Pro-apoptotic protein–protein interactions of the extended N-AChE terminus

Debra Toiber · David S. Greenberg · Hermona Soreq

Abstract The N-terminally extended “synaptic” acetylcholinesterase variant N-AChE-S operates to promote apoptosis; however, the protein partners involved in this function remain unknown. Here, we report that when microinjected to fertilized mouse oocytes, N-AChE-S caused embryonic death as early as the zygotic stage. To identify the putative protein partners involved, we first tried yeast two hybrid screening, but this approach failed, probably because of the N-AChE-S-induced lethality. In contrast, sequence analysis and a corresponding peptide array revealed possible partners, which were validated by co-immunoprecipitation. These include the kinases GSK3, Aurora and GAK, the membrane integrin receptors, and the death receptor FAS. Each of these could potentially modulate N-AChE-S-induced apoptosis with possible therapeutic value for the treatment of Alzheimer’s disease.

Keywords Acetylcholinesterase · Apoptosis, N-AChE-S · Neurodegeneration · Protein–protein interactions

Introduction

Molecular evidence points to two principal apoptotic pathways leading to neuron death in Alzheimer’s disease (AD). The amyloid cascade hypothesis attributes the neurodegenerative process observed in AD brains to a complex series of events triggered by abnormal processing of APP, the amyloid precursor protein of Aβ. This causes deposition of aggregates and neurotoxicity of the Aβ derivative of APP, disrupting calcium homeostasis. Production of free radicals and inflammation ensue leads to neuronal damage (Parihar and Hemnani 2003). However, neuron loss in AD is selective and occurs particularly in the cholinergic basal nuclei, hippocampus and neocortex (Coyle et al. 1983), and the amyloid hypothesis does not provide an explanation for the cholinergic loss in AD. Combination of the amyloidogenic and cholinergic pathways may thus provide an in-depth view of the processes leading to the initiation and progression of AD.

The cholinergic hypothesis states that the cognitive impairments in AD stem from the loss of cholinergic function (Terry and Buccafusco 2003). This hypothesis is supported by postmortem anatomical findings in AD patients, verifying substantial loss of cholinergic neurons. Dysfunction of the cholinergic system is indeed, by itself, sufficient to produce memory deficits in animal models, which are analogous symptoms to those observed in Alzheimer’s dementia (Gordon et al. 1995; Herholz et al. 2000, 2004; Ikarashi et al. 2004; Rees et al. 2003; Rees and Brimijoin 2003). Accordingly, AChE inhibitors serve to ameliorate symptoms by prolonging ACh availability (Mesulam 2004). Some argue for attenuation of the disease process under treatment with AChE inhibitors (Nordberg et al. 2006; Recanatini and Valenti 2004), others develop
alternative AD therapeutics, including inhibitors of the Tau kinase, glycogen synthase kinase 3 (GSK3) (Bhat et al. 2004; Huang and Klein 2006), or other key proteins of the apoptotic pathway, but it is still unclear if these different approaches reflect a single targeted cascade and if so, what triggers this cascade.

Intriguingly, the known principal AD-associated mutations all converge in impaired Ca\(^{2+}\) homeostasis which is also predicted to activate the AChE promoter. Specifically, this involves changes in calcium signaling through the CCAAT motif located −1,270 to −1, 248 from the start codon. This motif binds to the CCAAT binding factor (CBF/NF-Y) (Wan et al. 2006; Zhu et al. 2007c). Also, NFATc3 and NFATc4 which are activated by calcineurin during apoptotic signaling are responsible for AChE expression (Zhu et al. 2007a, b, c). Reciprocally, under thapsigargin-induced calcium imbalance and apoptosis, N-AChE-S was upregulated, whereas its silencing by siRNA prevented thapsigargin-induced cell death. Moreover, over-expression of the N-terminally extended N-AChE-S variant induces apoptosis both in primary cortical cells and in different cell lines of various tissue and species origins, attributing to this protein a conserved and ubiquitous role as a general trigger of apoptosis (Toiber et al. 2008). N-AChE-S causes cell death and morphological impairments in primary brain cultures by activating an apoptotic pathway that includes activation of GSK3, Bax and caspases. Moreover, N-AChE-S overexpression was upregulated in AD brains and showed positive correlation with hyperphosphorylated Tau (Toiber et al. 2008). However, the mechanism through which N-AChE-S initiates the apoptotic pathway remains uncertain.

The core domain of N-AChE-S is positioned extracellularly, whereas its extended N terminus protrudes into the cytoplasm (Toiber et al. 2008). Therefore, we hypothesized that N-AChE-S lethality stems from protein partners interacting with the extended N terminus. To challenge this hypothesis, we used two hybrid screening and peptide array screens, with co-immunoprecipitation for validation.

Materials and methods

Circular dichroism measurements

N-AChE peptide was dissolved in double-distilled water to a final concentration of \(1 \times 10^{-4}\) M. Direct CD spectra were recorded at room temperature using a CD J-810 spectropolarimeter (Jasco, Easton, MD) with a 100-QS 1-mm path-length quartz cuvette (Hellma, Müllheim, Germany). Recordings were at 0.5-nm intervals in the spectral range 185–260 nm.

Two-hybrid screen

The two-hybrid screen was carried out by Hybrigenics (Paris, France). A plasmid containing 66 amino acids of the unique N-AChE N-terminal was sent and cloned into their proprietary two-hybrid vectors.

Peptide array

The synthetic N-terminal peptide of mouse N-AChE was diluted in PBS to the final concentration of 500 µg/ml, and added to 96 wells in NUNC-IMMUNO PLATE (F96 MAXSORP Cat 442404) to a final volume of 100 µl, for overnight incubation. Supernatant (sup) was discarded and the plate washed three times in PBT [5 min, room temperature (RT)]. Protein extracts (100 µg) were added in a final volume of 100 µl for 2 h at RT. Following washes as above, blocking buffer (5% skim milk in PBT) was added (1 h at RT). Sup was discarded and primary antibody was added, diluted 1:100 in the blocking buffer (2 h, RT). Following an additional round of wash, secondary antibody diluted 1:10,000 in blocking buffer was added (2 h, RT). Detection followed repeated wash, with 100 µl 3,3′,5,5′-tetramethylbenzidine (TMB, light protected). When binding occurred, the TMB turned blue, reaction was stopped by addition of 50 µl 1 M H\(_2\)SO\(_4\). Absorbance at 450 nm served to evaluate the binding efficiency.

Results

Lethality of N-AChE-S in microinjected mouse embryos

Transgenic animals are commonly used as models to study the effect of enforced over-expression of genes in the live organism. To substantiate the lethality of N-AChE-S, we microinjected fertilized mouse oocytes; this generated relatively small litters (2–3 mice per female), with none of the newborn carrying the transgene [compared to a minimum 8% of transgenic animals when microinjected with control, non-lethal genes (Steward et al. 1982)]. In search of the developmental stage(s) where embryonic death took place, we analyzed the microinjected embryos before implantation. Most of the N-AChE-S injected embryos did not develop further than the two-cell stage and only one of them developed to a blastocyst; however, it presented massive morphological impairments (Fig. 1 and data not shown). For comparison, water-injected embryos all survived to reach morula and blastocyst stages. Together, this indicated that N-AChE-S over-expression is already lethal at the zygotic stage. Because the shorter AChE-S is not lethal and yields viable
transgenic mice (Beeri et al. 1995; Toiber et al. 2008), we suspected that the extended N terminus was the cause.

Sequence considerations of the N-AChE terminus

The human exon hE1e encodes the N-AChE extended N terminus, 66 amino acids. Using SwissProt, this protein sequence was screened for homology against all known proteins, as well as for structure predictions and protein modification sites. No significant homology was found for this new protein sequence in the database. Intriguingly, the hE1e translation product includes 15 positively charged residues (11 arginines, 1 lysines, 3 histidines), making it an extremely positively charged peptide, with a \( pI = 11.76 \).

Such high \( pI \) values are often found for histones and other nucleic acid binding proteins (http://www.expasy.org/tools/tagident.html).

The extended N-terminal domain translated from hE1e is in frame with the signal peptide (MRPPQCLLHTPSLAS PLLLLLLWLLGGGGVGA, position 1-31) of the regular AChE protein. This signal peptide is normally cleaved off during protein maturation (Fournier et al. 1992). The addition of the extended N terminus masks the signal peptide, potentially preventing its cleavage. This would result in a protein larger by 96 (66 + 30) amino acid residues, compared to the regular AChE. The very hydrophobic signal peptide would then become an alternative trans-membrane region, similar to the neurexin and cyclooxygenase proteins (Dean et al. 2003). The resultant N-AChE protein would then possess a 66 residues long protrusion at the other side of the plasma membrane from the enzyme’s core domain, similar to neureligins (Ichtchenko et al. 1996).

Sequence analysis revealed that the N-extended terminus of AChE carries multiple consensus sites for predicted protein–protein interactions and/or modifications that potentially participate in signal transduction. These include an SH3 interaction domain as well as binding sites

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**Fig. 1** N-AChE-S induced cell death in early embryonic stage. Mouse embryos were microinjected with an N-AChE-S expression vector or DDW for control. Results are presented as percent of cells in each cellular stage. N-AChE-S \( N = 43 \), and Ctrl \( N = 30 \).

**Fig. 2** Predicted protein motifs of the N extended AChE. The N-extended AChE tail forecasts interaction with cell signaling proteins involved in different cellular pathways (eukaryotic linear motif) http://elm.eu.org. Peptide sequence: MSCPDRTLVTKVRSHP SGNQRPRTRGGRSFHCRRGVRPRPAALRVLPRCPAFSDAA

**Fig. 3** Circular dichroism evidence for natural unfolding of the murine extended N terminus. a Prediction of the disorder domain of the N-AChE terminus. b Shown is the profile of the 46 amino acids of the N-extended terminus of mouse AChE. Note the characteristic shape of an unfolded protein. Y axis: delta E (Delta e = eL - eR), showing the difference in extinction coefficients for left- and right-handed circularly polarized light. X axis: wavelength (yellows, predicted unfolded structure, red N-peptide).
for GSK3, cyclin, 14-3-3, etc. (Fig. 2). These interaction motifs served for planning experiments to find new possible partners.

The N-extended protein is naturally unfolded

The N-extended terminus was predicted to be a naturally unfolded peptide, as was demonstrated by circular dichroism (CD). The secondary structure of a synthetic peptide carrying this sequence was determined by CD spectroscopy in the “far-uv” spectral region (190–250 nm). At these wavelengths the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. CD signals reflect an average of the entire molecular population and may report the existence of alpha-helix, beta-sheet and random coil structures, each

Table 1 Two-hybrid screening

| Gene or clone ID | # of clones | Category | Function | Processes | Component | Reference |
|------------------|-------------|----------|----------|-----------|-----------|-----------|
| HCN1             | 3           | B        | Sodium channel, voltage gated | Apical protein, ion transport | Cell membrane, axons, dendrites | GeneID: 348980 |
| ASH1L            | 1           | D        | DNA binding, RNA polyII transcription factor activity, metal binding | Chromatin modification | Nucleus, Golgi | GeneID: 55870 |
| Elf4A2           | 1           | D        | Translation Initiation factor, ATP binding | Translation | Cytosolic | GeneID: 1974 |
| GAK              | 1           | D        | Kinase activity, ATP binding, transferase activity | cell cycle, protein phosphorylation | Cytoplasm, Golgi, Cell junction | GeneID: 2580 |
| PPP3CA           | 2           | D        | Phosphatase activity, calcium binding, metal binding | Cell cycle, synaptic transmission, stress response | Nucleus | GeneID: 5530 |
| COP5S            | 15          | E        | Peptidase activity, Transcription co-activator, metal binding | Cell cycle, translation | Cytosol, Signalosome | GeneID: 10987 |
| RNFP123          | 5           | E        | Ligase activity, metal binding | Ubiquitin catabolic process | Cytosol | GeneID: 63891 |
| SNAPAP           | 7           | E        | soluble N-ethylmaleimide-sensitive factor attached protein receptor (SNARE) binding | Exocytosis, Synaptic transmission | Synaptic vesicles, synaptosome | GeneID: 23557 |
| SRI              | 2           | E        | Calcium channel regulator activity, calcium binding | Signal transduction, Heart development, transport | Cytosol | GeneID: 6717 |
| ST13             | 7           | E        | Protein binding, bridging | Protein folding | Cytosol | GeneID: 6767 |
| UBQLN1           | 5           | E        | Kinase binding | Protein modification process | Cytosol, Nucleus | GeneID: 29979 |

(A) Known transcripts, (B) unknown transcripts, for further details, see Entrez Genome NCBI database http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene. The category column notes the reliability of the results, as follows: A very high, B high, C good, D moderate confidence in the interaction identified through one unique prey fragment (singleton), E these interactions involve at least one domain of interaction that might be a potential false positive
with a characteristic shape and magnitude of the CD spectrum. The tested peptide emerged as a naturally unfolded protein (Fig. 3), which implies “promiscuous” protein–protein interaction features because of its flexibility.

To discover the protein partners interacting with the N terminus, we first attempted a yeast two hybrid screening; however, yeast cells expressing N-AChE terminus were unable to survive. Next, we used an optimized two-hybrid screening which again yielded very few possible protein partners with weak binding properties, possibly due to the lethality of this protein (Table 1). That none of those found partners showed tight interactions, called for alternative search technologies. The peptide array we employed next differs substantially from two-hybrid screening, which uses a chimeric protein, in a nuclear environment. Nuclear interactions with the chimeric domains (in both, the bait and the prey) may yield false positives, whereas, cytoplasmic interactions may be overlooked. In the case of toxic peptides, very few protein interactions would be observed in this in vivo system. The peptide array overcomes these limitations, because the synthetic environment is indifferent to toxic effects. False positive interactions were excluded by the use of negative controls, leading to some plausible partners for the N-AChE terminus.

ELISA-based reactions primarily tested N-AChE interactions with those target proteins predicted by the sequence analysis. A synthetic peptide comprising the mouse N-AChE terminal 46 amino acid residues was tested for its possible interactions with three candidate proteins: integrin, involved in signal transduction from the cell membrane, for example in AD (ffrench-Constant and Colognato 2004; Ho et al. 2005); Aurora kinase members, involved in

![Fig. 4 Peptide array for protein interactions: a Positive control. Note the first two bars detection compared to the negative controls. b Dose dependence. Different concentrations of the synthetic peptide were added to the plate, and detected by the N-specific antibody, showing the sensitivity of the assay. c Positive interaction](image)

![Fig. 5 Co-immunoprecipitation 293 cells were transfected with GFP or N-AChE-S and the protein extract used for Co-Ip a Negative control was without antibody. In each experiment four samples were used GFP input, N-AChE-S (NS) Input and GFP co-IP, NS Co-IP. b Westerns were probed with anti-AChE which yields a specific band at 75 kD. The IP was considered specific if the band appeared in the NS IP and not GFP IP. The lower stronger band is nonspecific. Antibodies that yielded positive interactions: GSK3, JAB1(CSN5), Fas, GAK and integrin](image)
cell cycle regulation (Kollareddy et al. 2008; Taylor and Peters 2008), and finally Apaf, a proapoptotic protein, part of the apoptosome complex (Fig 4) (Bao and Shi 2007). Integrin, Aurora kinase and APAF showed interaction, whereas three different proteins did not interact (not shown). Integrin which showed a promising interaction and other 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 further subjected to co-immunoprecipitation. Positive co-precipitation (Fig. 5) served as a validating test, supporting the notion of co-interaction with these tested partner proteins.

N-AChE-S protein multimers with integrin receptors were also observed. Integrin receptors are known to interact with many other cellular receptors and be involved in cell signaling, including the observed GSK3 activation and dephosphorylation of GSK3 by activation of Protein Phosphatase 2A (Ivaska et al. 2002). In addition, N-AChE-S showed a specific interaction with GSK3, fortifying the importance of this enzyme in the N-AChE-S apoptotic pathway (Toiber et al. 2008).

Discussion

Unusual AChE forms were reported in AD (Navaratnam et al. 1991) and in dementia (Shen 1997). In addition, some of the commercially available anti-AChE antibodies yield double bands around 66–70 kDa [see, for example (Belbeoch et al. 2003; Brenner et al. 2003; Meshorer et al. 2004; Morel et al. 2001)]. Sucrose gradient analyses also reveal yet unexplained diversity of the various AChE isoforms (Massoulié 2002). These observations could potentially reflect the new, longer member(s) of the AChE family; and the potent labeling of brain AChE-like protein with anti-N antibodies (Meshorer et al. 2004) suggests abundance of such variants.

The novel human N-AChE protein variant shows similarity to the murine protein, supporting the notion that it is physiologically relevant. In addition to the N-terminal extension, the new protein spans the very hydrophobic sequence of the former signal peptide, which becomes a trans-membrane domain in N-terminally extended AChE variants. Parallel examples include the asialoglycoprotein receptor (Spiess and Lodish 1986), the bovine adrenal dopamine β-monooxygenase (Taljandisz et al. 1989), α-neurexin (an extended form of β-neurexin) (Ushkaryov et al. 1994), and cyclooxygenase (Chandrasekharan et al. 2002). For the extended N-AChE variants, positioning of the “conservative” signal peptide in the membrane would enable direct docking of AChE to the cell membrane, independently of the structural PRiMA, or ColQ proteins necessary to adhere AChE-S tetramers to the synapse (Massoulié 2002) (Krejci et al. 1997).

Together with the N-terminal extension, the AChE protein is enlarged by 20%. However, its catalytic activity is unaffected, suggesting that the extended N terminus forms a structurally independent domain, distinct from the globular AChE structure (Sussman et al. 1991) and separated from it by the conservative signal peptide. This further implies that the role of the N terminus is not related to AChE’s catalytic characteristics.

The potential partners for the extended AChE N terminus, as deduced from the sequence analysis, tested in the peptide array or by co-immunoprecipitation experiments included Cyclin G associated kinase (GAK) which contributes to cell signaling and internalization of clathrin-coated vesicles, and which is probably involved in the mechanism by which N-AChE-S is internalized. Also included was JAB1/CSN5, which is involved in the regulation of p53, and could have a role in preventing N-AChE-S-induced cell death (Lee et al. 2006). GSK was previously shown by us (Toiber et al. 2008) to be involved with N-AChE apoptosis. Parallel heterodimers formation with other cell membrane receptors such as p75, TRK or FAS family members, can likewise, activate additional signaling mechanisms. FAS, which also interacts with N-AChE-S is a known death receptor, which interacts with caspases and with death ligands, inducing cell death from a variety of stimuli. The interaction of N-AChE-S with Fas could induce mutual activation, leading to known apoptotic cascades. Together, this data suggests possible protein partners initiating the N-AChE-S-induced apoptotic cascade and/or protection from it. Blockade of the apoptogenic partners to prevent the neuronal associated N-AChE-S cell death in AD should be considered.

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