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From Protein to Peptides: a Spectrum of Non-Hydrolytic Functions of Acetylcholinesterase

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Abstract: Acetylcholinesterase (AChE), a member of the α/β-hydrolase fold superfamily of proteins, is a serine hydrolase responsible for the hydrolysis of the well studied neurotransmitter acetylcholine (ACh). However, it is becoming clear that AChE has a range of actions other than this 'classical' role. Non-classical AChE functions have been identified in apoptosis, stress-responses, neuritogenesis, and neurodegeneration. Furthermore, these non-classical roles are attributable not only to the native protein, which appears to act as a mediary binding protein under a number of circumstances, but also to peptides cleaved from the parent protein. Peptides cleaved from AChE can act as independent signalling molecules. Here we discuss the implications of non-hydrolytic functions of this multi-tasking protein.

Keywords: Acetylcholinesterase, adhesion, apoptosis, neurogenesis, non-hydrolytic function, signalling peptide.

INTRODUCTION

Acetylcholine (ACh) is one of the major neurotransmitters both in the central nervous system (CNS) and in the peripheral nervous system at neuromuscular junctions. The hydrolysis of ACh was first demonstrated nearly 100 years ago [1] and the enzyme responsible is acetylcholinesterase (AChE, EC 3.1.1.7). Hydrolysis of ACh by AChE is one of the fastest known enzymatic reactions [2], producing choline and acetate [3]. The power of this enzyme is observed most dramatically upon administration of organophosphates as used in insecticides, and chemical weapons such as sarin gas; these chemicals inhibit AChE and are potentially lethal.

The first 3D X-ray structure of AChE from *Torpedo californica* obtained by Sussman *et al.* [4] revealed a charged area at the active site within a tertiary structural motif – an α/β hydrolase fold [4-6] – and thus is classified within the α/β-fold superfamily. In common with other serine hydrolases, AChE exhibits a conserved amino acid 'triad' at the catalytic site although in AChE this is comprised of serine, histidine and glutamate as opposed to the canonical serine-histidine-aspartate[7] Although the strong electrostatic forces generated by the distinct regions of positively and negatively charged residues around the enzymatic cleft were once thought to aid in the rapid rates of substrate binding and product removal, this does not appear to be the case [6- 8]. Since the active site of AChE is situated at the bottom of a relatively deep gorge, it is unclear how the catalytic reaction occurs so rapidly. However, site-directed mutagenesis suggests that disruption of the electrostatic field does not alter the reaction rate of AChE [9].

The human AChE gene is located on the long arm of chromosome 7 (7q22) [10]. The mammalian gene encodes 6 exons, alternative splicing of which produces a number of AChE variants. The 'synaptic' isoform (AChE-S; also known as 'tailed' AChE-T [11]) translates exons E4 and E6 at the C-terminus, while the 'erythrocytic' isoform (AChE-E; also known as 'hydrophobic' AChE-H [11]) translates exons E4 and E5. A 'read-through' isoform (AChE-R) employs the same exons as AChE-E, while retaining the intron between E4 and E5 [12]. These AChE protein variants differ at the C-terminus and thus exhibit different binding characteristics both between themselves and with other proteins. Due to the cysteine residue within the C-terminus of AChE-T, the protein is capable of forming dimers by disulphide bonding. Tetramers can also assemble by the association of two monomers to a dimer by hydrophobic interactions [13]. Tetramers can form bundles together of up to 12 AChE-T subunits *via* covalent interactions with the collagen-like protein[14] ColQ at neuromuscular junctions or PriMA in brain neurons, anchoring the bundles to cell surfaces, whereas monomers and dimers are soluble[15]. AChE-E dimers are anchored by glycophosphatidylinositol (GPI) linkage to the cell surface of erythrocytes [16]. AChE-R remains monomeric and soluble and does not appear to be capable of linking either to other AChE-R monomers or to other anchoring proteins [17].

Different tissue types express different molecular isoforms of AChE [18] and vary depending on the tissue requirements [19-20]; for example, increased expression of the AChE-R isoform has been shown to occur in response to cell stress [21] although the levels and relevance of expression are disputed [17]. AChE is expressed by numerous tissue types that are not innervated by cholinergic nerves and a growing body of research has concentrated on non-neuronal signalling of ACh [22-24] in controlling various cell functions including immune cell activation and epithelial cell adhesion and growth. The considerable diversity of protein products available from just one ACHE gene strongly suggests a diverse function for the AChE protein. It was first suggested that AChE may have functions other than hydro-

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lysing ACh at synapses, when a soluble, monomeric secreted form of AChE was identified in 1969[25]. Indeed, over the last 30 years research has shown AChE has a number of non-classical functions which do not involve the breakdown of ACh; the current research in these areas will be discussed.

NEURITOGENESIS AND CELL ADHESION

AChE is thought to regulate axonal growth by a mechanism independent of its enzymatic activity [26-31]. The involvement of AChE in neurite outgrowth has been inferred from the spatiotemporal expression pattern of AChE before the onset on synaptogenesis[32-34]. If synapses are not yet formed, ChAT is not present and hence ACh is not produced, so why is AChE expressed? The expression of AChE before synaptogenesis suggests that AChE performs a role quite distinct from that of ACh hydrolysis at the synapse. It was initially thought that membrane docking *via* the C-terminal was required by AChE for neuritogenesis; indeed, by expressing AChE without the C-terminus required for membrane docking, neurite promotion by AChE was abrogated[35]. However, it has now been shown that the C-terminal of AChE is not required and that all AChE variants are capable of stimulating neurite outgrowth [36] regardless of the ability of the C-terminus to bind membranes. Numerous studies now suggest that the cell-adhesive properties of AChE are responsible for stimulating neurite outgrowth.

Cell adhesion is a major mechanism controlling the direction of neuritic growth and the homology between AChE and other α/β hydrolases involved in protein-protein adhesion, such as neurologist [6- 37], suggests that the strong electrostatic dipoles within the α/β hydrolases domain are required for neurite outgrowth stimulation by AChE – a theory which has been confirmed by a number of studies [38-39]. The extracellular domain of neuroligins shares a large region of homology with AChE, however, the amino acid residues required for the catalytic activity of AChE are not conserved and hence neuroligin has no hydrolase activity[37]. Sharma *et al.*[38] have put forward two possible theories of how AChE modulates neuronal adhesion and outgrowth; firstly, that AChE mediates some form of intracellular signalling cascade and secondly, that AChE adhesion properties facilitate binding of other cell adhesion molecules classically involved in the process. One possible interacting protein is laminin-1, an extracellular matrix protein classically involved in neuronal differentiation and adhesion, which binds AChE at the cell membrane [40-41], although the exact function of this interaction is unclear.

APOPTOSIS

An involvement of AChE in developmental processes is closely linked with the role it plays in regulating apoptosis. Robitzki and colleges carried out studies with chick embryo retinal cells where they showed that decreasing BChE expression by gene knock-out caused and increase in AChE expression and cell death [42]. Studies in which the AChE gene was knocked out, but not the AChE-related butyrylcholinesterase (BChE), exhibited an increase in hematopoietic progeny cell number but with reduced differentiation [43-44], highlighting the necessity of AChE for differentiation of these cells. Apoptosis-associated DNA fragmentation is also reduced when AChE is knocked out [44], suggesting that AChE contributes to hematopoietic differentiation by increasing apoptotic markers in stem cells. In skeletal muscle formation expression of AChE has also been identified prior to other cholinergic signalling pathway components in mononuclear myoblasts during embryonic development [45]. Decreasing AChE expression reduces apoptosis – an essential process in myoblast proliferation and differentiation – while stimulating apoptosis increases AChE expression [45]. Thus it appears that AChE is involved in developmental pathways by modulating apoptotic signals.

During apoptosis, the localization of AChE changes, moving from the cytoplasm to the nucleus during late stage apoptosis [46-47]. The mechanism of translocation is unclear; the Ran binding protein (RanBP) binds AChE-T during translocation to the nucleus, although neither RanBP nor AChE contain a recognised nuclear localisation signal (NLS) [48]. Interaction with additional NLS-containing proteins must therefore be required. Inhibiting AChE-T expression with anti-sense ACHE-T [46-47] or blocking AChE activity using inhibitors [47] prevents apoptosis in various neuronal and non-neuronal cell lines. Apoptosis-induced increase in AChE-T expression has been shown to be mediated by glycogen synthase kinase-3β (GSK-3β) in PC-12 cells derived from rat adrenal pheochromocytomas (an *in vitro* model for neural cells) [49]. GSK-3β is a serine/threonine kinase which modulates the activity of various regulatory proteins, many of which are transcription factors [50]. Taken together, these data provide compelling evidence for the involvement of AChE-T in controlling apoptotic signals in certain types of tissue; apoptosis initiation signals an increase in AChE-T expression which is then directly involved with, and required for, the completion of apoptosis. Interestingly, silencing AChE-T expression inhibits interaction between Apaf-1 and cytochrome c [51], required for the assembly of the apoptosome complex responsible for the proteolytic cascade and cellular destruction [52]. Further studies are required to elucidate fully the involvement of AChE in these processes and answer questions such as how AChE is translocated to the nucleus and what the function within the nucleus is. Does AChE act as a transcription factor controlling apoptosis-related gene expression? Or is the role of AChE in apoptosis purely at the cell membrane? A number of pathologies are characterised by dysregulation of apoptosis, raising the interesting possibility that AChE may have previously undiscovered roles in a number of diseases due to the role AChE plays in mediating apoptosis.

TUMORIGENESIS

One such pathology characterised by a dysregulation in apoptosis is cancer. Although the involvement of AChE in tumorigenesis is unclear, the roles of AChE in apoptosis and development strongly suggest that the protein could be involved since these are the cellular functions that are perturbed. In brain tumours, changes in AChE have been identified in a number of cell types [53]. Levels of AChE expression alter depending on cell type; AChE is over-expressed in human glooms [54] but down-regulated in human meningiomas[55-56]. Expression of AChE variants also differs during tumorigenesis in some cell types with a shift from AChE-T to AChE-R expression observed in astrocytomas,

associated with an increase in tumour aggression [57]; however, whether the non-hydrolytic or hydrolytic functions of AChE are involved is ambiguous.

Elsewhere in the body, increases in AChE activity and expression have been observed in breast cancers [58-59] and ovarian carcinomas [60] but a decrease in activity in malignant lymph nodes [61] and colorectal cancer [62-64]. Amplification of the region in which the ACHE gene is located on chromosome 7 occurs in cancers of the lung [65] and prostate [66]. In brain tumours, expression of AChE variants changes during cancer progression in a tissue-specific manner. AChE variants expressed in malignant breast cancer do not appear to alter compared to healthy tissue [58], while in cancerous lymph nodes, which express AChE-T monomers, dimers and tetramers under normal conditions, a decrease in tetramers is observed [61]. In glioblastomas, the predominant AChE-T expression is replaced by an over-expression of AChE-R which promotes proliferation [67].

Interestingly, farmers exposed to AChE inhibitor-containing pesticides show an increased risk of leukaemia's [68] and glooms [69]. These cancers over-express AChE probably in order to counteract the enzyme inhibition [53]; however, it is unlikely that the enzyme function is involved in cancer development since the activity should remain similar overall. Could the increase in protein levels and therefore non-hydrolytic actions, regardless of enzymatic function, be involved? How disregulation of AChE expression or activity impacts on tumorigenesis is ambiguous; in breast cancer for example, tumour size appears to correlate with AChE amplification [59]; however, a contribution from non-neuronal ACh signalling must also be taken into account. Non-neuronal ACh signalling has been shown to control pathways that are vital in cancer progression such as cell adhesion and growth in epithelial cells of the breast, lung and colon, amongst others [22]; consequently, aberrant AChE expression or activity in these situations may promote tumorigenesis due to the impact on this signalling pathway. Non-hydrolytic AChE functions may be involved in conjunction with effects on non-neuronal ACh signalling, or may be solely responsible for tumorigenesis depending on tissue type. Again, this highlights the multi-functionality of the AChE protein, both as an enzyme and as a protein mediating cell signals itself.

STRESS-RESPONSE AND AChE-R

There is significant evidence for an involvement of the AChE-R splice variant in stress responses in brain, muscle, blood and testicular cells [21, 70-73]. The production of alternative AChE splice variants in response to stress appears to be an important component of this process: AChE-R(-/-) transgenic mice exhibit accelerated stress responses compared to those expressing AChE-R[74]. In addition to a long-term increase in expression of AChE-R in response to stress (lasting weeks after the initial stress-induction[75]), the AChE-R protein appears to be cleaved near the C-terminus, producing an AChE Read-through Peptide (ARP; 1-GMQG PAGSGWEEGSGSPPGVTPLFSP-26) that has been detected in mice following induced stress[76]. Experimentation with synthetic ARP has shown a role for this peptide in hematopoietic homeostasis following stress induction[76]. Alternate AChE mRNA splicing differs in a region-specific manner with hippocampal [21- 75- 77] and prefrontal cortical[78] neurons showing higher levels of AChE-R expression compared to other cortical neurons[17]. AChE-R binds the scaffold protein RACK1 [79], following which it can recruit protein kinase C (PKC) [79] and subsequently activate specific intracellular signalling pathways [79]. Recently, it has been proposed that microRNAs are responsible for controlling the change in AChE expression in the brain [80]. MicroRNAs were initially thought to be random, non-coding pieces of RNA with no particular function but were shown in C. elegans to be involved in gene downregulation [81] and are in fact a highly conserved and large class of riboregulators [82-83]. Soreq *et al.* have demonstrated that chronic and acute stress cause variations in microRNAs 134 and 183 expression, both of which target the splicing factor SC35 [80]. SC35 has been shown to shift the predominant splicing species from AChE-T to AChE-R under stress conditions [78]; hence Soreq and colleagues suggests that the increase in AChE-R expression under stress is controlled by multiple mechanisms [80].

AChE-T C-TERMINAL PEPTIDES

AChE[32] is expressed before ChAT during development, as is the ACh receptor (α7-nAChR)[84]. This not only suggests a role for the AChE molecule separate from its ACh-hydrolysis function such as those discussed above, but also raises the possibility of AChE and α7-nAChR constituting a signalling system independent of Ach [85], in which AChE provides the signalling molecule and α7-nAChR is the target receptor. A region of the AChE molecule has been identified that can mediate non-hydrolytic actions [86] and thus act as the signalling molecule. The C-terminal region of AChE shares a high level of homology with the β-amyloid precursor protein (APP), a protein which shares many similarities with non-hydrolytic AChE functions such as trophic actions[87], activation of macrophages[88-89] and enhancing calcium influx into neurons[90]. The bioactive homologous AChE-T C-terminal peptide (T14; AEFHRWSSYMVH WK) [86] associates with an allosteric site on the α7-nAChR [91-92]. Subsequently, a longer peptide incorporating T14 and the remainder of the AChE-T C-terminus (T30; KAEFH RWSSYMVHWKNQFDHYSKQDRCSDL) has been shown to be yet more potent [91]. The specificities of the effects of T14 and T30 were established using a variety of controls including a scrambled version of the bioactive peptide, a peptide from the equivalent region in BChE (BChE 573-586; AGFHRWNNYMMDWK) and the biologically inactive 15 amino acid sequence from the AChE-T C-terminus (T15; NQFDHYSKQDRCSDL) (Table **1**). T14 and T15 together, produce the T30 peptide, minus the lysine at the beginning of the T30 sequence.

Although the cleaved AChE peptide has not been identified *in vivo*, a monomeric form of AChE-T (G1) that lacks the C-terminal region (the T30 sequence) has been identified in both fetal bovine serum [99] and human umbilical vein endothelial cells [100]. Whether this region is cleaved from the full-length protein or is omitted by alternative splicing at the mRNA level is unclear. However, it does raise the possibility that G1 AChE-T is cleaved in the C-terminal region, releasing a peptide capable of acting as a signalling mole-

Table 1. The Effects of AChE-T C-terminal Peptides on Various Cell and Animal Models. Peptide Sequences: T14 AEFHRWSSYMVHWK; T30 KAEFHRWSSYMVHWKNQFDHYSKQDRCSDL.

Effects of Peptide Treatment	Experimental Model
(T14) Causes a decrease in the amplitude of depolarization induced when the glutamate receptor is activated and an increase in calcium spikes when the receptor is inhibited, by modulating calcium entry[93]	Guinea-pig hippocampal slices
(T14) Produces long-lasting potentiation of the EPSP in an α7-nAChR-dependent manner when the NMDA receptor is blocked[92]	
(T14) Causes neurotrophic effects over short periods of time (1hr). longer exposures cause reduced neurite outgrowth in a concentration-dependent manner by increasing calcium influx, leading to free radical production, apoptosis and necrosis[94, 95] in an α7-nAChR-dependent manner[92, 96]	Rat organotypic hippocampal cultures
(T14) Reduces NMDA-stimulated AChE release from neurons[97]	Rat substantianigra tissue punches
(T14) Increases ACh-induced membrane conductance in an α7-nAChR-dependent manner[92]	Xenopus oocytes transiently expressing α4- and α7-nAChR
(T14) Decreases metastatic activity in strong, but not weak, metastatic cell lines in an α7-nAChR-dependent manner[98]	Human breast tumour cell lines
(T14 and T30) Displaces α-BTX from the α7-nAChR binding site and causes upregulation of α7-nAChR mRNA and protein expression[91]	Rat pituitary tumour cell line stably expressing α7-nAChR
(T30) Causes damage to the blood brain barrier of the basal forebrain when injected into the region (unpublished work from Greenfield group)	Rat

cule. AChE-T C-terminal peptides exhibit effects similar to those seen during neurodegeneration, such as calcium influx and neurotoxicity (Table **1**). Studies outlined in Table **1** suggest a trophic-toxic role for AChE-T C-terminal peptides whereby during development, calcium influx and trophic responses are stimulated with toxic responses stimulated in aged cells. Interestingly, an increase in the G1 monomer is observed in Alzheimer's diseased brains[101], so there exists the possibility that a consequent increase in C-terminal peptides might mediate neurodegeneration *via* these toxic effects.

AChE-T C-TERMINAL PEPTIDES AS SYSTEMIC SIGNALLING MOLECULES?

Although choline is synthesised in small quantities within the body, the main source is the diet. Choline acts as an alternative primary ligand to ACh at α7-nAChR[102-103] and may hence constitute a more generalised, basic signalling system which may have preceded the more familiar transmission mediated by ACh. The combination of choline as the primary ligand with AChE-peptides modulating an allosteric site might act *via* α7-nAChR to constitute a parallel signalling system, independent of ACh and ChAT. Our group hypothesises that AChE-T C-terminal peptides could act as novel signalling molecules for a previously undiscovered, unifying system coordinating CNS, endocrine and immune responses: 'Para-Cholinergic' Signalling. Fig. **1** represents the hypothetical 'Para-Cholinergic' signalling pathway within the body.

A fundamental challenge in neurobiology is how brain processes impact on the rest of the body, and vice versa. How do the three major control processes in higher organisms - endocrine, immune and CNS - achieve the coordination that is essential for cohesive functioning? When perturbed, compromise of this coordination might be a vital, but as yet overlooked, factor in the aetiology of many disease states. However, despite the evident relevance to a wide range of biomedical areas, no single, unifying system has been identified that operates effectively both within and between endocrine, immune, and nervous systems. There are few systems that exhibit greater interaction between these systems than the hypothalamo-pituitary system (HPS). This ancient system uses both peptidergic non-synaptic transmission and direct neuroendocrine secretion: the HPS controls the hypothalamo-pituitary stress axis, which is influenced both by inflammatory cytokines and immune-modulating glucocorticoids[104]. Magnocellular neurons, projecting into the posterior pituitary, are rich in AChE though not ChAT, and signal to surrounding neurons and glia by calcium-stimulated dendritic peptide secretion[105]. Similarly, the anterior pituitary contains abundant AChE, but spatially separate from pituitary ChAT activity[106]. Magnocellular neurons also express α7-nAChR, the receptor responsible for the classic bursting firing of vasopressin neurons in response to choline[107]. In general the entry of calcium, in both neuronal and non-neuronal systems, might be the key trigger in cell development, differentiation, migration and programmed cell death. Consistent with the hypothesis of a common mechanism of 'para-cholinergic' signalling, this would be coordinated across the control systems of the body. Modulation of α7-nAChR activity is critical in numerous cell functions: in lung cancer, for example, activation of α7-nAChR by nicotine induces cell proliferation, invasion and epithe-

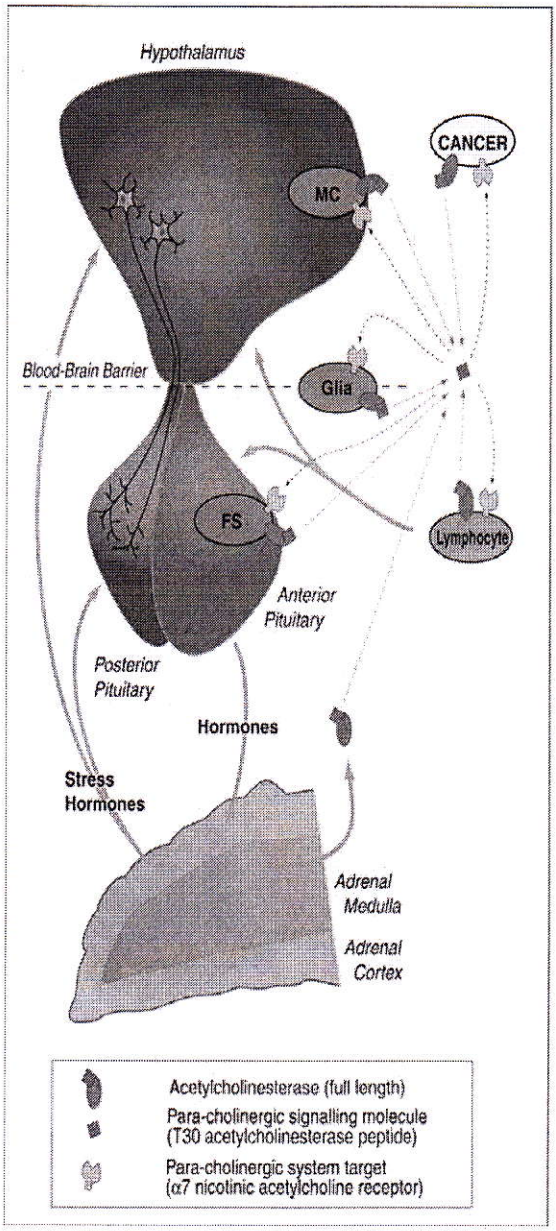

Figure 1. Para-cholinergic signalling: the schematic diagram outlines the theory of para-cholinergic signalling between neuronal, endocrine, and immune cells with potential interactions around the body *via* the release of the AChE C-terminal peptide. Green lines represent documented signalling pathways or release of AChE. Orange dotted lines represent theoretical release of AChE-T C-terminal peptide. Black dotted lines represent theoretical interaction with and subsequent activation from the peptide. In this way, the release of AChE-T C-terminal peptide could affect numerous cell systems around the body, with wide-spread adverse affects if the system were compromised. Magnocellular neurons (MC), folliculostellate cells (FS).

lial-mesenchymal transition[108]. Such observations raise the intriguing possibility that modulation of this receptor by para-cholinergic signalling could play a pivotal role in various cell functions within different cell systems. When perturbed this would have profound systemic consequences effecting endocrine, immune and nervous systems as well as affecting more local paracrine signalling systems in cells such as epithelia.

CONCLUSIONS

It was first suggested that AChE may have functions other than hydrolysing ACh at synapses, when a soluble, monomeric secreted form of AChE was identified in 1969[25]. Since then, the research into this highly conserved yet multi-functioning protein has often produced more questions than answers. The roles of the different AChE variants and how their expression is controlled is obviously a complicated mechanism, with the added difficulty that different tissues use AChE to control or mediate different cellular functions. The release of secondary signalling peptides, such as ARP from AChE-R and C-terminal peptides from AChE-T adds another level of complexity. AChE-T C-terminal peptide regulation of α7-nAChR signalling presents an interesting theory of how AChE may be involved in controlling cell functions around the body. This area of research clearly requires more work to fully elucidate all the different roles of AChE, not only non-hydrolytic functions, but also the role of AChE regulation in non-neuronal ACh signalling.

ABBREVIATIONS

ACh	=	Acetylcholine
AChE	=	Acetylcholinesterase
AChR	=	Acetylcholine receptor
ARP	=	Acetylcholinesterase read-through peptide
BChE	=	Butyrylcholinesterase
ChAT	=	Cholineacetyltransferase
HPS	=	Hypothalamo-pituitary system

CONFLICT OF INTEREST

The authors state no conflicts of interest.

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