row), 25.2 ± 8.4% and 75 ± 7.1%, respectively (**Figure 6C**). No significant induction of apoptosis was seen between sh-4 Lenti-infected Y79 cells incubated under 3.5 mg/ml glucose (**Figure 6B**, 4th row), and sh-4 Lenti infected Y79 cells, which remained under 1 mg/ml glucose (**Figure 6B**, 3rd row), 25.2 ± 8.4% and 36.5 ± 7.2%, respectively (**Figure 6C**). sh-5 was also effective in blocking hyperglycemia-induced apoptosis. The fraction of TUNEL positive cells was significantly ($p < 0.05$) reduced by sh-5 in Y79 cells incubated for 24 h in 3.5 mg/ml glucose (**Figure 6B**, 6th row) compared to sh-control cells incubated under the same conditions (**Figure 6B**, 2nd row); 52.8 ± 3.4% and 75 ± 7.1%, respectively (**Figure 6C**). No significant differences were found in the fraction of TUNEL positive cells between sh-5 Lenti-infected Y79 cells incubated in 3.5 mg/ml glucose for 24 h (**Figure 6B**, 6th row), compared to sh-5 Lenti-infected Y79 cells which remained under 1 mg/ml glucose (**Figure 6B**, 5th row), 52.8 ± 3.4% and 36 ± 8%, respectively (**Figure 6C**).

The TUNEL results were strengthened by measurements of PARP cleavage in nuclear extracts of the sh-Lenti infected lines; sh-control, sh-4, and sh-5. The western blot shown in **Figure 7A** and the densitometry results plotted in **Figure 7B**, show that PARP was cleaved by almost 3-fold in sh-control Lenti infected Y79 cells following 1 h incubation in 3.5 mg/ml glucose compared to cells remaining in 1 mg/ml glucose. In contrast, cleavage of PARP could not be detected in the sh-4 and sh-5 Lenti-infected Y79 cells, where AChE translation was blocked. The TUNEL and PARP results and the AChE protein expression data seen in **Figures 6** and **7**, suggest that inhibition of AChE protein expression prevents Y79 cells from entering into the apoptotic cascade.

DISCUSSION

Apoptosis is an essential process during development and differentiation, and following exposure to damaging insults. It removes unwanted cells, which may be injured or misplaced, thus preventing the spread of pathological processes from injured cells to healthy ones. This naturally occurring cell death is prominent in neurons of CNS including the retina during development (Young, 1984; Rodier, 1995). Apoptosis also occurs in the adult CNS tissue subjected to a variety of stressful events such as pathological changes observed in both Alzheimer's disease and other neurodegenerative disorders in brain (Sasaki et al., 1998). In the adult retina, apoptosis is observed after subjecting it to photic stress (Hafezi et al., 1999; Grimm et al., 2001), or to metabolic stress such as hyperglycemia (Barber et al., 1998; Mohr et al., 2002; Kusner et al., 2004; Xi et al., 2005).

Here, apoptosis was induced in a retinal Y79 cell line by exposure to hyperglycemia, and was assessed by PARP cleavage as a molecular indication for apoptosis process (**Figure 2**), and by positive TUNEL staining as indication of apoptotic cells (**Figure 1**). Part of this effect could be attributed to hyperosmolarity, as tested by comparing the effects of adding glucose to those induced by adding mannitol, but exposure to 3.5 mg/ml glucose induced apoptosis mainly by hyperglycemia (**Figure 2**). These results are comparable to previous findings in different cell types such as endothelial cells (McGinn et al., 2003), pericytes (Naruse et al., 2000), myocytes (Shizukuda et al., 2002), and neuronal cells (Lelkes et al., 2001), undergoing apoptosis when exposed to glucose, but not to mannitol until a specific osmolarity level was reached (0.33–0.8%), depending on the cell type.

It should be noted that 6 mg/ml glucose induced a lesser degree of apoptosis compared to 3.5 mg/ml glucose, as indicated by PARP cleavage (**Figure 2**), but not by TUNEL (**Figure 1**). This suggests different pathways for hyperglycemia-induced apoptosis compared to hyperosmolarity-induced apoptosis. Indeed, different mechanisms for apoptosis induction by hyperglycemia and hyperosmolarity were described (Igarashi et al., 1999), showing that glucose concentrations in the range 11–16.5 mM

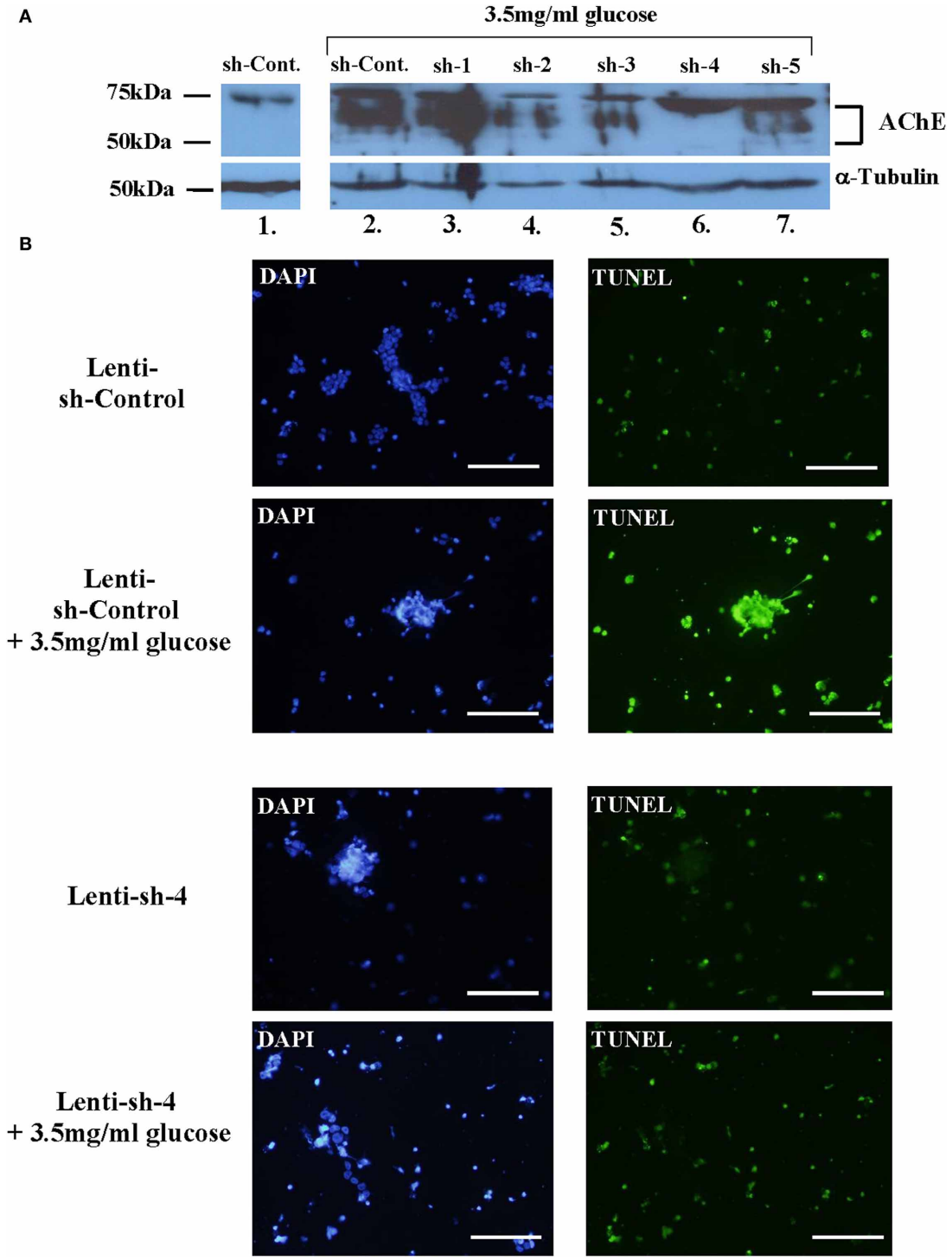

FIGURE 6 | Continued

(2–3 mg/ml, respectively), could activate p38 kinase associated with apoptosis, via a PKC-delta isoform-dependent pathway, while higher glucose or mannitol concentrations activated p38 kinase by hyperosmolarity via a PKC-independent pathway (Igarashi et al., 1999).

Although the molecular mechanisms underlying apoptosis have been extensively studied, these mechanisms and the interactions between the proteins which are involved seem to be very complex and much remains to be elucidated. Increasing body of evidence has shown that AChE may be involved in apoptosis

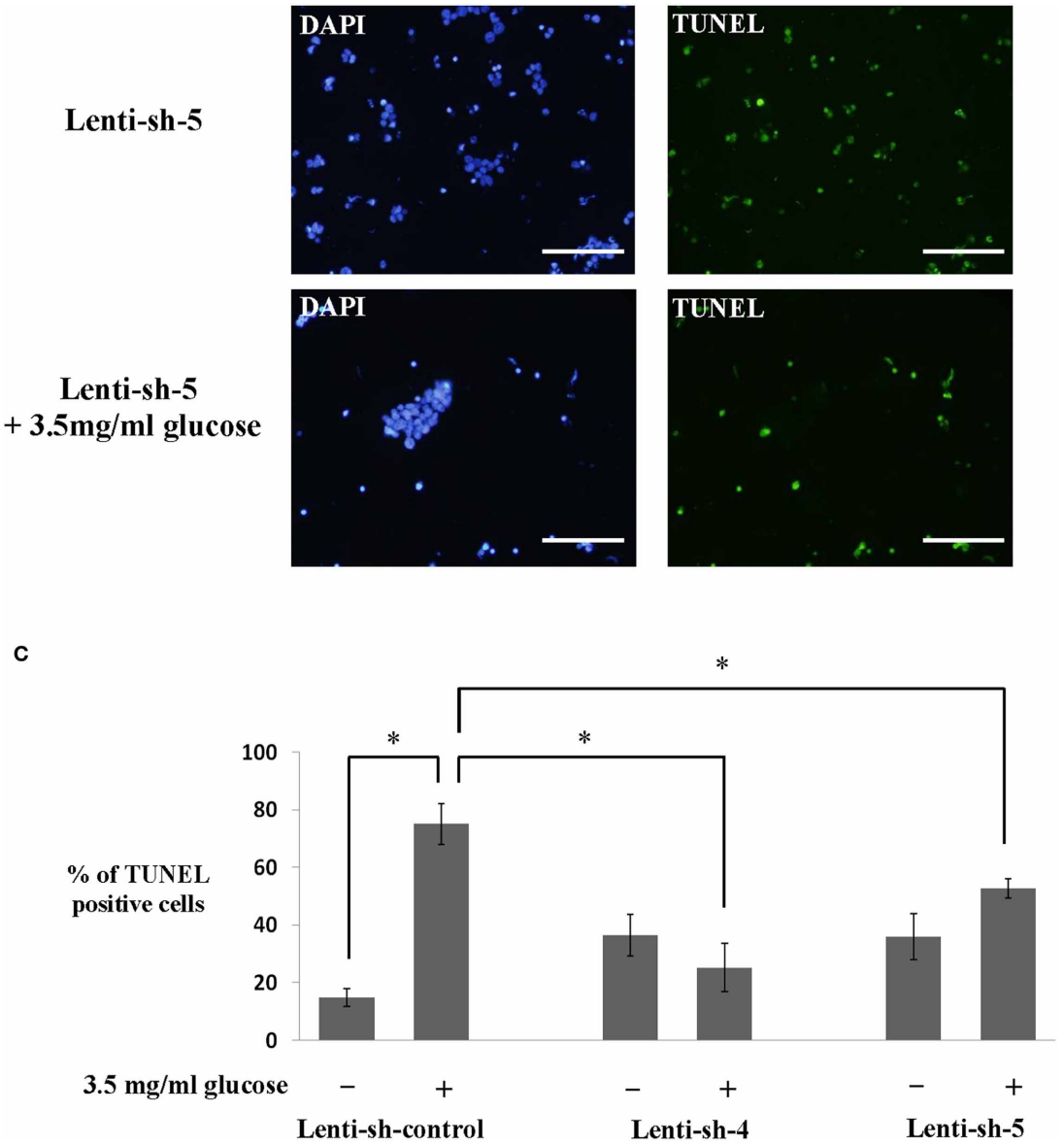

FIGURE 6 | Inhibiting AChE protein expression by different sh-RNA sequences reduces apoptosis in Y79 cells treated with glucose. (A) Y79 sh-RNA lenti-infected and selected cells (as described under Materials and Methods), were pre-treated in starvation medium containing 1% FBS and 1 mg/ml of glucose for 16–24 h and then treated with 3.5 mg/ml glucose for 1 h except for sh-control in lane 1. Cytoplasmic extracts were prepared and run for western blot analysis to detect AChE expression or anti α-Tubulin (for loading control) as described under Materials and Methods. The sample in lane 1 and those in 2–7 were run in separate lanes on the same gels. The western blot shown is one of two independent experiments. **(B)** Y79 sh-RNA lenti-infected and selected cells (as described under Materials and Methods), were pre-treated in starvation medium as described in **A** and then assayed by TUNEL (green) to detect apoptosis after 24 h in 3.5 mg/ml glucose. Nuclei were stained with DAPI (blue). Cells were photographed under an upright fluorescence microscope (Zeiss). Scale bar is 100 μm. **(C)** TUNEL positive Y79 sh-RNA lenti-infected and selected cells were counted and calculated as fold of control cells incubated under starvation medium, and plotted in histograms. Values are means ± SEM, ($N = 3$). $^*p < 0.05$.

(Zhang et al., 2002; Jin et al., 2004; Deng et al., 2006; Toiber et al., 2008). Under control conditions, AChE expression (**Figure 3**) and activity (**Figure 4**) in Y79 cells is very low, probably reflecting de-differentiation of this retinoblastoma-derived cell line. However, when Y79 cells were treated with 3.5 mg/ml glucose for an hour, AChE expression and activity (**Figures 3** and **4**, respectively) were significantly increased compared to control. AChE expression decreased almost back to the control level, when tested 2 h after exposure to 3.5 mg/ml glucose (**Figure 3**), reminiscent of an immediate early gene mRNA and protein expression (Kaufer et al., 1998). AChE expression was not increased in cells treated with 6 mg/ml glucose or in cells treated with mannitol although Y79 cells exposed to these conditions were shown to undergo apoptosis, probably as a result of hyperosmolarity (**Figures 2**, **3**). This may indicate that AChE induction is involved in apoptosis induced by selective stress types, e.g., hyperglycemia but not

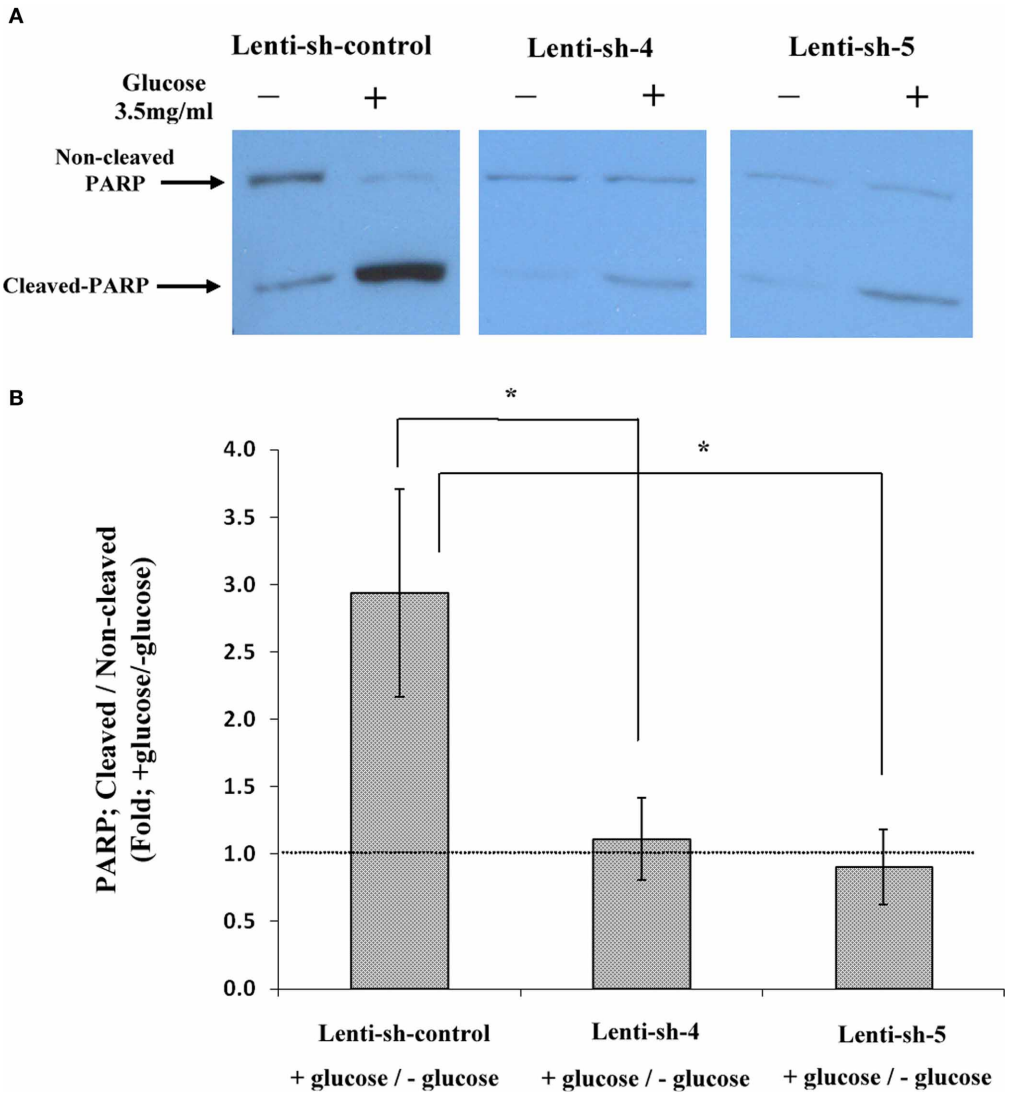

FIGURE 7 | Cleavage of PARP in nuclear extracts of sh-RNA lenti-infected Y79 cells treated by adding glucose. Nuclear extracts were prepared from starved Y79 sh-RNA lenti-infected and selected cells, after raising glucose concentration to 3.5 mg/ml glucose for 1 h or keeping the cells in control (1 mg/ml glucose) conditions (–). Extracts were run on SDS-PAGE, blotted and probed with anti- PARP as described under Materials and Methods. Five independent western experiments were conducted; one is shown in **(A)**. Densitometry values were calculated for cleaved to non-cleaved ratios, and then calculated as fold of cells incubated under starvation medium (containing 1% FBS and 1 mg/ml glucose) and plotted in histograms as shown in **(B)**. Values are mean ± SEM, ($N = 5$). $^{*}p = 0.05$.

by hyperosmolarity. These results shown involvement of AChE-stress-induced apoptosis, agree with multiple previous reports demonstrating stress-induced expression of AChE following a variety of stress episodes to the central nervous system, e.g., forced swimming (Kaufer et al., 1998), head trauma (Shohami et al., 2000), and photic stress to the retina (Kehat et al., 2007). The findings that apoptosis and AChE were induced by hyperglycemia can reflect two unrelated pathways linked by a common trigger or can reflect a single pathway in which AChE expression leads to apoptosis. To differentiate between these possibilities we blocked AChE expression with shRNA. We found that preventing AChE expression using shRNA was accompanied by prevention of apoptosis (**Figures 6** and **7**), thus supporting the hypothesis that AChE is involved in the apoptosis induced by hyperglycemia in Y79 cells.

Our RT-PCR studies indicated that hyperglycemia-induced the expression of an N-extended AChE variant and an AChE-R variant (**Figure 5**). The trivial conclusion is that hyperglycemia (3.5 mg/ml glucose) induced the expression of an N-extended AChE-R variant and of an AChE-R variant with regular N-terminus. The former is found in the membrane fraction and the latter in the cytoplasm fraction. This conclusion is contrary to recent findings suggesting that the N-extended AChE-S is the detrimental variant causing apoptosis (Toiber et al., 2008, 2009). These differences may be related to the type of stress and/or to the stressed tissue, but the common feature of the

two variants, the N-extended terminus, probably includes elements that are involved in cellular apoptosis. This is supported by a previous retinal study demonstrating that photic stress induced the expression of an N-extended AChE variant that was involved in cell apoptosis, but its specific C-terminus type could not be identified at the protein level (Kehat et al., 2007). In the same study, *in-situ* hybridization showed up-regulation of AChE-R mRNA (Kehat et al., 2007). It should be noted that our findings cannot rule-out that hyperglycemia induced the expression of a low-level of N-extended AChE-S that was sufficient to cause apoptosis, but was not detected by the RT-PCR experiments. A potential mechanism for the role of AChE in the induction of apoptosis was suggested recently emphasizing the role of the extended N-terminus (Toiber et al., 2008, 2009). According this suggestion, the extended N-terminus protrudes into the cytoplasm, while the core domain is exposed to the extracellular compartment (Toiber et al., 2008). Several proteins, known to be involved in the apoptotic signaling cascade, i.e., GSK3, GAK (cyclin G associated kinase), JAB1/CSN5, integrin, and Fas (TNF receptor super-family, member 6), were found to interact with the extended N-terminus of AChE-S (Toiber et al., 2009), thus probably linking this AChE variant to apoptosis. It should be stressed that this hypothesis emphasizes the role of the extended N-terminus in apoptosis and not of the C-terminus. Therefore, it is possible that, depending upon the studied cells, the N-extended AChE-R or the N-extended AChE-S can equally play a role in inducing cell apoptosis. Therefore, the protein partners, mentioned above, would be very interesting to test in our *in vitro* system. Our *in vitro* findings on retinoblastoma cell line are consistent with the hypothesis that AChE expression pattern in the mammalian retina is altered by hyperglycemia in a manner affecting its roles. A previous study reported a reduction in AChE catalytic activity in the diabetic rat retina probably due to loss of the hydrophobic variant of AChE (Sanchez-Chavez and Salceda, 2001). Here we showed, in a retinoblastoma cell line, that hyperglycemia altered the expression pattern of AChE causing up-regulation of the AChE-R and the extended N-terminus AChE variants. The latter variant is the prime candidate to induce the observed cell apoptosis, reminiscent of retinal cells' apoptosis that was observed during early stages of diabetes (Barber et al., 1998), before any vascular alteration. These findings need to be expanded to identify with certainty the AChE variant involved in hyperglycemia-induced apoptosis, and to test this effect *in vivo*, in experimental diabetic models, before new therapeutic approaches can be suggested.

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Molecular assembly and biosynthesis of acetylcholinesterase in brain and muscle: the roles of t-peptide, FHB domain, and N-linked glycosylation

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Acetylcholinesterase (AChE) is responsible for the hydrolysis of the neurotransmitter, acetylcholine, in the nervous system. The functional localization and oligomerization of AChE T variant are depending primarily on the association of their anchoring partners, either collagen tail (ColQ) or proline-rich membrane anchor (PRiMA). Complexes with ColQ represent the asymmetric forms (A_{12}) in muscle, while complexes with PRiMA represent tetrameric globular forms (G_4) mainly found in brain and muscle. Apart from these traditional molecular forms, a ColQ-linked asymmetric form and a PRiMA-linked globular form of hybrid cholinesterases (ChEs), having both AChE and BChE catalytic subunits, were revealed in chicken brain and muscle. The similarity of various molecular forms of AChE and BChE raises interesting question regarding to their possible relationship in enzyme assembly and localization. The focus of this review is to provide current findings about the biosynthesis of different forms of ChEs together with their anchoring proteins.

Keywords: acetylcholinesterase, butyrylcholinesterase, assembly, membrane trafficking, glycosylation

INTRODUCTION

Cholinesterases (ChEs) are serine hydrolases that preferentially act on choline esters. Vertebrates possess two types of cholinesterases (ChEs), corresponding to two distinct genes: acetyl cholinesterase (AChE, EC 3.1.1.7) and butyryl cholinesterase (BChE, EC 3.1.1.8). These two enzymes are distinguished on the basis of the substrate specificities and their sensitivities to selective inhibitors (Mendel and Rudney, 1943; Austin and Berry, 1953). The primary function of AChE is to efficiently hydrolyze the neurotransmitter acetylcholine (ACh) at cholinergic synapses (Massoulié et al., 1993), whereas the physiological function of BChE in vertebrates remains a question of different speculations. Studies of AChE knockout mice suggested that BChE can partially compensate for the absence of AChE in the nervous system (Xie et al., 2000; Duysen et al., 2001); therefore BChE could hydrolyze acetylcholine functionally. Poisoning by ChE inhibitors, such as insecticides or nerve gas, results in accumulation of ACh, and uncontrolled activation of cholinergic receptors, which causes cholinergic crisis and potentially leads to death (Feyereisen, 1995; Bajgar, 2004). On the other hand, controlled treatment with ChE inhibitors are used in therapeutics for patients suffering from myasthenia gravis (Brenner et al., 2008; Mehndiratta et al., 2011), Alzheimer's disease (Giacobini, 2000; Stone et al., 2011), and Parkinson's disease (Hutchinson and Fazzini, 1996; Emre et al., 2004).

In vertebrates, *ACHE* gene produces several types of coding sequences differing in an alternative choice of splice acceptor sites in the 3′ region. This process generates different AChE isoforms, named $AChE_R$, $AChE_H$, and $AChE_T$ (Massoulié, 2002). They contain the same catalytic domain, but are associated with distinct C-terminal peptides. In contrast, *BCHE* gene produces single type of transcript and generates single type of isoform $BChE_T$ (Blong et al., 1997). AChE and BChE are well-known for their multiple molecular forms that have their specific localizations: $AChE_R$ is a soluble monomer that is up-regulated in the brain under stress stimulation (Kaufer et al., 1998; Perrier et al., 2005); $AChE_H$ is a glycosylphosphatidylinositol-anchored dimer that is mainly expressed in red blood cells (Li et al., 1991); $AChE_T$ and $BChE_T$ are present in collagen-tailed forms at the neuromuscular junction (nmj) and hydrophobic-tailed forms in the brain (Legay et al., 1995; Blong et al., 1997; Massoulié et al., 2005). The molecular forms of $AChE_T$ and $BChE_T$ in brain and muscle are of particular interest because they are associated with their anchoring proteins: collagen Q (ColQ) or proline-rich membrane anchor (PRiMA). Complexes with ColQ represent the collagen-tailed or asymmetric (A) forms in muscle (Krejci et al., 1997), while complexes with PRiMA represent membrane-bound tetrameric globular form (G_4), mainly in brain (Perrier et al., 2002, 2003; Xie et al., 2009) and muscle (Xie et al., 2007). In addition, mixed cholinesterases, ColQ-linked AChE–BChE A_{12} hybrid enzyme (Tsim et al., 1988a) and PRiMA-linked AChE–BChE G_4 hybrid enzyme (Chen et al., 2010) that contain both AChE and BChE homodimers in a single molecule are being found in avian system. Here, we summarized the recent studies on the assembly of oligomeric AChE and BChE, as well as the regulation of AChE and/or BChE biosynthesis in neurons and muscles.

Abbreviations: A, asymmetric; ACh, acetylcholine; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChEs, cholinesterases; ColQ, collagen tail; Endo H, endoglycosidase H; ER, endoplasmic reticulum; FHB, four-helix bundle; G_4, tetrameric globular form; nmj, neuromuscular junction; PRAD, proline-rich attachment domain; PRiMA, proline-rich membrane anchor; WAT, tryptophan amphiphilic tetramerization.

MOLECULAR FORMS OF AChE AND BChE IN BRAIN AND MUSCLE

PRiMA-LINKED GLOBULAR FORMS OF ChEs

In *Xenopus* oocytes, COS-7 cells, neuroblastoma cells, and muscle cells, the expression of PRiMA has been identified as a limiting factor in organizing G_4 AChE and targeting it to the cell membrane (Perrier et al., 2002; Xie et al., 2007), as well as in directing its membrane raft localization (Xie et al., 2010a). In the absence of PRiMA, the G_4 AChE, or the G_4 BChE, could not be formed. In the brain, the catalytic subunit contained in G_4 AChE is $AChE_T$ (Inestrosa et al., 1994). The expression of PRiMA mRNA and protein are increased with an increment of G_4 AChE during the development of brain and spinal cord (Leung et al., 2009; Xie et al., 2010b). The PRiMA-linked AChE is also present at the nmj, in which both motor neuron and muscle are the major suppliers. In rat muscles, the protein expression of PRiMA and $AChE_T$, as well as the G_4 AChE, are dramatically increased during development (Leung et al., 2009). The parallel expression of PRiMA and G_4 AChE during the development strengthens the importance of PRiMA in directing, and/or regulating the formation of G_4 enzyme. The PRiMA-linked G_4 BChE however has not been well studied, and therefore the expression profile of which in brain or muscle are not fully revealed.

ColQ-LINKED ASYMMETRIC FORMS OF ChEs

Asymmetric forms of AChE and BChE are characterized by the presence of collagen-like tail, which is formed by the triple helical association of three ColQ subunits (Feng et al., 1999). The cDNA encoding ColQ has been cloned in *Torpedo* (Krejci et al., 1991), rat (Krejci et al., 1997), human (Ohno et al., 1998), quail, and chicken (Ruiz and Rotundo, 2009a). The presence of the collagen-tailed forms of ChEs has been found in all classes of vertebrates, but not in invertebrates. They are specifically expressed in muscles and regulated by physiological activity (Sketelj and Brzin, 1985; Deprez et al., 2003; Lau et al., 2008). In human and rat, *COLQ* gene has two transcripts, ColQ-1 and ColQ-1a: they are differentiatedly expressed in slow-twitch (ColQ-1) and fast-twitch (ColQ-1a) muscles (Ohno et al., 1998; Krejci et al., 1999). The differentiated expression patterns may account for the synaptic and non-synaptic expression profile of ColQ-linked AChE in fast slow-twitch and fast-twitch muscles, respectively (Lee et al., 2004; Crne-Finderle et al., 2005; Choi et al., 2007).

EXISTENCE OF AChE–BChE HYBRID ChEs

Most of our knowledge concerning ChEs derives from the studies on the classical AChE and BChE homogenous oligomers. However, the sequence similarity between $AChE_T$ and $BChE_T$ has been further emphasized regarding the existence of hybrid A_{12} forms in new-born chicken muscle (Tsim et al., 1988a), as well as a hybrid G_4 from in the brain (Chen et al., 2010) and embryonic muscles of chicken (Chen et al., 2009). In these hybrid enzymes, both $AChE_T$ and $BChE_T$ are attached to the same anchoring protein, ColQ for A_{12} form and PRiMA for G_4 form (**Figure 1**). The hybrid enzyme exists as a single type, with equivalent number of AChE and BChE catalytic subunits in a single molecule. Interestingly, the expression of these hybrid molecules in chicken brain and muscle was found to be developmentally regulated. In 1-day-old chicken muscle, the predominant form of AChE is A_{12} hybrid form; however, the proportion of BChE subunits in the hybrid molecules progressively disappear during the muscle development from embryonic, hatching to adult, and the homogeneous asymmetric AChE becomes the sole form upon the muscle maturation (Tsim et al., 1988b). On the other hand, a continuous increase of AChE–BChE G_4 hybrid expression was observed in chicken brain, while an obvious decrease was found in the leg muscles. To date, we do not have conclusive idea about the underlining regulatory mechanism of these hybrid enzymes. We believe that AChE–BChE hybrid enzymes could take part in cholinergic functions, which includes (i) they carry the catalytic activities of AChE and BChE in hydrolyzing ACh; (ii) they are associated with the anchoring protein, PRiMA or ColQ, which can anchor the hybrid enzymes onto the plasma membrane in the brain or nmj in the muscles; and (iii) the level of $BChE_T$ indirectly regulates the expression of homogenous G_4 AChE.

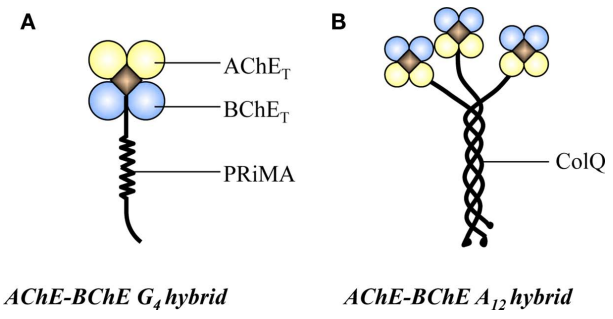

FIGURE 1 | Two types of AChE–BChE hybrid enzyme. (A) The PRiMA-linked G_4 AChE–BChE hybrid in chicken brain. **(B)** The ColQ-linked A_{12} AChE–BChE hybrid in chicken muscle.

In fact, the notion of having the existence of AChE–BChE hybrid enzyme is not new. An abnormal ChE species in the serum of patients suffering from carcinomas was reported (Zakut et al., 1988). This abnormal ChE species in human serum was inhibited by both AChE inhibitor BW284c51 and BChE inhibitor iso-OMPA. In addition, a collagen-tailed asymmetric hybrid AChE has been found relatively abundant in young chicken muscle (Tsim et al., 1988b), but which tends to disappear at the adult stage (Tsim et al., 1988b). Moreover, a hybrid tetramer having AChE and BChE activity was also found in cyst fluids derived from a human astrocytoma (García-Ayllón et al., 2001). However, none of the physiological function of these abnormal ChEs has been elucidated.

ASSEMBLY MECHANISM OF AChE AND BChE

The presence of G_4 and A_{12} hybrid ChEs raised interesting questions about the organization of the subunits in a hybrid ChE complex. The assembly of AChE and BChE in cells could provide a good model in revealing the protein assembly of oligomers. By using DNA transfected cell cultures, the organization of different subunits in the PRiMA-linked ChE tetramers has been studied (Chen et al., 2010). Interestingly, $AChE_T$ and $BChE_T$ could not form hybrid dimer in the absence of anchoring protein; on the

other hand, a single type of hybrid tetramer was clearly observed when the two catalytic subunits were co-expressed with PRiMA. Therefore, a "2 + 2" model is proposed for the organization of the four catalytic subunits in the PRiMA-linked ChE tetramers (**Figure 2**). After protein synthesis, $AChE_T$ and $BChE_T$ spontaneously form AChE homodimer and $BChE_T$ homodimer first. When two dimers (two $AChE_T$ dimers, or two $BChE_T$ dimers, or one $AChE_T$ dimer plus one $BChE_T$ dimer) encounter the anchoring protein, PRiMA, and thus ChE tetramers, e.g., G_4 AChE, G_4 BChE, and G_4 AChE/BChE hybrid, are formed.

T-PEPTIDE IS NECESSARY FOR THE OLIGOMERIZATION OF $AChE_T$ AND $BChE_T$

$AChE_T$ and $BChE_T$ have a catalytic domain of approximately 500 amino acids, followed by a C-terminal t-peptide of 40 and 41 residues, respectively. The t-peptide on $AChE_T$ and $BChE_T$ presents a considerable sequence similarity, with 24 identical residues, including seven aromatic residues and one cysteine near the C-terminus, which are conserved in human, cat, rabbit, mouse, cow, rat, chicken, and *Torpedo* (Massoulié et al., 1993).

The t-peptide was reported to play an important role in the biosynthesis of ChEs, particularly in the protein folding and exportation. The presence of aromatic residues in the t-peptide induces the misfolding of newly synthesized $AChE_T$ polypeptides, and this effect depends on the hydrophobic character of these residues, because the same effect occurs when they are replaced by leucines (Falasca et al., 2005). In the absence of a proline-rich attachment domain (PRAD)-containing anchoring protein, the t-peptide enhanced a pool of $AChE_T$ molecules toward endoplasmic reticulum (ER)-associated degradation (Belbeoc'h et al., 2003).

The major function of t-peptide is directing the assembly of tetramers of $AChE_T$ (Bon and Massoulié, 1997) and $BChE_T$ (Blong et al., 1997), as well as the association with the structure proteins, ColQ and PRiMA (Krejci et al., 1997; Perrier et al., 2002; Bon et al., 2004). The t-peptide is also named as tryptophan (W) amphiphilic tetramerization (WAT) domains, which contains a sector with seven aromatic residues that are strictly conserved between $AChE_T$ and $BChE_T$. The association between $AChE_T$ or $BChE_T$ catalytic subunits and anchoring proteins, ColQ and PRiMA, is mostly based on the interaction between four WAT domains on the t-peptides and a PRAD on ColQ or PRiMA (Bon et al., 1997; Dvir et al., 2004; Noureddine et al., 2007). A crystallographic analysis of the complex of synthetic t-peptide and PRAD peptide indicated that four α-helical t-peptides form coiled-coil structure around the PRAD, which is arranged in a poly-proline II helix (Dvir et al., 2004). In addition, the formation of disulfide bonds through the cysteine residues near the end of the t-peptides stabilizes the quaternary association, and in fact this association appears to be critical in the case of AChE dimer formation. Dimers could be hardly observed after mutagenesis of the C-terminal cysteine residues in H or T-peptides of *Torpedo* and rat AChE (Morel et al., 2001; Chen et al., 2010).

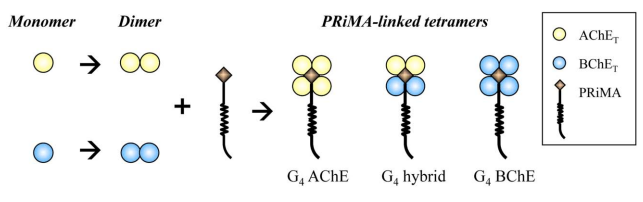

FIGURE 2 | Model for the assembly of PRiMA-linked AChE and BChE tetramers. When $AChE_T$ and $BChE_T$ subunits are expressed together, they form their own homodimers spontaneously. PRiMA recruits two homodimers together to form a PRiMA-linked tetramers, e.g., G_4 AChE, G_4 BChE. G_4 hybrid.

The importance of t-peptide in the assembly of $AChE_T$ and $BChE_T$ was also reported by DNA mutagenesis studies. The truncated mutants, $AChE_{\Delta T}$ and $BChE_{\Delta T,}$ in which the t-peptides were deleted from the catalytic subunits, produced only monomers (Duval et al., 1992). $AChE_T$ and $AChE_{BChE\text{-}T}$, $BChE_T$ and $BChE_{AChE\text{-}T,}$ in which the catalytic domain of each enzyme was swapped with the t-peptide of each other, presented similar assembly ability to form oligomers (Liang et al., 2009; Chen et al., 2010).

THE FHB DOMAIN IS INVOLVED IN THE SELECTION OF CATALYTIC SUBUNITS DURING DIMERIZATION

Although the nature of $AChE_T$ and $BChE_T$ oligomers depends on the presence of the t-peptides, the catalytic domains also influence the oligomerization patterns. The X-ray crystallography studies of *Torpedo* AChE dimers showed that the contact zone between two $AChE_H$ subunits could be a "four-helix bundle" (FHB), formed by two α helices from each catalytic domain (Sussman et al., 1991). In addition, rat $AChE_T$ was also demonstrated to dimerize through FHB inter-subunit contact zone (Morel et al., 2001). Based on these findings, we compared the predicted FHB sequences that are responsible for the dimeric contact zone of $AChE_T$ and $BChE_T$ from different species. Indeed, FHB domains are highly conserved across different species for either AChE or BChE, including human, mouse, rat, chicken, and *Torpedo* (**Figure 3**). On the other hand, the similarity between FHB domains of $AChE_T$ and $BChE_T$ is very low. An inter-species hybrid dimer could be formed between human $AChE_T$ and chicken $AChE_T$, but not between mammalian $AChE_T$ and $BChE_T$, in transfected HEK293T cells (Chen et al., 2010). The selectivity of dimerization seems to be based on the feature that the FHB domains of AChE are highly conserved among different species of vertebrates, but are distinguished from vertebrate $BChE_T$s. Moreover, another hybrid dimer, between human $AChE_{BChE\text{-}T}$ and chicken $AChE_{T,}$ which contained the similar FHB but different t-peptides, was formed when they were co-expressed together in HEK293T cultured cells (Chen et al., 2010). This further confirmed that the catalytic domains, possibly the FHB domains, should play a critical role in the selection of subunits during the dimerization of ChEs.

According to our current knowledge, the oligomerization of $AChE_T$ or $BChE_T$ with their associated anchoring proteins could rely on three types of interactions (**Figure 4**): (i) the FHB interaction for the formation of dimer through hydrophobic interaction; (ii) intercatenary disulfide bonds between the t-peptide of $AChE_T$ or $BChE_T$ subunits; and (iii) tight hydrophobic interaction between the WAT domains of $AChE_T$ or $BChE_T$ subunits and the PRAD on PRiMA or ColQ.

	AChE		BChE	
	FHB-1	FHB-2	FHB-1	FHB-2
	352 362	506 520	390 401	540 561
Human	D**L**AAE**AV**V**L**H**Y**	A**Q**ACA**FW**NR**FLPKLL**	**S**EFGK**E**SIL**FH**Y	T**KLRA**Q**QC**R**FW**TS**FFPKVLEMT**
Mouse	D**L**AAE**AV**V**L**H**Y**	A**Q**TCA**FW**NR**FLPKLL**	**S**RLGK**E**AVL**FY**Y	S**KLRA**P**QC**Q**FW**RL**FFPKVLEMT**
Rat	D**L**AAE**AV**V**L**H**Y**	A**Q**T**C**A**FW**NR**FLPKLL**	**S**SLGK**E**AIL**FY**Y	S**KLRA**P**QC**Q**FW**RL**FFPKVLEIT**
Chicken	E**L**AAE**AV**V**L**H**Y**	T**Q**I**C**A**FW**TR**FLPKLL**	**S**KLAI**E**SII**F**Q**Y**	T**KLRA**Q**QC**R**FW**NM**FFPKVLEMT**
Torpedo	D**L**GLD**AV**T**L**Q**Y**	V**Q**M**C**V**FW**NQ**FLPKLL**		

FIGURE 3 | Comparison of FHB sequences of $AChE_T$ and $BChE_T$ among different species. The sequences of the two alpha helices (FHB-1 and FHB-2) forming the dimeric contact zone of AChE and BChE are shown. The residues conserved across species are highlighted in bold. The amino acid sequences of human, mouse, rat, chicken, and *Torpedo* AChE catalytic subunits were deduced from nucleotide sequences accessed from GeneBank™ AAA68151, CAA39867, EDM13278, P36196, and CAA27169, respectively. The amino acid sequences of human, mouse, rat, and chicken BChE catalytic subunits were deduced from nucleotide sequences accessed from GeneBank™ AAA99296.1, AAH99977, NP_075231, and NP_989977, respectively.

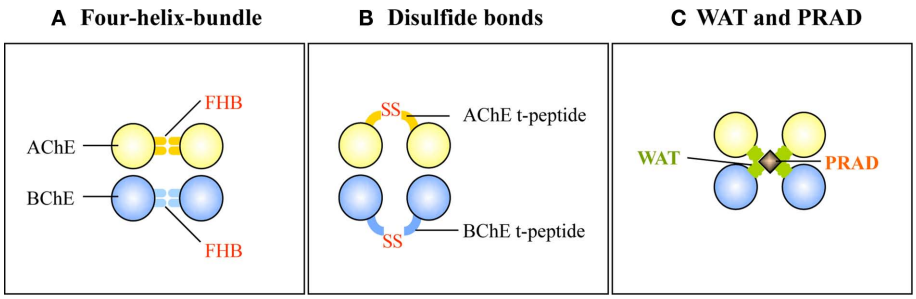

FIGURE 4 | Three types of interaction involved in the oligomerization of AChE and BChE. (A) The FHB domains of $AChE_T$ or $BChE_T$ direct the dimer formation. **(B)** The t-peptides of $AChE_T$ or $BChE_T$ form intercatenary disulfide bonds. **(C)** WAT domains of $AChE_T$ and $BChE_T$ interact with PRAD on PRiMA or ColQ.

N-GLYCOSYLATION IS NOT REQUIRED FOR THE ASSEMBLY OF $AChE_T$ BUT IS REQUIRED FOR THE MEMBRANE TRAFFICKING

$AChE_T$ and $BChE_T$ are well-known highly glycosylated enzymes, which carry various amounts of N-linked carbohydrate side chains attached to their core polypeptides (Liao et al., 1992; Kolarich et al., 2008). Mature human $AChE_T$ monomer possesses three potential N-linked glycosylation sites (Soreq et al., 1990; Velan et al., 1993). Mature human $BChE_T$ carries nine potential N-linked glycosylation sites (Lockridge et al., 1987). These N-linked glycosylation sites, both in AChE and BChE, are highly conserved in mammals, which implies the physiological importance of these glycans for ChEs.

Glycosylation is proposed to be used as a marker for the progression of ChE forms through different subcellular compartments, since it is known that the glycans added in ER are remodeled and matured in Golgi apparatus. In chicken muscles, the assembly of catalytically active dimer and tetramer occurred in the rough ER, with a subset of tetramers being further assembled with ColQ in Golgi apparatus into asymmetric forms (Rotundo, 1984). Once assembled, these catalytically active AChE oligomers were stable, acquired complex oligosaccharides in Golgi apparatus, and were transported to plasma membrane or secreted into medium (Rotundo, 1984). All of these exported AChE molecules contain complex oligosaccharides, because they could bind to lectins such as wheat germ agglutinin and ricin, and were endoglycosidase H (Endo H) resistant (Rotundo et al., 1989). In contrast, 70–80% of the newly synthesized AChE polypeptide chains in chicken muscle appeared to be catalytically inactive and Endo H sensitive, and they were degraded intracellularly with a half-life of about 1.5 h (Rotundo, 1988). Recently, Ruiz and Rotundo (2009a,b) reported that the expression of AChE in quail muscles is regulated by muscle activity through post-translational controls: the over-expression of ER molecular chaperons, such as calnexin, ER protein 72, and protein disulfide isomerase results in an increase of catalytic active ColQ-linked AChE in quail muscles.

The biological function of the glycans on ChEs was elucidated by site mutagenesis studies. Elimination of *N*-glycosylation sites did not interfere with the ability of $AChE_T$ to form a soluble dimer (Velan et al., 1993), or to assembly with PRiMA to form a PRiMA-linked AChE tetramer (Chen et al., 2011). It appears therefore that the oligosaccharide side chains do not affect the structural elements that are responsible for the interaction of different subunits. Indeed, none of the *N*-glycosylation sites on AChE is close to or within the FHB domain implied in dimerization of AChE subunits (Sussman et al., 1991; Morel et al., 2001), or the t-peptide that allows the oligomerization of $AChE_T$ with the anchoring

proteins, e.g., ColQ and PRiMA (Bon et al., 2004). Moreover, the glycosylation of $AChE_T$ can greatly affects the protein folding and membrane trafficking. When the glycosylation is eliminated, the folding of $AChE_T$ fails, leading to a severe lost of the enzymatic activity (Chen et al., 2011). In the absence of glycosylation, the secreted G_1 and G_2 AChE are dramatically reduced in transfected cells, and the PRiMA-linked G_4 AChE is retained in ER and fails to be exported to plasma membrane (Chen et al., 2011).

The importance of *N*-glycosylation in the biosynthesis of AChE could explain the abnormality of glycosylation status in some pathological conditions. Proper glycosylation of AChE is important for normal brain function. Accumulation of molecular forms of AChE with altered patterns of glycosylation has been observed in the brain and cerebrospinal fluid of Alzheimer's patients (Sáez-Valero et al., 1999, 2000). Moreover, characteristics of AChE found in the senile plaques are different from those in normal brain with a higher degree of glycosylation, which is proposed to be one of the factors facilitating formation of amyloid fibrils in the senile plaques (Mimori et al., 1997). These abnormalities in the glycosylation of AChE are very specific for Alzheimer's disease and are not detected in other dementia illness, which suggests that glycosylation of AChE may have a diagnostic value.

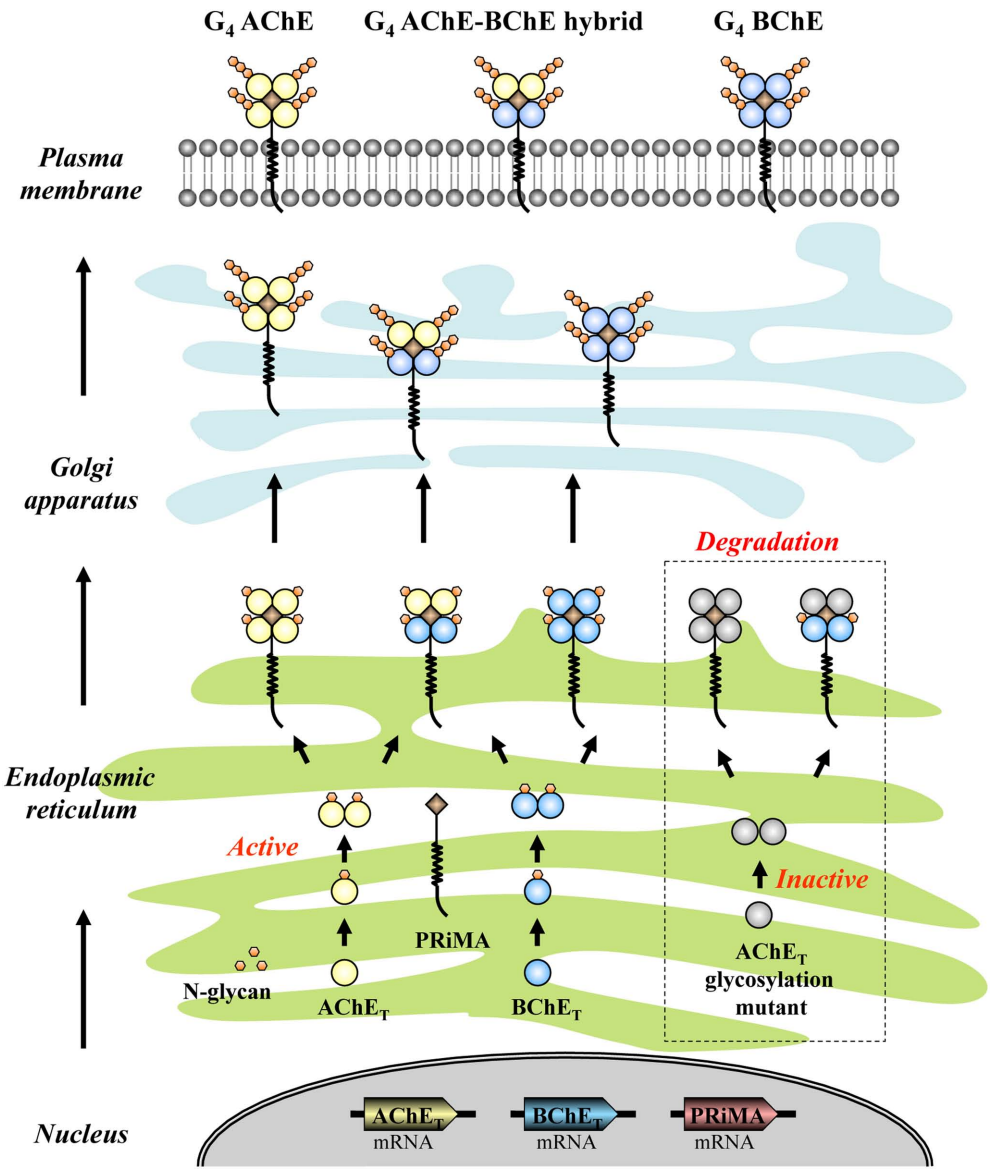

FIGURE 5 | Proposed model for the assembly and membrane processing of G_4 ChEs. G_4 AChE, G_4 BChE, and AChE–BChE G_4 hybrid molecules are assembled in ER where both $AChE_T$ and $BChE_T$ subunits have initial glycosylation. These G_4 complexes are subsequently transported to Golgi apparatus where the catalytic subunits can have further glycosylation, and finally anchored onto the plasma membrane. The $AChE_T$ glycosylation mutant, in which the glycosylation is completely abolished, is still able to assembly with PRiMA and $BChE_T$ to form G_4 AChE and G_4 hybrid. However, both of them are retained in ER, which possibly will be subjected to the degradation pathway.

To summarize the assembly and membrane processing of G_4 ChEs, a model is being proposed as **Figure 5**. After the mRNAs are translated into peptides, $AChE_T$ and $BChE_T$ polypeptides undergo initial glycosylation, presumably in a co-translation manner. Shortly after the synthesis, the glycosylated $AChE_T$ and $BChE_T$ are assembled into homodimers spontaneously. When these homodimers encounter PRiMA, they form PRiMA-linked G_4 AChE, or G_4 BChE, or AChE–BChE G_4 hybrid in ER. Afterward, PRiMA targets these G_4 complexes to Golgi apparatus where $AChE_T$ and $BChE_T$ subunits have further glycosylation. This trafficking allows the G_4 enzymes to be fully functional and finally become anchored onto the plasma membrane. During the whole process, the proper glycosylation of $AChE_T$ is the key point for the membrane targeting. Without glycosylation, $AChE_T$ polypeptides cannot fold properly, resulting in inactive AChE molecules. These un-glycosylated and inactive $AChE_T$ molecules can still form PRiMA-linked G_4 AChE and AChE–BChE G_4 hybrid, but both of them are retained in ER, failing to be exported to Golgi apparatus, and finally they are subjected to degradation most possibly.

SUMMARY

The assembly of ChEs constitutes a fascinating model to study numerous biological processes, such as post-translational modification, protein–protein interactions, membrane trafficking, and protein degradation. The physiological function of ChEs depends on the catalytic property of the enzymes and the restricted subcellular localization. In brain and muscles, ChEs display extremely rich molecular polymorphisms, possessing soluble, membrane-bound and basal lamina-anchored forms, and additionally, hybrid ChEs containing both AChE and BChE catalytic subunits also curiously exist. During the assembly of these ChE complexes, dimer is believed to be the precursor for the PRiMA-linked tetramers and ColQ-linked asymmetric forms. The dimer formation of AChE or BChE depends on recognition between the FHB domains in their catalytic domains, and the assembly of tetramers with PRiMA or ColQ requires the interaction of WAT domain on the C-terminal t-peptides with the PRAD domain on PRiMA or ColQ. N-linked glycans of AChE are employed as both maturation and quality control tags that dictate the destination of the enzyme being exported or not, which inspires that the control of glycosylation may be as a means in regulating the level of functional AChE in pathological conditions. However, there are still several questions out there, which have not been resolved. These questions are: (i) the possible control mechanism in directing the formation of different forms of ChEs; (ii) the regulatory mechanism for the protein trafficking of different states of ChEs; (iii) the fate of the active and inactive ChEs; and (iv) the possible non-cholinergic function of different forms of ChEs.

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Acetylcholinesterase involvement in apoptosis

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To date, more than 40 different types of cells from primary cultures or cell lines have shown AChE expression during apoptosis and after the induction apoptosis by different stimuli. It has been well-established that increased AChE expression or activity is detected in apoptotic cells after apoptotic stimuli *in vitro* and *in vivo*, and AChE could be therefore used as a marker of apoptosis. AChE is not an apoptosis initiator, but the cells in which AChE is overexpressed undergo apoptosis more easily than controls. Interestingly, cells with downregulated levels of AChE are not sensitive to apoptosis induction and AChE deficiency can protect against apoptosis. Some tumor cells do not express AChE, but when AChE is introduced into a tumor cell, the cells cease to proliferate and undergo apoptosis more readily. Therefore, AChE can be classified as a tumor suppressor gene. AChE plays a pivotal role in apoptosome formation, and silencing of the AChE gene prevents caspase-9 activation, with consequent decreased cell viability, nuclear condensation, and poly (adenosine diphosphate-ribose) polymerase cleavage. AChE is translocated into the nucleus, which may be an important event during apoptosis. Several questions still need to be addressed, and further studies that address the non-classical function of AChE in apoptosis are needed.

Keywords: acetylcholinesterase, apoptosis, tumor

The human gene that encodes acetylcholinesterase (AChE) is located on chromosome 7q22 (Ehrlich et al., 1992; Getman et al., 1992). Acetylcholinesterase is a type B carboxylesterase that rapidly hydrolyzes the neurotransmitter acetylcholine (ACh) at cholinergic synapses, as well as at neuromuscular junctions (Grisaru et al., 1999; Sperling et al., 2008). Additional splice variants involving both the 5′ and 3′ ends of the gene have also been reported. Among these splice variants are three distinct AChE variants, each with a different carboxyterminal sequence, called the "synaptic" (S), "erythrocytic" (E), and "readthrough" (R) AChE isoforms (Soreq and Seidman, 2001; Meshorer and Soreq, 2006). "Synaptic" AChE-S constitutes the principal multimeric enzyme in brain and muscle; soluble, monomeric "readthrough" AChE-R has been reported in embryonic and tumor cells and is induced under psychological, chemical, and physical stress; and finally, glypiated dimers of erythrocytic AChE-E are associated with red blood cell membranes (Grisaru et al., 1999). In addition to its catalytic function of the hydrolysis of acetylcholine, AChE has been shown to be involved in many non-cholinergic functions, such as cell growth, stem cell differentiation (Sperling et al., 2008), neuritogenesis, cell adhesion (Paraoanu and Layer, 2008), synaptogenesis, activation of dopamine neurons, tumorigenesis, amyloid fiber assembly (Inestrosa et al., 1996; Alvarez et al., 1997), hematopoiesis, and thrombopoiesis (Greenfield, 1996; Layer, 1996; Small et al., 1996; Soreq and Seidman, 2001). Almost a decade ago, work by our group revealed that AChE-S expression can be induced during apoptosis in various cell types, including cells that did not originate from the nervous or hematopoietic system (Zhang et al., 2002). It has also been shown that AChE exerts its pivotal role in apoptosis by its participation in the formation of the apoptosome (Park et al., 2004). In this review we will summarize the functional evidence for the role of AChE during apoptosis, despite the fact that our understanding of the apoptotic role of AChE is still not complete.

Abbreviations: AChE, acetylcholinesterase; AChE-E, erythrocytic acetylcholinesterase; AChE-S, synaptic acetylcholinesterase; AChE-R, readthrough acetylcholinesterase; AML, acute myeloid leukemia; BuChE, butyrylcholinesterase; HCC, human colorectal carcinoma; Icv, intracerebroventricular; RA, refractory anemia; TGF-β, transforming growth factor-beta; TGF-α, transforming growth factor-alpha; TgS mice, transgenic mice overexpressing AChE-S.

AChE LEVELS ARE INCREASED IN APOPTOTIC CELLS AFTER APOPTOTIC STIMULI BOTH *IN VITRO* AND *IN VIVO*

AChE is expressed in cholinergic neurons, neuromuscular junctions, hematopoietic cells, including erythrocytes (Grisaru et al., 1999) and megakaryocytes (Paulus et al., 1981; Tranumjensen and Behnke, 1981), as well tissues that are not innervated by cholinergic neurons (Small et al., 1996). There is mounting evidence, however, that AChE expression is induced during apoptosis in a variety of other cell types (Zhang et al., 2002). Human lung fibroblast (HLF) cells and rat kidney (NRK) cells do not normally express AChE, but have been shown to express high levels of AChE when undergoing apoptosis or when moving from an inactive to an active state (Zhang et al., 2002; Jin et al., 2004). In general, cells that normally express low levels of AChE, such as PC-12, showed increased levels of AChE and AChE activity whilst undergoing apoptosis (Yang et al., 2002; Jing et al., 2008). Steinritz et al. (2007) reported that

increased AChE activity was found in sulfur mustard (SM)-treated A549 cell culture, as determined by Ellman assay and Western blotting. Interestingly, AChE activity showed a strong correlation with the number of TUNEL-positive cells. Furthermore, AChE activity may be a potential marker of apoptosis in A549 cells after SM injury (Steinritz et al., 2007). Other diseases with a possible apoptotic role in disease pathogenesis such as Alzheimer's disease and diabetes also show cholinesterase involvement. Allam et al. observed that butyrylcholinesterase and AChE-related proteins were found to be common to both Alzheimer's disease and diabetes; these proteins may play an etiological role via their influence on insulin resistance and lipid metabolism (Allam et al., 2006). Toiber et al. (2008) showed that in transfected primary brain cultures, an alternative N terminal extended transcript of AChE (N-AChE-S) induced cell death, morphological impairments and caspase-3 activation. In addition in cortical tissues from AD patients, N-AChE-S overexpression coincides with Tau hyper-phosphorylation. In other pathologies, Hu et al. (2009) showed increased expression levels of AChE and caspase-3 in the brain and peripheral immune system of focal cerebral ischemic rats. Xiao et al. (2006) utilized AChE with Bax, c-fos, and p53 genes as markers of apoptosis. Huang et al. (2005) have developed a novel assay that utilizes fluorochrome-tagged physostigmine (Ph-F) *in situ* to detect active AChE that has been induced during apoptosis. Cells that show an increase in AChE activity during apoptosis are summarized in **Table 1**.

CELL SENSITIVITY TO APOPTOSIS IS RELATED TO AChE EXPRESSION LEVELS

AChE is not a universal activators of apoptosis as it has been well-established that cholinergic neurons and neuromuscular junctions that express AChE do not initiate apoptosis under normal conditions without an apoptotic stimulus. However, AChE-positive cells, such as PC-12 (Yang et al., 2002; Jing et al., 2008) as well as neurons have been shown to respond more sensitively to apoptotic stimuli than AChE-negative cells (Jin et al., 2002, 2004). To investigate this, a stable cell line that overexpressed AChE was established from NRK cells. Overexpression of AChE was shown to inhibit cell proliferation and promote apoptosis in these cells. Transgenic mice which overexpress catalytically active AChE-S and have a higher levels of AChE hydrolytic activity than control mice, show increased levels of cell labeling by both bromodeoxyuridine and caspase-3; which reflects increased neural apoptosis (Cohen et al., 2008) though these transgenic mice also showed increased neuronal survival through an as yet unidentified compensatory mechanism. AChE was also shown to suppress cell proliferation via catalytic hydrolysis of acetylcholine in human colorectal carcinoma (HCC) (Montenegro et al., 2006).

Zhang et al. (2002) showed that the downregulation of AChE expression with antisense inhibited apoptosis, and they suggest that AChE is potentially a marker and a regulator of apoptosis. Park et al. (2004) explored the role of AChE in apoptosis by silencing the *AChE* gene. Silencing of *AChE* abolished the expression of AChE and prevented caspase-9 activation, decreased cell viability, nuclear condensation, and poly(adenosine diphosphate-ribose) polymerase cleavage. Animal models of degenerative diseases were found to be more easily established in $AChE^{+/+}$ mice than $AChE^{+/-}$ mice. Espallergues et al. (2010) showed that the intracerebroventricular injection of amyloid-beta (25–35) failed to induce learning deficits in $AChE^{+/-}$ mice, but impaired learning in $AChE^{+/+}$ controls. The peptide showed reduced toxicity in the forebrain structures of $AChE^{+/-}$ mice, as an increase in lipid peroxidation levels was measured in the hippocampus of $AChE^{+/+}$ but not $AChE^{+/-}$ mice. They concluded that the increase in cholinergic tonus observed in $AChE^{+/-}$ mice did not result in increased memory functions but allowed a significant prevention of the deleterious effects of muscarinic blockade or amyloid toxicity. Ye et al. (2010) recently reported that AChE is expressed during ischemia–reperfusion (I/R)-induced apoptosis *in vivo*. Their study demonstrated that AChE may be a pro-apoptotic factor and that the inhibition of AChE reduces the degree of renal I/R injury.

AChE AND TUMOR SUPPRESSION

Papers reporting the presence of AChE in tumors have first been reported almost 40 years ago (Gearhart and Mintz, 1974). The exact role of AChE in oncogenesis or tumor progression is still unclear and beyond the scope of this review. However a number of studies (Gearhart and Mintz, 1974; Takahashi et al., 1995; Johnson and Cotter, 1997) have shown an genetic alterations in the long arm of chromosome 7 the genetic locus of the AChE gene, in several tumors. Furthermore, the agricultural use of AChE inhibitors is known to induce several types of tumors (Dich et al., 1997; Cabello et al., 2001; Abou-Donia, 2003). Stephenson et al. (1996) investigated gene copy-number alterations at these genes in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). These data raise the intriguing possibility that the proaptotic role of AChE could play a role in tumor suppressor. (Stephenson et al., 1996).

Cholinesterases are downregulated in HCC (Montenegro et al., 2006), squamous cell carcinoma, and retinoblastoma (Qavi and Al-Rajhi, 2009). Zhao et al. (2011) demonstrated that AChE was significantly downregulated in the cancer tissues of 69.2% of HCC patients, and the low level of AChE expression in HCC was correlated with tumor aggressiveness, the elevated risk of postoperative recurrence, and a low survival rate. Both the recombinant AChE protein and the enhanced expression of AChE significantly inhibited HCC cell growth *in vitro* and tumorigenicity *in vivo*. The studies describe the function of AChE as a tumor growth suppressor that acts in the regulation of cell proliferation, relevant signaling pathways, and the chemosensitivity of HCC cells. AChE is a promising independent prognostic predictor for HCC recurrence and the survival of HCC patients (Montenegro et al., 2006). AChE-R was shown to increases germ cell apoptosis (Mor et al., 2008).

LOCALIZATION OF AChE TO THE CELL NUCLEUS AND APOPTOTIC BODIES MAY PLAY A ROLE IN APOPTOSIS INDUCTION

Zhang et al. (2002) have shown by cytochemical staining and immunohistochemistry that the presence of AChE is not homogeneous in either the cytoplasm or in the nucleus. To establish whether this heterogeneity is due to the stage of

Table 1 | Summarized cell lines in which AChE expression or activity were increased by the apoptotic stimuli with various apoptotic inducers.

Cell type	Apoptotic inducer	Increase of AChE expression or activity	Reference
A549	Sulfur mustard	Yes	Steinritz et al. (2007)
Animal brain	HBP hypobaric hypoxia (HBH)	Yes	Muthuraju et al. (2009)
Brain tissue of rats	Ischemia	Yes	Hu et al. (2009)
BRL	Detachment	Yes	Zhang et al. (2002)
C57BL/6J beta cells	Streptozotocin	Yes	Zhang et al. (2012)
Dami	TGFβ	Yes	Zhang et al. (2002)
EC prim. bovine endothelial cell	TGFβ	Yes	Zhang et al. (2002)
EC prim. swine endothelial cell	Detachment	Yes	Zhang et al. (2002)
HEK293T	H_2O_2, cisplatin	Yes	Zhang et al. (2008), Gong et al. (2009)
HEL	Huangqi (Hex)	Yes	Cheng et al. (2004)
HeLa	A23187, thapsigargin, topotecan, H_2O_2, TNFα + CHX	Yes	Zhang et al. (2002), Huang et al. (2005); Gao et al. (2009), Zhu et al. (2007a,b,c)
HFL-1	Aging	Yes	Zhang et al. (2002)
HL-60	Topotecan, H_2O_2, Daunorubicin	Yes	Zhang et al. (2002), Huang et al. (2005)
HLF	Aging	Yes	Zhang et al. (2002), Jin et al. (2002)
HOS	TGFβ	Yes	Zhang et al. (2002)
HT-29	Etoposide	Yes	Park et al. (2004, 2008)
HUVEC	Serum-starved medium	Yes	Xie et al. (2011)
IL-3-deprived (murine) bone marrow derived mast cells	A nitric oxide donor	Yes	Park et al. (2008)
Jarkat Cell	Topotecan, H_2O_2	Yes	Huang et al. (2005)
K562	Huangqi (Hex)	Yes	Cheng et al. (2004)
Lymphocytes	Post partum	Yes	Pick et al. (2004)
M07e	Minus GM-CSF	Yes	Zhang et al. (2002)
Malme-3M	Etoposide	Yes	Park et al. (2008)
MDA-MB-435s	A23187, thapsigargin	Yes	Zhu et al. (2007c)
Meg-01	TGFβ	Yes	Zhang et al. (2002)
Mouse hippocampal granule cells from Tgs mice	Tgs	Yes	Cohen et al. (2008)
Myoblast	Staurosporine	AChE-R	Pegan et al. (2010)
NIH/3T3	Detachment	Yes	Zhang et al. (2002)
MIN6 cells	Streptozotocin	Yes	Zhang et al. (2012)
NRK	G418	Yes	Jin et al. (2004)
Osteoblast	Long-term culture	Yes	Gu et al. (2002)
PC-12	H_2O_2, A23187, thapsigargin	Yes	Jing et al. (2008), Jiang et al. (2007)
PC-3	TGFβ	Yes	Zhang et al. (2002)
Primary cortical neuron	Sodium selenite	Yes	Xiao et al. (2006)
Primary cultured rat articular chondrocytes	Infected with 100 MOI adenoviral TRAIL	Yes	Park et al. (2008)
Rat kidney	Ischemia/reperfusion	Yes	Ye et al. (2010)
Rat smooth muscle	TGF-β	Yes	Zhang et al. (2002)
Raw264.7	SIN-1, a nitric oxide donor	Yes	Park et al. (2008)
Retinal	Light-induced retinal damage	Yes	Kehat et al. (2007)
SH-SY5Y	Tertiary butylhydroperoxide	N-AChE	Kehat et al. (2007)
SK-MEL-5	Etoposide	Yes	Park et al. (2008)
SK-N-SH	TNFα + CHX	Yes	Zhang et al. (2002)

(Continued)

Table 1 | Continued

Cell type	Apoptotic inducer	Increase of AChE expression or activity	Reference
SW620 cells	Etoposide, excisanin A	Yes	Deng et al. (2006)
TE671	Etoposide	Yes	Park et al. (2008)
U373MG	Etoposide	Yes	Park et al. (2008)

HUVEC, human umbilical vein endothelial cells.

apoptosis induction, the authors examined the time course of AChE expression in the leukemic Meg-01 cell line after treatment with transforming growth factor (TGF)-β. Cytochemical staining showed AChE initially appears in the cytosol and afterward in the nucleus. AChE-positive staining follows the nuclear morphological changes such as condensation and fragmentation. Eventually, when the cell membrane ruptures, AChE is released. They also examined the expression of AChE by transmission electron microscopy. At 2 h after TGF-β treatment, AChE was observed only in the cytosol but by 4 h had moved entirely to the nucleus. Therefore, during the induction of apoptosis, AChE is first synthesized in the cytosol and then accumulates in the nucleus. In general AChE is present in the nucleus at the mid- and late-stages of apoptosis (Yang et al., 2002; Jin et al., 2004; Xie et al., 2011). Santos et al. (2007) initially investigated the expression and subcellular localization of acetylcholinesterase (AChE) in endothelial cells and showed the expression of a 70-kDa AChE in both the cytoplasmic and nuclear compartments. The levels of this novel isoform are decreased in response to vascular endothelial growth factor via the proteasome pathway. They found that AChE was downregulated in human leukemic T cells compared with normal T cells, which suggested that the decreased expression of the 55-kDa AChE protein may contribute to an angiogenic response and be associated with tumorigenesis. Importantly, nuclear expression is not specific to endothelial cells, but is also present in non-neuronal and neuronal cells. Xie et al. (2011) showed that the 55-kDa AChE protein was induced during apoptosis and was negatively regulated by the Akt pathway. PC-12 express AChE at basal levels in normal cells, but a 55-kDa AChE protein can be found during the progression of apoptosis. This 55 kDa AChE protein was temporally expressed after the activation of caspases and was caspase-dependent. The inhibition of protein synthesis with CHX indicated that the 55-kDa AChE protein resulted from the cleavage of the 68-kDa AChE (Xie et al., 2011). There are still questions regarding AChE that need to be addressed, including its function when it enters the nucleus. Furthermore, whether AChE entry into the nucleus is an active or passive event, how is it transported and what is the signal that allows it to enter the nucleus are all questions which still need to be resolved.

AChE COULD PLAYS A PIVOTAL ROLE IN THE FORMATION OF APOPTOSOMES

The role of AChE in apoptosis is a key question in this field. Park et al. (2004) showed that silencing of the *AChE* gene inhibited the interaction between Apaf-1 and cytochrome *c*. They concluded that AChE plays an important role in apoptosome formation. Their data showed that an antisense AChE oligonucleotide showed a similar effect by silencing the *AChE* gene. Small interfering RNA (siRNA) against the cytochrome *c* gene blocked the interaction of AChE with Apaf-1, whereas siRNA targeting the *Apaf-1* gene did not block the interaction of AChE with cytochrome *c*. These findings indicated that the interaction of AChE with cytochrome *c* is required for the interaction between cytochrome *c* and protease-activating factor-1. Park et al. (2008) also showed that the interactions of AChE with caveolin-1 and, subsequently with cytochrome *c*, appear to be indispensable for the formation of apoptosomes. To date, however, these findings have only been shown in one study and more evidence is required to confirm how AChE participates in the formation of the apoptosome.

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