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PRINCIPAL INVESTIGATOR: Hermona Soreq, Ph.D.

CONTRACTING ORGANIZATION: Hebrew University of Jerusalem
91904 Jerusalem, ISRAEL

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6. AUTHOR(S)
Hermona Soreq, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Hebrew University of Jerusalem
91904 Jerusalem, ISRAEL

E-Mail: soreq@shum.huji.ac.il

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13. ABSTRACT (Maximum 200 Words)
The effect of stress and exposure to anti-cholinesterases on the cholinergic system were tested in vitro and in vivo. Rapid muscle fatigue was identified and electrophysiologically characterized in mice with elevated levels of AChE-S, the synaptic variant of acetylcholinesterase (AChE). Anxiety responses were observed in mice that over-expressed the stress-associated variant, AChE-R. The binding partner of AChE-R in mouse neurons was identified as a component of the protein kinase-C signaling system. The role of AChE-R in the symptoms of experimental myasthenia gravis in rats has been documented. As the response to chronic exposure to organophosphate anticholinesterases provokes a stress-like response from the cholinergic system, the effect of such exposure in humans on electroencephalographic abnormalities and their origin in the brain was studied. Future research will be assisted by the development of an anti-AChE-S polyclonal antibody, and a mouse strain that produces an endogenous anti-AChE-R antisense reagent upon feeding of doxycycline. These experiments are building a case for the involvement of unregulated AChE-R production in long-term stress response.

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Introduction

The annual report of research results is arranged according to the tasks undertaken in the grant application:

task 1: characterize the sensory, cognitive and neuromotor consequences of a transgenic excess in AChE variants. Progress on this task is reported under the heading,

- Interaction of "readthrough" acetylcholinesterase with RACK1 and PKCβII correlates with intensified fear-induced conflict behavior

task 2: employ transgenic mouse models with up to 300-fold differences in peripheral AChE levels for demonstration of direct correlation between AChE dosage and protection from stress and chemical warfare agents and to test their responses to pyridostigmine administration. Progress on this task is reported under the heading,

- Stress-induced alternative splicing of acetylcholinesterase results in enhanced fear memory and long-term potentiation

task 3: develop RT-PCR tests in peripheral blood cells of model animals, and additional surrogate markers, for follow-up of responses and protection. Progress on this task is reported under the headings,

- Anxiety scores in the HERITAGE Family Study associate with polymorphisms in the acetylcholinesterase locus
- Peripheral blood cell RT-PCR tests. Nucleated blood cells were prepared by established procedures from whole blood samples of 20 individuals in an effort to establish a RT-PCR procedure that would serve as a surrogate marker for expression of the *ACHE* gene and its variant mRNAs. Parallel tests involved biochemical measurement of enzyme activity in serum (see an example in our HERITAGE study report). The outcome of the RT-PCR tests revealed a considerable variability in *ACHE* gene expression, with some tests being unsuccessful due to contaminating proteins and inefficient RNA extraction. Enzyme activity measurements, which were performed on a considerably larger scale (over 400 individuals) confirmed this variability, attributing it to individual's age, gender, ethnic origin and body mass index (HERITAGE study). Moreover, data analysis of the biochemical tests demonstrated a possible association with the tested individuals' state and trait anxiety phenotype. In view of these findings, and the considerably lower cost of the biochemical, as opposed to the RT-PCR tests, it was decided to focus on enzyme activity measurements as surrogate markers for *ACHE* gene expression in blood. Future tests should, however, include additional analyses, e.g. non-denaturing gel electrophoresis followed by staining of gels for enzymatic activity and/or immunoblot analysis with variant-specific antibodies. This would yield, in addition to enzyme activity, information on the specific AChE variants being expressed, especially monomers vs. dimers and tetramers, and AChE-R/AChE-S ratios.

task 4: adapt such tests to use in humans following accidental exposure to agricultural anti-AChEs. Progress on this task is reported under the headings,

- Electrophysiological alterations in individuals under low levels exposure to organophosphates

task 5: employ the transgenic mouse models to test effects of sudden changes in AChE levels at all the above sites and functions. Progress on this task is reported under the heading,

- Protection of mice against paraoxon and lipopolysaccharide intoxication by a mouse AChE-R-specific antisense reagent: preliminary experiments

task 6: delineate the protein partners through which AChE exerts non-catalytic signals which lead to delayed symptoms. Progress on this task is reported under the above-mentioned heading,

- Two-hybrid approach to the intracellular function(s) of readthrough acetylcholinesterase

task 7: develop tetracycline-inducible animal models in which AChE activity can be induced or antisense-suppressed at will. Progress on this task is reported under the above-mentioned heading,

- Conditional suppression of AChE *in vitro* and *in vivo*

task 8: continue the search for promoter sequence polymorphisms that lead to natural variations in human AChE levels and correlate them with responses to anti-ChEs. Progress on this task is reported under the above-mentioned headings,

- A rare *ACHE–PON1* haplotype increases the risk of insecticide-induced Parkinson's disease
- *ACHE* and *PON1* genotypes and their relation to acetylcholinesterase and paraoxonase activity levels in humans

task 9: expedite transgenic models for production from milk of recombinant human AChE, as a potential scavenger. There is no progress to report this year

Interaction of "readthrough" acetylcholinesterase with the PKC scaffold protein RACK1 facilitates antisense-suppressible contextual fear response

Physiological stress induces rapid, robust signaling processes in mammalian brain neurons. Neurophysiological signaling cascades elicit stress-related suppression of long term potentiation (LTP, Vereker et al., 2000), augment stress-induced long term depression (LTD, Xu et al., 1997) and induce the release of synaptic vesicles, potentiating neurotransmission (Stevens and Sullivan, 1998). At the longer term, these signal transduction cascades eventually attenuate the stress response, protecting the organism from being excessively affected by a stressful experience. This is associated with modulation of neuronal dendrite branching (Sousa et al., 2000) and synapse re-organization (McEwen, 1999). However, the molecular pathway(s) leading from electrophysiological to behavioral processes that enable the adjustment to stressful stimuli are not yet known. This called for searching for potential candidate molecules that could serve as the mediators that transduce physiological stress signals into rapidly initiating yet long-lasting changes in behavioral responses.

The "readthrough" acetylcholinesterase variant, AChE-R, is a promising candidate for this role (reviewed in Soreq and Seidman, 2001). AChE-R is exceedingly rare in the adult, non-stressed brain. Various stress insults induce AChE-R overproduction through alternative splicing of the pre-mRNA. Its levels rise rapidly under acute psychological stress (Kaufer et al., 1998) or chemical neurotoxication (Shapira et al., 2000) and stay high for over two weeks following head injury (Shohami et al., 2000) or forced swim (Meshorer et al., 2002). Being a secretory protein (Soreq and Seidman, 2001), AChE-R fulfills the extracellular function of reducing the stress-induced acetylcholine levels. In addition, however, it accumulates in neuronal cell bodies (Birikh et al., 2003; Sternfeld et al., 2000), where acetylcholine hydrolysis is an unlikely function. Intriguingly, AChE-R possesses a unique C-terminal domain, which does not participate in acetylcholine hydrolysis for which the core domain, common to all of the AChE variants is sufficient (Duval et al., 1992). This suggests for the AChE-R protein distinct stress-related signal transduction function(s).

Using a yeast 2-hybrid screen (Chien et al., 1991) we have recently shown that AChE-R forms tight, co-immunoprecipitable triple complexes with the PKC scaffold protein, RACK1 (Birikh et al., 2003).

RACK1 belongs to the family of WD proteins, which can simultaneously bind different partners to various regions in their multi-blade rings (Smith et al., 1999). For example, RACK1 interacts with β-integrin (Liliental and Chang, 1998), cAMP phosphodiesterase (Yarwood et al., 1999), phospholipase C-γ1 (Disatnik et al., 1994), src kinase (Chang et al., 2001) or the β-adrenergic receptor (Rodriguez et al., 1999). In its role as an intracellular receptor for activated protein kinase C (Ron et al., 1994), RACK1 interacts with activated PKCβII and facilitates its subcellular movement under exposure to phorbol ester, dopamine $β_2$ agonists or ethanol (Ron et al., 1999; Ron et al., 2000). Therefore, we initiated experiments aimed at exploring RACK1's distribution in the murine brain. Our findings were compatible with the assumption that RACK1 facilitates stress-induced, PKCβII accumulation associated with prolonged contextual fear responses (Birikh et al., 2003). Therefore, we have tested whether the formation of neuronal AChE-R/RACK1/PKCβII complexes tilts the balance towards extended contextual fear responses in

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transgenic mice with neuronal AChE-R overexpression and examined if this phenotype is suppressible by antisense destruction of AChE-R synthesis in non-transgenic FVB/N mice.

Materials and Methods

Laboratory animal experiments were approved by the Hebrew University's Committee for Animal Experimentation. Female FVB/N and transgenic AChE-R (TgR) mice, 6-8 weeks old, were tested as naive or injected with saline (0.2 ml, 0.9%, *i.p.*) to induce mild psychological stress. Mice were tested, deeply anesthetized with Pental (pentobarbitone sodium, CTS Chemical Industries, Petach Tikva, Israel, 100 mg/Kg) and sacrificed 24 hr post-injection. For neuroanatomical analyses, anesthetized mice were transcardially perfused with 4% (weight/vol) paraformaldehyde and processed as in (Birikh et al., 2003).

Immunohistochemical analyses were essentially as previously described using rabbit anti-AChE-R (Sternfeld et al., 2000) 1:100, and mouse anti-RACK1 (Transduction Laboratories) 1:200. Sections were incubated with the primary anti-AChE-R antibody, then with biotin-conjugated donkey anti-rabbit antibody (Chemicon, Temecula, CA, 1 hr, room temp., overnight at 2-8 °C). RACK1 staining was further preceded by trypsin type II treatment (Sigma), 1 µg/mL with $CaCl_2$ 0.001%, 0.001% soybean trypsin inhibitor (Sigma), 2 min, room temp. Detection was with horseradish peroxidase-conjugated goat anti-mouse antibody (1:100 dilution, Sigma), followed by incubation in 0.0125% diaminobenzidine, 0.05% nickel ammonium sulfate, and 0.00018% hydrogen peroxide. Anti-RACK1 pre-incubation with 10 µM RACK1 (1 h, room temp) totally eliminated anti-RACK1 staining, demonstrating specificity.

Emergence into an open field. A 9 x 10 x 11 cm tilt bin box (Arnon Caine, IL) was placed in the center of a black painted plywood 100 x 100 cm open field with 50 cm walls. A 32 x 14 cm stainless steel cage top (Techinplast, Milano, Italy) leaning on this box formed a platform reaching 5.5 cm above the open field floor. Latencies were measured for exit from the tilt-bin box to the platform and for descending from the platform to the open field floor.

Statistical analyses: Two-factor analysis of variance subjected to post-hoc analyses involved transgenic overexpression (TgR vs. FVB/N) and stress (saline injection vs. no injection).

Results

Stress induces neuronal co-accumulation of immunoreactive AChE-R and RACK1. To explore the *in vivo* relevance of AChE-R/RACK1 interactions, we tested the expression patterns of RACK1 in normal and post-stress mouse brain. Immunohistochemical analyses with anti-RACK1 antibodies demonstrated selective neuronal expression of this protein and highlighted its presence in the cytoplasm and closely proximal processes of pyramidal neurons, both in the mouse cortex and in CA1 neurons of the posterior hippocampus (Fig. 1B,1 and 3). In particular, RACK1 expression was prominent in layers 3 and 5 of the frontal and parietal cortex, in both superficial and deep layers of the piriform cortex (Fig 2), and in regions CA1-3 of the hippocampus. A subset of these neurons also over-expresses AChE-R under acute psychological stress (Kaufer et al., 1998; Kaufer et al., 1999). Moreover, RACK1 displayed stress-induced increase in parietal cortex layer 5 (compare Fig. 3, 3 to 5 and 7 to 9). AChE-R- positive neurons, like those expressing RACK1, were more prominently labeled under stress as compared with control conditions in cortical layer 5 as well as in hippocampal CA1 (Fig. 1). Unlike RACK1,

AChE-R antibodies also stained cells with glial morphology. Also, in some regions, such as hippocampal CA1, RACK1 staining formed an almost continuous pattern, whereas AChE-R was localized to a subset of the pyramidal neurons. For both AChE-R and RACK1, uneven perikaryal accumulation and increased neurite labeling were observed under stress (Fig. 3). Thus, RACK1 and AChE-R co-accumulate in large neuronal populations of the mammalian brain under psychological stress.

Increased contextual fear response in TgR mice: To investigate the physiological relevance of AChE-R/RACK1 interactions we tested the behavioral effects of stress on naive and AChE-R overproducing transgenic mice (TgR). Contextual fear response was measured for exit from a sheltered box to a higher platform. A second phase of this test involved descent from this platform to an unfamiliar, and therefore threatening open field floor. Contextual fear was manifested in repeated episodes of approach to the edge of the platform and retreat back to the box, rather then descend. The stress response associated with saline injection prolonged the increased latencies to exit and to descend from the platform to the open field floor (Table 1). At 24 hr post-injection, saline exerted main effect on exit latency [$F_{(1,33)}=7.74$; $P<0.001$], suggesting that it exerted a stress response, but had no interaction with AChE-R overexpression, compatible with the hypothesis that saline injection induces short-term AChE-R over-expression. However, TgR mice with chronic AChE-R overproduction displayed yet longer exit and descent latencies. The effect of chronic AChE-R overproduction on these responses was measured by saline injection of TgR mice. This induced a significant main effect on both exit latency [$F_{(1,33)} = 11.36, P<0.002$] and descent latency [$F_{(1,34)} = 51.8, P< 0.0001$]. Transgenic overexpression did not show an interaction with the prolonged exit latency; However, it increased descent latency following saline injection [$F_{(1,34)}= 7.03, P<0.02$]. This yielded a significant interaction of transgenic overexpression with injection stress [$F_{(1,34)} = 4.69, P<0.04$], with the outcome that saline-injected TgR mice presented greater descent latency compared to saline-injected FVB/N mice ($P<0.005$). Thus, TgR mice displayed prolonged contextual fear response following a stressful event.

Antisense suppression of the contextual fear response: To test whether the delayed descent time in stressed FVB/N mice was due to AChE-R accumulation, injected saline was replaced with the antisense oligonucleotide EN101, which attenuates AChE-R accumulation, or with the inversely oriented oligonucleotide INV102 (Brenner et al., 2003) as negative control. In the emergence test 24 h later, we observed antisense suppression of exit latency (a significant main effect, [$F_{(3,26)} = 3.35, P<0.04$]) in post hoc analysis compared to saline treatment ($P<0.03$). EN101 suppressed descent latency [$F_{(3,26)} = 2.91, P<0.05$] compared to saline treatment ($P<0.04$ post hoc analysis). In contrast, INV102-injected FVB/N mice behaved similarly to saline-injected ones, demonstrating sequence specificity for the EN101 effect. Thus, the extended contextual fear response in FVB/N mice depended on AChE-R overproduction, which (in the limbic system) we have found to be associated with RACK1 accumulation.

Discussion
In mice, the stress-induced splice variant, AChE-R, co-accumulates after mild stress with the PKC scaffold protein RACK1. This occurs in neurons of layers 3 and 5 of the entorhinal and parietal cortex, in both superficial and deep layers of the piriform cortex, and in regions CA1-3 of the hippocampus. AChE-R interacts with the scaffold protein RACK1 and through it, with its

target protein kinase PKCβII, known to be involved with fear conditioning (Weeber et al., 2000). Therefore, we tested the physiological relevance of these interactions in FVB/N mice, where mild injection stress extended contextual fear responses. Transgenic FVB/N mice overexpressing AChE-R displayed yet more extended conflict behavior, demonstrating that the extended fear response was associated with AChE-R overproduction. Moreover, EN101, an antisense oligonucleotide attenuating AChE-R accumulation, diminished this effect, suggesting causal relationship.

Our search for stress-induced neuronal signal transduction revealed that AChE-R overproduction tilts the balance of contextual fear responses away from the excitatory tendency to escape towards the inhibitory freezing phenotype. This hypothesis points to RACK1 as a novel component in the molecular cascade that controls physiological stress responses and may explain the long-known behavioral effects of neuroactive agents that modulate cholinergic neurotransmission (Muller et al., 2002; Ichikawa et al., 2002)

Behavioral antisense effects: Two lines of evidence demonstrate association between intraneuronal AChE-R and conflict behavior. First, transgenic mice overexpressing neuronal AChE-R present extended contextual fear responses in the open field-emergence test; second, EN101, but not a negative control agent, suppressed this accumulation, sustaining a normal emergence phenotype in injected FVB/N mice.

The *in vivo* efficacy in the brain of the peripherally administered EN101 antisense agent was a surprise, although we had recently shown that EN101 reduces AChE-R mRNA and AChE-R protein levels in the brain and suppresses stress-induced locomotor impairments (Cohen et al., 2002). To suppress AChE-R-induced contextual fear responses following injection stress, at least part of the injected EN101 should first penetrate the blood-brain barrier. 2-O-methyl protection of EN101 promotes such penetrance: minor, but detectable, levels of peripherally administered 2-O-methyl protected oligonucleotides were observed by imaging techniques in the brain of rhesus monkeys (Tavitian et al., 1998). Once in the brain, EN101 should induce sufficient destruction of AChE-R mRNA to reduce AChE-R below a threshold level and induce a contextual fear response. Based on a molecular weight of 6×10^3 and an estimated 30 μl free extracellular volume for the murine brain, 10 ng (0.1% of the injected 10 μg/mouse) yields 50 pM. This is within the maximal range for inducing AChE-R mRNA destruction, yet low enough to avoid non- specific upregulation of AChE gene expression (Galyam et al., 2001; Sazani et al., 2002).

In TgR females, the chronic excess of AChE-R, RACK1 and PKCβII, attributes to AChE-R interactions, a contextual fear effect that likely reflects combined contribution of strain and sex-specific properties (Ohno et al., 2001; Trullas and Skolnick, 1993). That the extended emergence latency of FVB/N mice was attenuated by antisense suppression of AChE-R production, and that TgR displayed yet longer latency to emerge, compared to mice from their parent strain, FVB/N, supports this interpretation; The mild stress employed and the "prophylactic" treatment mode likely contributed to this behavioral response.

RACK1 labeling highlights neural circuits that modulate stress responses. In the deep cortical layers, stratum oriens and stratum radiatum of CA1 hippocampal neurons, RACK1 may facilitate the transduction of signals across synapses between axons coming from outside the region. In

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neuronal cell bodies, RACK1 staining only partially overlaps the AChE-R labeling patterns, suggesting relevance of each of these two proteins to both distinct and common functions.

Mapping AChE-R-filled neurons with both punctiform PKCβII and RACK1 labeling revealed neuronal pathways associated with inhibitory behavior (Herman and Cullinan, 1997). These were located, as expected, in the stress-responsive cortical upper layers (Feng et al., 2001; Hamner et al., 1999), hippocampal CA1 region (Gould and Tanapat, 1999), lateral septum and basolateral amygdala (Amorapanth et al., 2000; Garcia et al., 1999). The amygdala, considered excitatory under fear and anxiety, also includes stress-inhibitory neurons (Dudai, 2003; Shumyatsky et al., 2002). These presumably suppress the stress-excitatory basolateral amygdala neurons; therefore, their intensified labeling is consistent with the limited stress-related neuropathology hallmarks in the brain of TgR mice (Sternfeld et al., 2000).

AChE-R and/or PKCβII accumulation may also be relevant for impairments in numerous processes where fear conditioning is involved. In particular, this refers to processes in which PKCβII plays a major role, e.g. post-traumatic stress disorders (Newport and Nemeroff, 2000), panic attacks (Battaglia, 2002), or post-stroke phenomena.

Each of the components of the triple AChE-R/RACK1/ PKCβII complexes represents one out of several variants. AChE-R is one out of three AChE splice variants, each with its own C-terminal peptide and demonstrated different interactions. RACK1 interacts with PKCε as well (Besson et al., 2002), whereas other PKC variants may interact with different shuttling proteins. This would exert distinct effects on the complex physiological phenomena that follow traumatic experiences. Finally, RACK1 operates as a shuttling vector to many other proteins. Further screening efforts should shed more light on the potential participants in this interaction.

In conclusion, the intracellular interaction of the stress-induced AChE-R variant with RACK1 and PKCβII adds a new dimension to our understanding of the interrelationship between cholinergic gene expression and the complex phenotype of contextual fear responses.
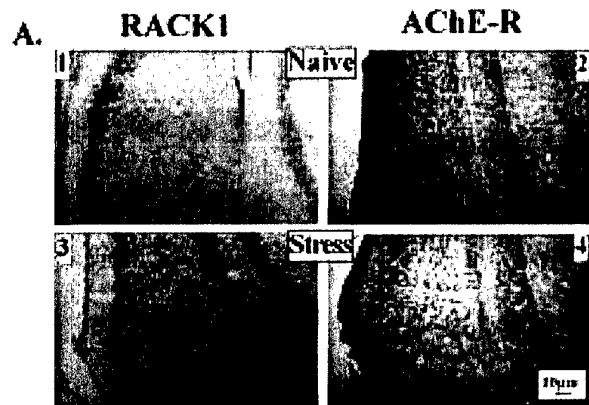
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A.

Fig. 1. RACK1 overexpression under stress in AChE-R positive entorhinal cortex and CA1 neurons

(A) Shown are entorhinal cortex sections stained with anti-RACK1 (left) or anti-AChE-R antibodies (right). (B) CA1 hippocampal sections stained with anti-RACK1 (left) or anti-AChE-R antibodies (right). RACK1 labeling of hippocampal CA1 neurons increases to include proximal processes following stress (inset).
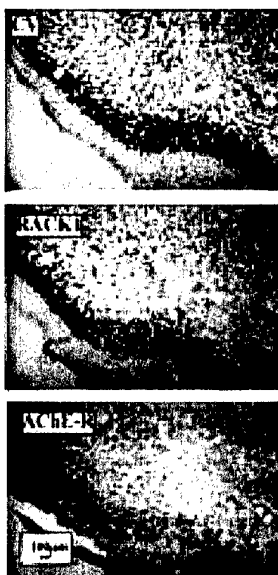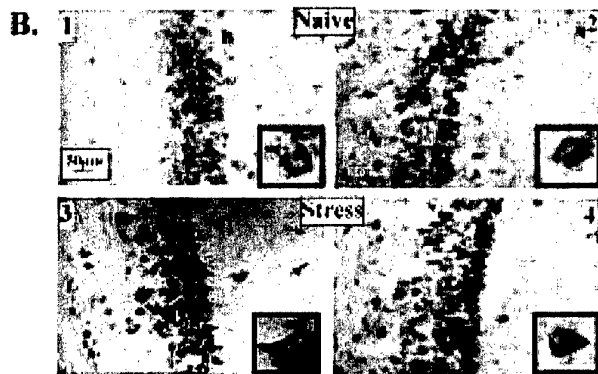
B.

Fig. 2. RACK1 and AChE-R co-expression in piriform cortex
Shown are piriform cortex sections stained with cresyl violet (top) or with anti-RACK1 (middle) or anti-AChE-R antibodies (bottom).
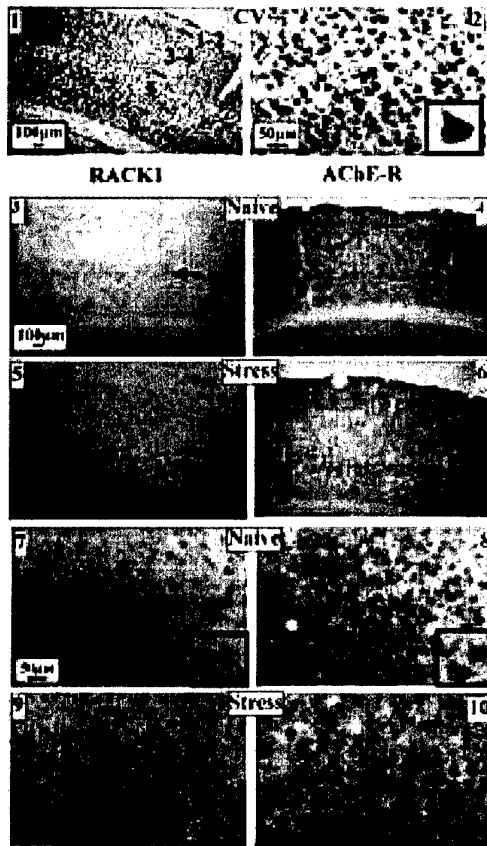

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Fig. 3. RACK1 and AChE-R co-overexpression in parietal cortex and CA1 neurons under stress

Shown are parietal cortex sections stained with cresyl violet (1,2, top) or with anti-RACK1 (left) or anti-AChE-R antibodies (right). Note asymmetric labeling patterns of both proteins in the cytoplasm and proximal processes of individual pyramidal neurons in the parietal cortex and hippocampus CA1 (insets). RACK1 and AChE-R expression increases in layers 5 (arrows) of the parietal cortex under stress.

Table 1: Post-stress exit and descent latencies into an open field.

| | FVB/N | | TgR | |
	exit	descent	exit	descent
Naïve	23.5±3	111.5±25.9	20.62±2.4	93.37±13.15
Saline	65.67±28.6 $P>0.05$ $P>0.03$	323.83±74 $P>0.06$ $P>0.04$	34.63±5 $P>0.002$	783.17±116.46 $P>0.001$
EN101	17.34±3.4	64.67±18.6	ND	ND
INV102	33.17±10.2	281.5±170.9	ND	ND

Latency times (sec) of exit from a box following contextual habituation time, and descent over a tilted cage cover into an open field ($n= 6$ naive and 6 saline-injected FVB/N mice). Experiment was repeated 24 hr post-injection with EN101 or the inverse INV102 oligonucleotides and with transgenic mice, whch overexpress AChE-R.

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Stress-induced alternative splicing of acetylcholinesterase results in enhanced fear memory and long-term potentiation

Acute stress intensifies the evolutionarily advantageous memory of events that are potentially threatening to the organism (Kim and Diamond, 2002). A major challenge in neurocognition is to identify molecular mechanisms that underlie the enhanced formation of memory following stress exposure. The hippocampus is a critical component of the neuroanatomical stress circuit (McEwen, 1994), which is also involved in forming episodic, spatial and contextual memories (Huerta et al., 2000; Jensen and Lisman, 2000; Phillips and LeDoux, 1994; Rolls et al., 2002; Wilson and McNaughton, 1993). Contextual fear conditioning, a procedure in which an animal learns to associate the neutral context of the training chamber with an aversive foot shock involves the hippocampus, which participates in the storage of the memory representation of the context (Barrientos et al., 2002; Moita et al., 2003; Phillips and LeDoux, 1994; Sanders et al., 2003). Stress-induced changes in hippocampal functioning require protein and RNA synthesis (Xu et al., 1998), are associated with differential expression of immediate early genes, e.g. c-fos (Cullinan et al., 1995), and involve alternative splicing of numerous transcripts, including K^+ channels (Kaufer et al., 1998) and acetylcholinesterase (AChE) (Meshorer et al., 2002). Unlike the abundant AChE-S "synaptic" variant, the stress-induced AChE-R possesses a hydrophilic C-terminus that is expected to incapable of supporting adherence. Neuronal AChE-R accumulation is accompanied by long-lasting hyperexcitation of glutamatergic activity (Cohen et al., 2002) and prolonged conflict behavior (Birikh et al., 2003). To explore the possibility that changes in alternative splicing are critically involved in the stress-induced consolidation of fear memory, we tested the potential interrelationship between neuronal overproduction of the "readthrough" AChE-R variant (Soreq and Seidman, 2001), stress-enhanced fear memory and facilitated long-term potentiation (LTP) in hippocampus (Blank et al., 2002). Here, we report that in murine hippocampus, the stress-induced overproduction of AChE-R mRNA underlies enhanced contextual fear memory by facilitating synaptic plasticity in a process, which involves the interaction of PKCβII with the C-terminal peptide of AChE-R.

Results
Immobilization stress induces transient alternative splicing of AChE in hippocampal neurons: Staining of hippocampal CA1 neurons from BALB/c mice with an anti-AChE-R antibody (Sternfeld et al., 2000) revealed significantly increased labeling intensity 1, 2 and 3 h after 1 h immobilization as compared to naïve mice (Fig. 1a and b), compatible with the rapid post-stress increase of hippocampal AChE activities (Kaufer et al., 1998). Staining was maximal 2 h after immobilization and returned to baseline within 24 h (Fig. 1b), demonstrating the transient nature of this response.

Enhanced memory of contextual fear depends on stress-induced AChE-R elevation: Following immobilization stress, RT-PCR analysis demonstrated a 3-fold increase of AChE-R mRNA, using HPRT mRNA as control (Fig. 2a). To study the implications of AChE-R mRNA up-regulation for fear conditioning, we employed mEN101, an antisense oligonucleotide inducing AChE-R mRNA downregulation (Cohen et al., 2002). When intracerebroventricularly (i.c.v.) injected 15 min before immobilization, mEN101 selectively limited the stress-induced accumulation of AChE-R mRNA and protein to less than half of its full scale (Figs. 2a and b and 3). In contrast, neither mEN101 nor the inversely oriented oligonucleotide invEN101 or the vehicle alone had any effect on AChE-S mRNA or the non-relevant mRNA encoding the homologous protein butyrylcholinesterase (BuChE) (Figs. 2a and b). Likewise, invEN101 or vehicle did not affect the level of the AChE-R mRNA or protein as tested in RT-PCA and immunoblot analysis of hippocampal homogenates (Figs. 2a and b and 3).

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mEN101 diminishes whereas the C-terminal peptide of mouse AChE-R intensifies contextual fear: We have recently found that contextual fear conditioning was elevated if mice were trained 2 and 3 h after exposure to a stressful stimulus and assessed for memory 24 h later (Blank et al., 2002). Our index of memory was the conditioned freezing response, absence of all movement except for respiration and heart beat. Freezing was shown in mice returned to the training context in which they were previously exposed to a footshock (Phillips and LeDoux, 1992). Injection of mEN101 totally prevented the increased freezing response after stress, unlike invEN101 or vehicle injection that were ineffective (Fig. 4).

A synthetic version of the C-terminal peptide unique to human AChE-R, hARP, has been shown to mimic the stress effect on proliferation of myeloid progenitor cells (Grisaru et al., 2001). Therefore, we analysed whether elevated contextual fear observed under AChE-R overexpression could be mimicked by mARP, a synthetic peptide with the sequence of the mouse AChE-R C-terminus. FVB/N mice were microinjected with rhodamine labeled mARP to the lateral ventricle. Cortical and hippocampal neurons near the injection site displayed rhodamine fluorescence signals, likely reflecting ARP diffusion or local uptake (Fig 5a and b). However, neurons in areas distant from the injection site, as the parietal cortex and the basal forebrain were stained as well, suggesting endocytosis and retrograde transport of ARP (Fig 5c to e). Alternative labeling group did not affect the neuronal accumulation of ARP (Fig 5f). Moreover, a negative control peptide representing the C terminus of murine AChE-S (mASP) displayed a different labeling pattern. While mARP is localized in the cytoplasm, mASP accumulated in neuronal nuclei (Fig 5g). Thus, injected mARP demonstated a potential capacity to transduce signals to neuronal cytoplasmic element(s). At the behavioral level intrahippocampal (i.h.) mARP injection (2 nM) 2 h before conditioning resulted in an elevated freezing response when mice were re-exposed to the conditioning context as compared to vehicle-treated controls. As a negative control peptide we used the insect Pheromone Biosynthesis Activating Neuropeptide (PBAN), with a molecular weight similar to mARP. PBAN, which had no effect on myelopoietic proliferation (Grisaru et al., 2001) also showed no effect on contextual fear conditioning (Fig. 4).

The elevated fear response involves enhanced hippocampal LTP: To test if AChE-R mRNA and protein up-regulation after acute stress enhance conditioned fear by altering synaptic plasticity, we measured hippocampal long-term potentiation (LTP). In the present experiments, the Schaffer collateral-pathway was stimulated to record theta-burst induced LTP (TBS-LTP) from the CA1 stratum radiatum of hippocampal slices from stressed animals 2 h after 1 h immobilization. TBS-LTP was enhanced when compared to the response in brain slices from naïve animals. Facilitation of TBS-LTP was not detectable in slices from stressed mice pretreated with mEN101, whereas invEN101 was ineffective in blocking stress-mediated LTP facilitation (Figs. 6a and b).

Persistent AChE-R overexpression induces a long-lasting upregulated LTP: Repeated forced swim episodes promote the dendritic translocation of hippocampal AChE-R mRNA in a long-lasting manner (Meshorer et al., 2002). This finding raised the question if persistent AChE-R up-regulation may contribute to enduring LTP facilitation and if such facilitation would persist in the maintenance phase, thought to be particularly relevant for long-term memory consolidation (Stanton, 1995). Therefore, we used high-frequency tetanic stimulation (HFS) to induce LTP in the stratum radiatum of naïve FVB/N transgenic mice overexpressing human AChE-R (Birikh et al., 2003; Sternfeld et al., 2000). LTP was stably elevated by 40% even 100 min after LTP induction when compared to LTP of the parent strain mice (Fig. 6c).

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Enhanced PKC signal transduction in AChE-R-overexpressing mice: Neuronal AChE-R was recently found to tightly interact, through the scaffold protein RACK1, with PKCβII, the alternative splicing product of PKCβ, to increase its enzymatic activity and enlarge its density in hippocampal neurons (Birikh et al., 2003). In the hippocampus of AChE-R-overexpressing transgenic mice immunohistochemical labeling of areas rich in PKCβII appeared as punctiform staining of higher cluster densities than in strain-matched FVB/N controls at the circumference of CA1 and dentate gyrus hippocampal neurons (Figs. 7a and b). Confocal microscopy revealed in CA1 hippocampal neurons from these transgenic mice enlarged intracellular clusters composed of both AChE-R and PKCβII (Fig. 7c). This finding was compatible with the assumption that the elevated LTP maintenance in transgenic mice may require interaction between AChE-R and PKCβII. We therefore investigated by activating PKC with phorbol di-butyrate (PDBu, 5 μM) whether the intensified staining of PKCβII correlated with enhanced PKC signaling in AChE-R transgenic mice. Indeed, 20 min administration of PDBu facilitated the synaptic field potential more dramatically in hippocampal slices from AChE-R transgenic mice than from control FVB/N mice (Fig. 7d). Further tetanic stimulation (20 min after PDBu wash) did not result in a significant additional potentiation, confirming that the synapses were fully potentiated by PKC activation in both WT and AChE-R Tg mice in accordance with previous studies, (for example (Stanton, 1995)). The observed enhanced potentiation of synaptic transmission after phorbol ester treatment and tetanic stimulation in transgenic slices suggest that excess AChE-R leads to an increased strengthening of CA1 synapses due to higher PKC activity.

DISCUSSION

This study provides evidence for a tight linkage between stress-induced alternative splicing in the hippocampus and the corresponding facilitation of fear conditioning. At the level of neuronal physiology, our study is compatible with the assumption that hippocampal LTP participates in the formation of contextual fear following a stressful experience.

Immunohistochemical staining indicated maximal expression of the stress-associated AChE splice variant AChE-R 2 h after immobilization. Mice conditioned at this time point revealed highest levels of contextual fear during the retention test. This fear enhancement was prevented by AChE-R mRNA antisense treatment, attributing a central role in stress-mediated contextual fear conditioning to AChE-R. In both acutely stressed and animals persistently overexpressing AChE-R, LTP was intensified. However, HFS-LTP could only be elicited in slices from AChE-R transgenic mice, but not in slices from stressed mice where it was inhibited (Blank et al., 2002). This implies that additional components, other than AChE-R participate in impeding HFS-LTP in slices from stressed mice. The involvement of additional, yet unidentified proteins in fear memory consolidation is further indicated from the alternative pre-mRNA splicing at the ERK-MAP kinase pathway (Schafe et al., 2000), which is activated during fear conditioning (Sananbenesi et al., 2002; Schafe et al., 2000).

The core domain of the AChE-R protein suffices for up-regulating acetylcholine hydrolyzing activity (Massoulie et al., 1999), whereas its C-terminal peptide does not possess such activity (Soreq and Seidman, 2001). Our finding that the synthetic peptide with the sequence of the mouse AChE-R C-terminus, mARP, mimicked the stress-associated effect of AChE-R demonstrates that the C-terminal domain is sufficient for promoting fear memories. In our current study, we indeed present endocytosis and active intracellular distribution of ARP. Thus, the involvement of AChE-R in eliciting fear memory emerges as a non-enzymatic activity, possibly an intracellular one. Interestingly, the half-life of both neuronal AChE-R mRNA and its protein product appeared to be particularly short (compatible with Chan et al., 1998 in hematopoietic cells), as would be expected of a modulator protein that is responsible for adjusting the individual's responses to a changing environment. The enhanced enzymatic

AChE activity found shortly after stress (Kaufer et al., 1998) thus appears to be mainly responsible for clearing the elevated levels of acetylcholine released after acute stress (Stillman et al., 1997). Because the C-terminus of AChE-R interacts intracellularly with the scaffold protein RACK1 and through it with PKCβII (Birikh et al., 2003), PKCβII might also contribute to enhanced fear conditioning after stress. In agreement, contextual fear conditioning of rats is associated with activation of hippocampal PKC and the translocation of PKCβII from the cytosol to the membrane (Young et al., 2002), and genomic disruption of mouse PKCβII causes inherited deficits in contextual fear responses (Weeber et al., 2000). However, PKCβII-deficient mice present apparently normal robust hippocampal LTP, albeit less sensitive to phorbol ester than that of control mice (Weeber et al., 2000). Thus, AChE-R-inducible PKCβII may serve as an important, but dispensable LTP modulator.

In summary, these data suggest that alternative splicing eliciting the accumulation of AChE-R and PKCβII is used by the hippocampus for mediating the effects of stress on fear conditioning and neuronal plasticity. It has been proposed that the neural mechanisms mediating adaptive fear are of clinical significance (Bouton et al., 2001; Gorman et al., 2000; Rosen and Schulkin, 1998). Thus, dysfunction of AChE-R or PKCβII or both are expected to be relevant to pathological conditions such as posttraumatic stress disorder (PTSD) and mood disorders.

Methods
Immunohistochemistry
Animals were deeply anaesthetized and transcardially perfused at several time-points (0, 1, 2, 3 or 24 h) after the end of the stress session. After elimination of endogenous peroxidase activity and a preincubation step, 50 μm-thick coronal sections were incubated at 4 °C with the rabbit anti-ARP antibody (1:600) for 48 h. Subsequently, sections were incubated with a biotinylated goat anti-rabbit antibody (1:200; Vector ABC kit) and with the ABC complex (Vector ABC kit). For visualization, DAB was used as a chromogen (Sigma fast tablet set). Sections were examined using light microscopy. The anteroposterior (AP) coordinates relative to bregma of the areas (Franklin and Paxonos, 1997) included for detailed analysis were AP −1.34 (Nijholt et al., 2002).

Western Blotting
Hippocampi were dissected out and immediately homogenized at 4 °C with a plastic homogenizer in homogenization buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 4 mM EGTA, 15 mM sodium phosphate, 100 mM β-glycerophosphate, 10 mM sodium fluoride, and a protease inhibitor cocktail tablet (Boehringer Mannheim, Germany). The insoluble material was removed by centrifugation at 13,000 x g for 10 min at 4 °C. Protein concentrations were determined by the Bradford method (BioRad, Munich, Germany). Equal amounts of protein for each group were separated on a 10% SDS gel and transferred to an Immobilon-P membrane (Millipore Corporation, Bedford, MA). The blot was probed using an anti-ARP antibody (Sternfeld et al., 2000). Western blots were developed using the chemiluminescence method.

Antisense oligonucleotides
mEN101 is a 20-mer oligodeoxynucleotide (5'-CTGCAATATTTTCTTGCA*C*C*-3', stars denote 2'-oxymethyl groups) complementary to a sequence in exon E2 of mouse AChE mRNA (Cohen et al., 2002). InvEN101 is the inverted sequence (negative control oligodeoxynucleotide). 5 μM oligodeoxynucleotides were combined with 13 μM of the lipophilic transfection reagent DOTAP (Boehringer Mannheim, Germany) in an artificial cerebrospinal fluid (aCSF) and incubated for 15 min at 37 °C prior to injection (of 25 ng in 1 μl).

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Cannulation
Double guide cannulae (C235, Plastics One, Roanoke, VA) were implanted into both lateral brain ventricles, using a stereotactic holder at Bregma AP 0 mm, lateral 1 mm, depth 3 mm. Alternatively, cannulae were directed toward both dorsal hippocampi, AP −1.5 mm, lateral 1 mm, depth 2 mm (Franklin and Paxonos, 1997). Bilateral injections were performed using an infusion pump (CMA/100, CMA/Microdialysis, Solna, Sweden) at a constant rate of 0.33 μl/min. Cannula placement was verified *post hoc* in all mice by injection of methylene blue. For electrophysiological experiments double cannula placement was verified by unilateral methylene blue injection (Blank et al., 2002).

Peptide
Mouse (m)ARP (GRRMEWGEQGMHKAARVGRRGERWGAKHRV) was synthesized manually on 0.05 mmol of the 5-(4-*N*-Fmoc-aminomethyl-3.5-dimethoxyphenoxy)valeryl (PAL) substituted polyethyleneglycol-polystyrene resin (Applied Biosystems) using *N*-α-Fmoc protected amino acids with the following side-chain protection: Arg(Pbf), Asn(Trt), Gln(Trt), Glu(OtBu), His(Trt) and Trp(Boc) obtained from Novabiochem. Couplings were carried out with 4-fold excess of 9-fluorenylmethoxycarbonyl (Fmoc) amino acid in the presence of 1 equivalent of 1,3-diisopropylcarbodiimide (DIC, Aldrich) and 2 equivalents of hydroxybenzotriazole (HOBt, Novabiochem) in freshly redistilled *N,N*-dimethylformamide (DMF). Coupling was monitored by bromophenol blue staining. The peptide was cleaved from the resin with 95/2.5/2.5 (v/v/v) trifluoroacetic acid/water/triisopropylsilane for 4 h and products were purified by preparative reverse-phase HPLC on a Vydac C8 column using a gradient of acetonitrile in 0.1% aqueous TFA. The purified product was characterized by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Voyager Mass Spectrometer using α-cyano-4-hydoxycinnamic acid matrix: mass calculated 3543.8, mass found 3544.2. PBAN (Grisaru et al., 2001) was from Phoenix Pharmaceuticals (Belmont, CA).

Fluorescent peptide microinjection
All surgical treatments were performed under pentobarbital-sodium anesthesia. Hamilton syringe was directed toward the right lateral ventricle, using a stereotactic holder at Bregma AP 0.1 mm, lateral 1.2 mm, depth 2.2 mm. Injections were performed manually at a constant rate of ca. 0.33 μl/min. 5 h were allowed for endocytosis and peptide transport before sacrifice period, followed by transcardially perfusion with 4% paraformaldehyde. 10 mm coronal brain sections were cut with a crayostat. Labeled peptides (NEN, Zaventhen, Belgium) were RITC-ARP (GRRMEWGEQGMHKAARVGRRGERWGAKHRV) and FITC-ASP (DTLDEAERQ WKAEFHRWSSYMVHWKNQFDHYSKQDRCSDL). Sections were inspected by means of confocal or fluorescent microscopy. Biotinylated ARP was detected by rhodamine-labeled streptavidin, of 6 h streptavidin incubation.

Drug treatment
mARP and PBAN were dissolved in aCSF solution and 0.25 μl of a 100 μM solution were injected per side.

Immobilization stress
An acute immobilization stress of mice consisted of taping their limbs to a plastic surface for 1 h (Smith et al., 1995).

Fear conditioning
The single training trail consisted of placing the animal in a novel context (180 s), and administering a tone (30 s, 10 kHz, 75 dB SPL, pulsed 5 Hz) followed by single footshock (US, 0.7 mA, 2 s, constant current). Under these conditions, the context served as background

stimulus. Background contextual fear conditioning but not foreground contextual fear conditioning, where the tone is omitted during training, has been shown to involve the hippocampus (Phillips and LeDoux, 1994). Freezing was recorded 24 h later in the same fear conditioning box for 180 s without tone presentation (Blank et al., 2002).

Slice preparation and electrophysiology (Blank et al., 2002)
Hippocampal slices were obtained from 3 to 6 month-old male BALB/c, FVB/N or transgenic mice. Animals were sacrificed by cervical dislocation. Brains were rapidly removed and placed in cold (2 °C) artificial CSF (aCSF) consisting (in mM) of 124 NaCl, 5 KCl, 26 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 1.3 MgSO$_4$, 2.4 CaCl$_2$, and 10 glucose (equilibrated with 95% O$_2$/5% CO$_2$). A bipolar, stimulating electrode placed in the stratum radiatum of the CA3 region of the slice (400 μm) was used to activate Schaffer collateral/commissural fiber synapses onto CA1 pyramidal cells. Extracellular field potentials were recorded with an aCSF-filled glass microelectrode placed in the stratum radiatum (electrode resistance up to 5 MΩ). Presynaptic fiber stimulation was set to evoke baseline fEPSPs ~50% of the maximal fEPSP amplitude. HFS-LTP was induced by three trains of 1 s, 50 Hz tetanic stimulation with 20 s inter-train interval, pulse width doubled to 0.1 ms in the tetanus. TBS consisted of 5 x 100 Hz bursts (five diphasic pulses per burst) with a 200 ms interburst interval. All values are reported as mean ± SEM of all slices tested in the corresponding paradigm.

Fluorescence double labeling for confocal microscopy of PKCβII and ARP
The primary staining solution contained 0.3% Triton X-100, 0.05% Tween 20, 2% normal goat serum, 2% normal donkey serum, rabbit anti-ARP (1:100), and mouse-anti-PKCβII (Sigma), diluted 1:500. Secondary antibody solutions and preparation for microscopy were as detailed (Birikh et al., 2003). Slices were scanned by using a Bio-Rad MRC-1024 scanhead (Hemel Hempsted Herts.,U.K.) coupled to an inverted Zeiss Axiovert 135M microscope with a 40X oil immersion objective (N.A. 1.3). Excitation was at 488 nm (using 10% of a 100 mW laser). Fluorescence emission was measured by using a 580df32 bandpass interference filter (580 nm ± 16 nm) for detecting tetra-methyl-rhodamine and a 525/40 filter for detecting fluorescein. The confocal iris was set to 3 mm. Conditions of scanning took into consideration the overlap of fluorescein fluorescence with the rhodamine filter (as was determined by control experiments). Images were then further processed with Image Pro Plus 4.01 program (version 4.0, Media Cybernetics, Silver Spring, MD).

Statistics
Statistical comparisons were made by using unpaired Student's *t*-test and ANOVA. Significance was determined at the level of $P < 0.05$ or 0.01.
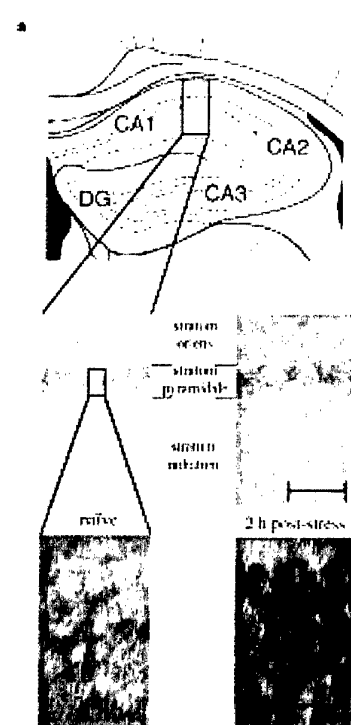
Fig.1. Acute immobilization stress induces AChE-R upregulation in hippocampal CA1 neurons. (a) Top, schematic representation of the analyzed hippocampal brain area. Middle, shown is AChE-R immunoreactivity in 50 μm coronal sections from the hippocampal CA1 area of naïve (left) and stressed (2 h after 1 h immobilization) mice (right; scale bar = 100 μm). Bottom, higher magnification images of the framed regions (scale bar = 25 μm). (b) Bars represent densitometric analysis (mean ± SEM; both hippocampi of *n* = 5 per group) of AChE-R-positive cells in the hippocampal CA1 area as seen in the middle segment under (a). Statistically significant differences: * $P < 0.05$ vs naïves.
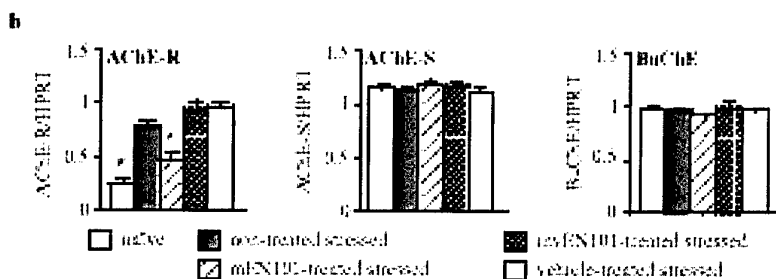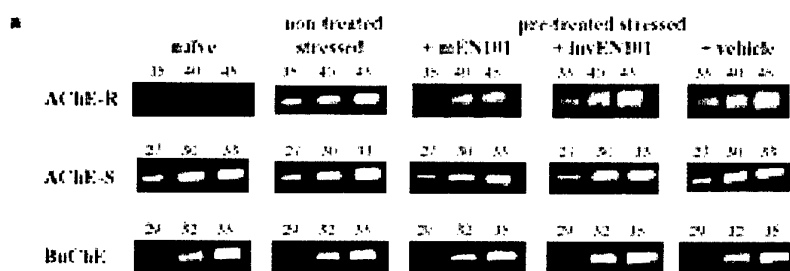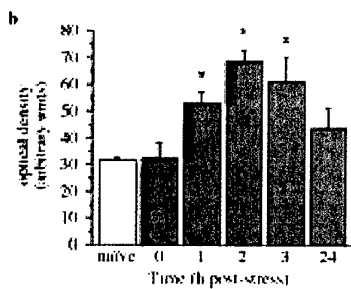

Fig. 2. Antisense prevention of stress-induced AChE-R mRNA accumulation. RT-PCR analysis was performed on RNA extracted from the hippocampus of naïve or stressed mice, and from the hippocampus of mice that were injected 15 min before the stress session with the antisense agent (mEN101) that induces destruction of AChE-R mRNA, or with an inversely oriented sequence (invEN101) or vehicle. Each reaction mixture contained a set of primers specific for

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the cDNA of hypoxantine-phosphoribosyl-transferase (HPRT), an enzyme constitutively expressed at a low and constant level in the central nervous system, and widely used as internal control. (a) Bands reflect levels of AChE-R mRNA, AChE-S mRNA and BuChE mRNA 2 h after the end of stress exposure. Numbers above each band indicate cycle number. (b) Bar graphs show the ratio of mRNA band intensities calculated from densitometric analysis of a single cycle (AChE-R: 45, AChE-S: 33, BuChE: 35) verified to be within the linear range of product accumulation, divided by those of the co-amplified HPRT product (mean ± SEM; 9-11 mRNA samples per group). Statistically significant differences: * P < 0.01 vs stressed animals.
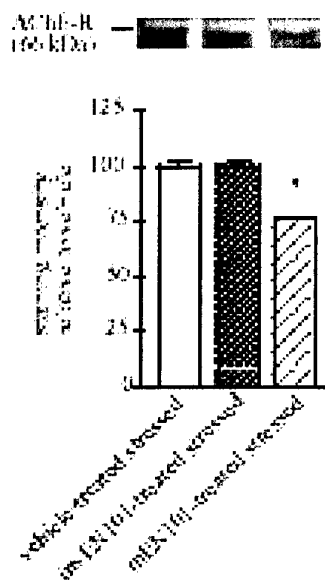

Fig. 3. Down-regulation of post-stress AChE-R protein levels by antisense treatment. Shown is a representative immunoblot reflecting AChE-R protein levels in hippocampi homogenates from stressed animals injected with mEN101, invEN101 or vehicle 15 minutes before stress exposure. Hippocampi were removed and homogenized 2 h after the end of the stress session. Bars represent mean band intensities ± SEM for hippocampal homogenates ($n = 5$, *P < 0.001).
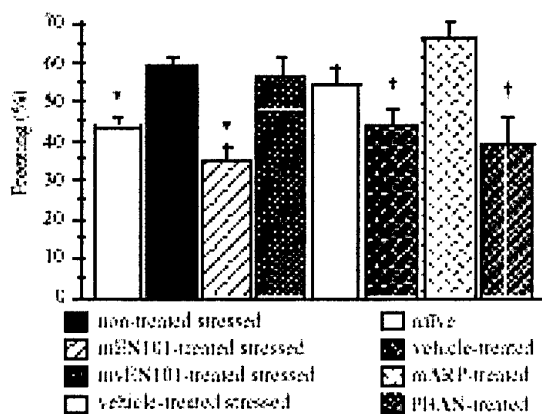

Fig. 4. AChE-R elevation enhances contextual fear conditioning. Naïve (n = 27), stressed (n = 25) and mice injected i.c.v. 15 min before stress exposure with mEN101 (n = 11), invEN101 (n = 11) or vehicle (n = 9), were trained 2 h after the end of the stress session or 2 h after intrahippocampal injection of vehicle (n = 8), mARP (n = 8) or PBAN (n = 5) in the context-dependent fear conditioning paradigm. Freezing was measured in the retention test performed 24 h after training. Statistically significant differences: *P < 0.01 vs stressed animals, $^{\dagger}P$ < 0.01 vs mARP treated animals.
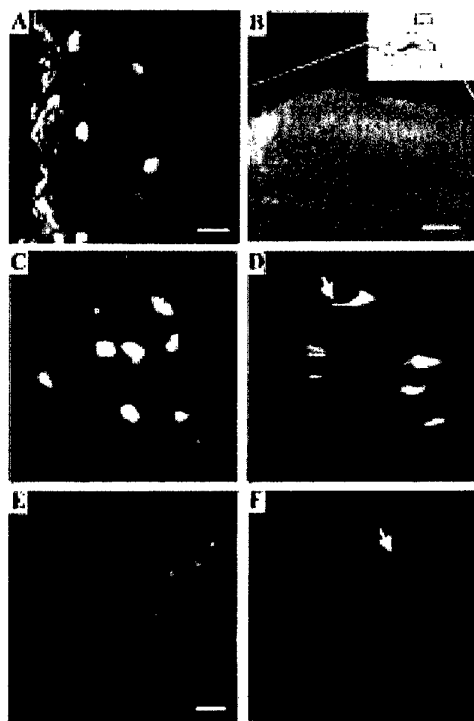
Fig. 5. Neuronal endocytosis and retrograde transport of mARP microinjected into brain. A. Confocal brain section of cortex in which the injection needle penetrated through (arrowheads). Near by cells are stained for RITC labeled ARP, probably via peptide diffusion. B. Fluorescence microscope analysis of distant labeled areas from the injection site, for example the parietal cortex. C-D display confocal microscope samples of stained cells from the basal forebrain; olfactory tubercle (C) and nucleus basalis of Meynert (D). Note neural processes labeling with mARP (arrowhead). E. Biotin and FITC labeled mARP of simultaneous injection to the same animal (biotin is visualized with RITC streptavidin). In the nucleus basalis of Meynert, double labeled cells appear yellow. F. Simoultaneous injection of RITC labeled mARP and FITC labeled mASP. While mARP is localized in the cytoplasm, mASP is localized in the nucleus. Note a cell labeled solely for RITC mARP (arrowhead). Scale bars: A,C,D,F- 25 μm; B- 200 μm; E-100 μm.
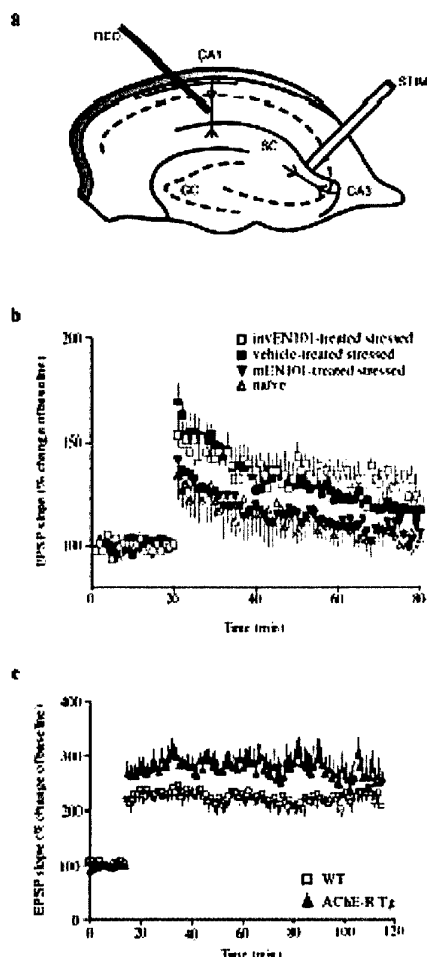

Fig. 6. AChE-R facilitates LTP induction and maintenance in the mouse hippocampus. (a) Setup for hippocampal extracellular field recordings (REC.) in the CA1 stratum radiatum upon stimulation (STIM.) of the Schaffer collateral pathway (SC). (b) TBS-LTP elicited in slices from mice that were sacrificed 2 h after 1 h immobilization (■) was significantly enhanced when compared to LTP induced in slices from naïve mice (Δ; 7 slices, 5 mice; $P < 0.001$). There was no statistical difference between stressed mice and mice that were pre-injected with vehicle before stress exposure (6 slices, 5 mice) (data not shown). The stress-induced enhancement was significantly attenuated when stressed mice were pre-injected with mEN101 (▼; 5 slices, 5 mice; $P < 0.001$). InvEN101 pre-injection had no effect on stress-induced LTP facilitation (□; 6 slices, 5 mice). (c) Chronic effect of AChE-R overexpression. Shown are fEPSP changes from baseline (%) of hippocampal slices from transgenic mice (Tg; ▲; 9 slices, 6 mice) reaching a value of 272 ± 10% potentiation extent, significantly higher than the 228 ± 8% potentiation extent in age and strain-matched FVB/N control mice (□; 12 slices, 9 mice, $P < 0.05$).
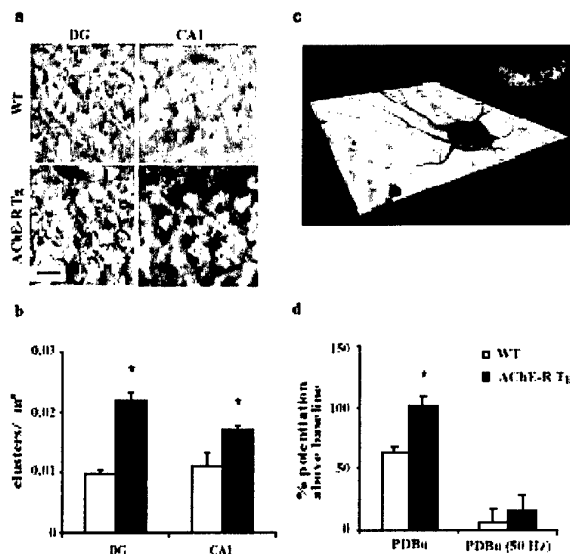
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Fig. 7. AChE-R facilitates LTP induction and maintenance in the mouse hippocampus. (a) Setup for hippocampal extracellular field recordings (REC.) in the CA1 stratum radiatum upon stimulation (STIM.) of the Schaffer collateral pathway (SC). (b) TBS-LTP elicited in slices from mice that were sacrificed 2 h after 1 h immobilization (■) was significantly enhanced when compared to LTP induced in slices from naïve mice (Δ; 7 slices, 5 mice; $P < 0.001$). There was no statistical difference between stressed mice and mice that were pre-injected with vehicle before stress exposure (6 slices, 5 mice) (data not shown). The stress-induced enhancement was significantly attenuated when stressed mice were pre-injected with mEN101 (▼; 5 slices, 5 mice; $P < 0.001$). InvEN101 pre-injection had no effect on stress-induced LTP facilitation (□; 6 slices, 5 mice). (c) Chronic effect of AChE-R overexpression. Shown are fEPSP changes from baseline (%) of hippocampal slices from transgenic mice (Tg; ▲; 9 slices, 6 mice) reaching a value of 272 ± 10% potentiation extent, significantly higher than the 228 ± 8% potentiation extent in age and strain-matched FVB/N control mice (□; 12 slices, 9 mice, $P < 0.05$).

Anxiety scores in the HERITAGE Family Study associate with polymorphisms and expression variabilities in the acetylcholinesterase locus

Anxiety is a ubiquitous and unavoidable experience of life, defined as a feeling of fear that is out of proportion to the nature of the threat (Weinberger, 2001). Anxiety disorders are the most prevalent of psychiatric disorders (Lepine, 2002), which led to an extensive search of the mechanisms and/or genomic elements underlying these phenomena. Several genes were reported to be potential contributors to the genetic variance of anxiety-related traits or modifiers of the phenotypic expression of pathologic anxiety. (Hariri et al., 2002) However, molecular genetics and/or biochemistry so far failed to identifiey variation(s) that are consistently associated with the increased psychologically-measurable susceptibility to anxiety feelings in generally healthy subjects.

Variance in personality traits, is often considered to involve complex interactions of environmental and experience-derived factors with several gene products and neurotransmission circuits, e.g. serotonergic (Stein et al., 2002), GABAergic and cholinergic (Degroot and Treit, 2003). Neurotransmission mediated by acetylcholine (ACh) in particular contributes to numerous physiological functions (Borovicka et al., 1997) as well as to memory, learning and panic responses (Battaglia, 2002; Everitt and Robbins, 1997). Anxiety provokes cholinergic hyper-arousal, e.g. sweating, intestinal or gastric constrictions etc. (Mayer et al., 2001; Pohjavaara, 2003). In addition, AChE is a target of pesticides and human exposure to them, or to the closely related chemical warfare agents, depletes both AChE and the homologous enzyme butyrylcholinesterase (BChE) (Taylor et al., 2000). Polymorphisms in the corresponding *ACHE* and *BCHE* genes could hence affect both the environmental and the experience-related elements of anxiety. Furthermore, the paraoxonase (*PON1*) gene is adjacent to the *ACHE* gene on chromosome 7 and its PON protein product can also affect AChE activity by destroying environmental toxins that target AChE. (Davies et al., 1996) Polymorphisms in the *ACHE*, *BCHE* and *PON1* genes could therefore affect both the environmental and the experience-related elements of anxiety.

In mice, we observed rapid, yet long-lasting neuronal over-production of the "readthrough" AChE-R mRNA splice variant and its rapidly migrating monomeric protein product following both psychological and environmental stress stimuli (Kaufer et al., 1998). This response acts in the short term to reduce excess ACh following stress but at a longer term is associated with glucocorticoid-regulated neuronal hyper-arousal and extreme sensitivity to anti-AChEs (Meshorer et al., 2002). Transgenic over-expression of AChE in mice intensifies conflict behavior (Birikh et al., 2003), another phenomenon associated with anxiety (Gray, 2000). In view of these findings, we initiated a study aimed at testing whether genomic polymorphisms in the *ACHE-PON1* locus and corresponding changes in serum AChE and PON activities could serve as predictors of the anxiety scores of healthy humans, providing an objective assessment tool to this complex phenotype.

Material and Methods
Study sample description: The HERITAGE Family Study was designed to investigate the role of diverse risk factors in the genotype on responses to regular exercise. (Bouchard et al., 1995). Measures of state and trait anxiety were obtained on a subset of the families as part of the study

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questionnaire. A total of 461 individuals (198 men, 263 women) from 150 two-generation families of African-American (172), or Caucasian origin (289) with complete data were available for this study.

Genotyping: Genomic DNA was prepared from permanent lymphoblastoid cells as described (Rankinen et al., 2002). PCR amplification using Taq polymerase (Sigma, St. Louis, MO) was followed by Exo-Sap enzymatic purification (USB, Cleveland, OH) of the PCR product (Table 1). Standard automated sequencing utilized the BigDye Terminator cycle sequencing chemistry, ABI 3700 DNA Analyzer and Data collection and Sequence Analysis software (Applied Biosystems, Foster City, CA). The 55L/M and 192Q/R polymorphisms in *PON1* were detected using single nucleotide primer extension and the SNaPshot methods (SNaPshot ddNTP Primer Extension kit, ABI), using as probes 5'-GGCAGAAACTGG CTCTGAAGAC-3' for 55L/M and 5'-GATCACTATTTTCTTGACCCCTACTTAC-3'for 192Q/R. Following extension and calf intestine phosphatase treatment (Amersham Biosciences, Freiburg, Germany), products were electrophoresed on a 3700 ABI analyzer and results analyzed with Genescan software.

Serum analyses: Blood samples were collected at baseline in the morning after a 12-hour fast. Questionnaires were completed later that morning. Serum was prepared by blood centrifugation at 2,000 x g (15 min, 4 °C). Aliquots of 2 ml in cryogenic tubes were frozen at 80°C until use. Nondenaturing gel and catalytic activity measurements of AChE were as described (Kaufer et al., 1998). For BChE activity, butyrylthiocholine served as substrate (10 mM, Sigma). Iso-OMPA (tetraisopropylpyrophosphoramide, Sigma) was used to block BChE activity in serum samples and polyacrylamide gels ($5x10^{-5}$ M). 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51, Sigma) was used ($1x10^{-5}$ M) to block AChE activity. Addition of both inhibitors reduced hydrolysis to the rate of spontaneous hydrolysis measured in control reactions lacking enzyme or substrate, attesting to the specificity of these serum activities. Spontaneous substrate hydrolysis was subtracted. Serum PON activity was determined as described (Kudchodkar et al., 2000) with serum diluted 1:10. Cortisol levels were assayed using a radioimmunoassay kit (Diagnostic Systems Laboratories Inc., Webster, TX).

Statistics: P values for the difference between the genotypes of the subjects in distinct trait anxiety subgroups were calculated using the likelihood ratio test. The *P* value was the exact conditional tail probability given the marginal as was assessed by 100,000 Monte Carlo simulations. *P* values for the differences between AChE, BChE and PON activities were calculated using 2-tailed Student's t-test.

Multiple regression analysis was performed using R, statistical software (Ihaka and Gentleman, 1996). Classification trees were grown using the R tree library (Breiman et al., 1984). The tree function in the R software was used to define a sequence of binary partitions of the population into subsets based on age, gender and the different enzyme activities. Classification trees were "grown" such that at each step the resulting subsets were the most homogeneous with respect to the membership in the top 20% state anxiety group. The tree was than "pruned" to a number of subsets, or "nodes", which is determined by minimizing the misclassification error by a 20-fold cross-validation. The process is automatic after selection of the relevant variables for the analysis.

Results

The psychological phenomenon of anxiety that is experienced by individuals at a certain time (state anxiety) differs from their general susceptibility to anxiety (trait anxiety). Both parameters are commonly measured by the self-reported questionnaires of the state-trait anxiety inventory (STAI, Spielberger et al., 1983), To investigate the role of cholinergic regulation in these measures we used DNA, sera and STAI scores from 451 healthy individuals from the HERITAGE Family Study (Bouchard et al., 1995).

The *ACHE-PON1* locus as a candidate site

Mean STAI scores for the Heritage cohort were 35±12 (range 20-80) for trait anxiety and 35±9 (range 16-73) for state anxiety. Others reported similar values, albeit for far smaller groups with disease-associated anxiety symptoms (Seki et al., 2003; Wolf et al., 2003). Potential genomic correlates for this tendency were sought in the *ACHE-PON1* locus. The extended human *ACHE* promoter includes a functional glucocorticoid response element (GRE), suggesting overexpression under stress. Israeli subjects frequently carry at this region an activating deletion associated with elevated blood AChE activity and acute anti-AChE hypersensitivity (Shapira et al., 2000). Because this deletion is exceedingly infrequent in the U.S. population -- 0.34% vs. 3.64% allele frequency in Israelis -- we focused as a linkage marker on *ACHE*'s biochemically ineffective P446 polymorphism, with 11.3% allele frequency in the HERITAGE subjects, compatible with findings of others (Bartels et al., 1993).

Our research was extended to two more genes and enzymes that contribute to balanced ACh regulation: the AChE-related ACh hydrolyzing enzyme butyrylcholinesterase, BChE, (and *BCHE* gene) and the organophosphate hydrolyzing enzyme paraoxonase (PON) (and *PON1* gene).We genotyped three known *PON1* promoter polymorphisms (indicated by distance in nucleotides from translation start site at 0): -108C/T, -162G/C, -126C/G, contributing to 22.4%, 2.4% and none of the variation in *PON1* expression, respectively. In the *PON1* coding region, we genotyped the substitutions (indicated by amino acid number and symbol) of L55M (TTG into ATG), reducing PON protein and mRNA levels and Q192R (CAA into CGA), which affects PON's catalytic efficiency (Costa et al., 2003). Of the numerous *BCHE* mutations, we genotyped the D70G substitution yielding the "atypical" BChE variant, with enzymatic activity 30% lower than the wild type enzyme (Neville et al., 1990). Homozygous carriers of this polymorphism display extreme anxiety following exposure to anti-AChEs (Loewenstein-Lichtenstein et al., 1995). The *PON1* and *ACHE* genes both map to the long arm of chromosome 7, separated by 5.5 Mb, whereas the *BCHE* gene is located on the long arm of chromosome 3 (Fig 1A). Therefore, joint effects of *PON1* and *ACHE* but not *BCHE* polymorphisms, could reflect *cis* effects.

Population admixture considerations

Because of the presumed multigenic origin of anxiety phenotypes (Lepine, 2002; Weinberger, 2001) and the modest differences between the anxiety symptoms in healthy individuals, we did not expect drastic associations. Nevertheless, significantly different genotype frequencies were found in the analyzed sites between subjects with a trait but not state anxiety score in the highest and lowest decile. Members of the high trait anxiety group included significantly more subjects heterozygous for the P446 polymorphism in *ACHE* (16 vs. 7 subjects, $P<0.02$), whereas lower trait anxiety group members included significantly more subjects heterozygous for the PON-108 polymorphism (25 vs. 12 subjects, $P <0.06$ respectively, χ^2 test). Both the average anxiety trait

and the allele frequencies differed significantly between populations (African-Americans 36.1±9.5, Caucasians 33.7±8.4, T-test, P <0.006 for trait anxiety and P <1.4x10^{-10}, P <5.6x10^{-9}, P <0.001, P <0.008, P <0.00001 for P446, PON108, PON162, PON55 and PON192 respectively, χ^2 test). Therefore, we considered the possibility of false positives due to population stratification combined with trait and allele differences. Significant differences between the top and bottom trait anxiety deciles were maintained in African-American subjects in the *PON1* gene, in spite of the considerably smaller group size, supporting the relevance of this analysis. Next, we randomly selected single siblings from each of the African-American and Caucasian families (a total of 163 individuals), and divided then evenly into subjects with high or low trait anxiety scores. Caucasian siblings showed significant anxiety-associated differences in *BCHE* (P<0.02), suggesting relevance in genetically independent subjects. Based on this cumulative evidence, we jointly considered polymorphism pairs for the entire analyzed population. Significant contributions to the trait anxiety score emerged for *ACHE* P446 with PON108 (P <0.006), or with PON192 (P <0.001), as well as for PON126 with PON108 (P <0.007) or with PON162 (P <0.006) (Fig. 1B). Polymorphisms in the *ACHE-PON1* locus thus appeared to be significant, albeit ethnic origin-dependent predictors of trait anxiety, either due to the modified phenotype they caused or because of linkage disequilibrium to other polymorphisms.

Serum enzyme analyses as surrogate measures
Significantly higher activities of AChE and BChE (T-test, P <0.006 and P <0.0002) but not PON (P >0.2) were found in females as compared with males. Serum AChE activity was significantly higher in individuals of Caucasian origin (P <0.002), PON activity was significantly higher in African-Americans (P <3x10^{-10}) and BChE activity showed no differences between these populations (P >0.5). AChE and BChE levels increased with age and body mass index (BMI), whereas PON activity declined with age. Trait, but not state anxiety scores decreased with age, suggesting that one's experience and/or age provide better protection from trait, but not state anxiety (Fig. 2, and data not shown). Compatible with these changes, an inverse correlation was found between AChE, but not BChE or PON activities and trait but not state anxiety (R^2=0.93, Fig. 3A), suggesting a trait anxiety predictive role for serum AChE activities. Cortisol levels, however, did not correlate with trait anxiety scores, in agreement with the apparent equivocal relationship between emotional distress and cortisol (Vedhara et al., 2003). Intriguingly, PON but not BChE or AChE activity displayed an inverse association with state anxiety (R^2=0.89).

Alternative splicing of *ACHE* gene products yields at least 3 distinct proteins with ACh hydrolytic activity. Of these, the primary AChE-S variant forms tetramers, the erythrocytic AChE-E protein appears as glycophosphoinositide-bound dimers and the stress-induced AChE-R variant remains monomeric (Soreq and Seidman, 2001). Non-denaturing gel electrophoresis followed by activity staining revealed, in all serum samples, active tetramers with very small amounts of dimers; however, active monomers were over-represented in serum samples from subjects with lowest trait anxiety scores as compared to those with highest scores (Fig. 3B). Immuno-labeling of serum protein blots suggested that serum AChE monomers represent AChE-R (data not shown) (Brenner et al., 2003). supporting the notion that individuals' capacity to respond to external stimuli by over-producing monomeric AChE-R associates with reduced trait anxiety scores.

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Statistical analyses

Correlations between serum biochemical markers and trait anxiety were statistically calculated for all subjects after normalizing the effect of age, ethnic origin, gender and BMI. Individuals with top 20% trait anxiety scores were tested against the remaining population using a generalized linear model. Significant effects emerged for both the genotyped polymorphisms (P <0.013) and cumulative serum enzyme activities (P <0.022) on the trait anxiety score. Regression analysis of trait anxiety scores revealed a clear effect of all the polymorphisms genotyped together with gender, age and ethnic origin on PON serum activities (P <5.4x10^{-7}). A smaller, but significant effect was observed on AChE (P <0.03) and BChE activities (P <0.04). Thus, the inherited parameters contributed significantly to the measured enzyme activities, predicting a considerable fraction of the quantified trait anxiety scores with significant power.

The measured effects were largely independent of family links. Out of the whole data set, 92 informative families with at least two siblings in each family were further analyzed. Differences between the serum and anxiety variables of two randomly chosen siblings revealed low correlations within families both for anxiety and for serum variables, indicating a major contribution of environmental and experience-derived factors. The population subset with top 20% anxiety scores was further classified using the regression tree method (Breiman et al., 1984). Serum AChE activity was found to protect from the trait anxiety phenotype and interact with other enzyme activities. Thus, for example subjects 40 years old and above with lower than 184 nmol hydrolyzed substrate/min/ml PON activity but AChE and BChE activities higher than 6175 and 343 nmol /min/ml, respectively, would have only 2% chance of belonging to the 20% top trait anxiety group, regardless of their gender (Fig. 4A).

Biochemical prediction of state anxiety scores

Last, but not least, we addressed the difference between trait and state anxiety. In subjects with increased serum AChE activity and elevated serum AChE monomers, the *ACHE* gene should be close to its maximal expression capacity, predicting limited ability to react to a changing environment by further overproducing AChE to suppress the induced stress (Kaufer et al., 1998). This predicted elevated risk for state anxiety in individuals with higher than expected serum AChE activities, unless other conditions are met which compensates for this property. To test this working hypothesis, we calculated the difference between, an individual's measured state anxiety and the expected average value based on the demographic parameters and plotted it as a function of the parallel differences between their measured and predicted serum AChE and PON activities. This yielded a significant two dimensional interaction (at P <0.004, ANOVA, Fig. 4B), implying that subjects with exceptionally low PON activities may be at greater risk of developing state anxiety under conditions that fail to trigger AChE overproduction. This risk would be larger, for example, in aged subjects due to their considerably lower PON activities. Compatible with the corresponding increases in AChE activities, this may explain their sustained state anxiety measures. In conclusion, serum AChE activities and their interaction with PON activities displayed distinct predictive associations with both trait and state anxiety scores.

Discussion

Our findings identified previously unperceived interrelationships between anxiety feelings, serum AChE, BChE and PON activities, and their corresponding genotypes. Anxiety **trait** scores, affected by demographic parameters, reflected inherited genotype properties combined with the

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corresponding enzyme activities. **State** anxiety, the capacity to respond to changing conditions, was however reflected by higher then expected serum AChE levels. These are less likely to appear in subjects with high basal activity of serum AChE (e.g. aged or overweight individuals), because there is a maximal expression level for this gene that is likely independent of demographic parameters. PON activity may determine the requirement for AChE overproduction, compatible with its association with the difference between the observed and predicted activity values of AChE . That these interrelationships predict distinctly and significantly one's trait and state anxiety scores may open these psychological parameters for genetic and biochemical tests.
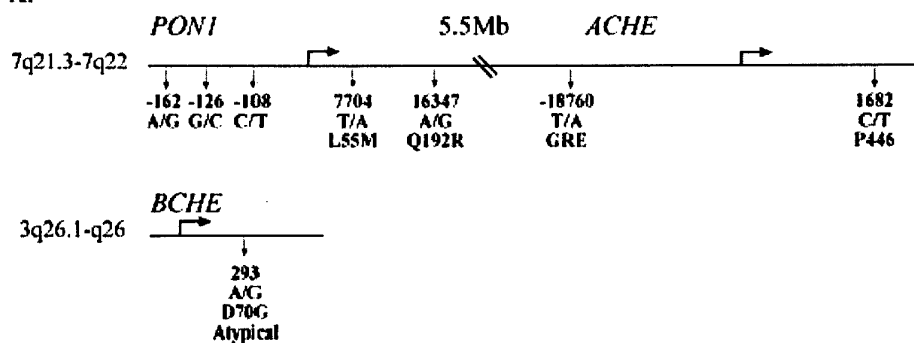
The fact that our laboratory findings correlated significantly with the psychologically examined anxiety scores of the HERITAGE Family Study subjects, and that such correlations were common for individuals of diverse ethnic origins, supports the notion that these biochemical and genetic factors are causally related to anxiety: either they reflect inherited elements which cause or mediate anxiety, or, when enzyme activities are involved, anxiety causes them. That anxiety is affected by polymorphisms in the *PON1* and *BCHE* genes, both having an AChE protective function, likely emphasizes the stress placed on AChE by environmental challenges. These results add cholinergic regulation and the *ACHE-PON1* locus to the findings of others of genetic components to anxiety (Hariri et al., 2002). Attributing to the difference between observed and expected AChE activity a causal role in state anxiety is further compatible with our recent findings that the suppression of the stress-induced AChE-R variant obliterates conflict behavior (Birikh et al., 2003).

The contribution of *PON1* polymorphisms toward serum enzyme activities and as risk factors for various diseases has been extensively discussed (Costa et al., 2003). The *ACHE* and *PON1* genes are closely positioned on the long arm of chromosome 7 and may be co-regulated. Therefore, polymorphisms in one gene may affect the other by a *cis* mechanism (Balciuniene et al., 2002). Because both AChE and PON are targets of organophosphates -- PON hydrolyzes them and AChE is inhibited by them (Furlong et al., 2000) -- subjects with high PON activity may less frequently need to overproduce AChE, e.g. under exposure to anticholinesterases in fresh crops (McGehee et al., 2000) then those with low PON levels. Also, PON confers protection from oxidative stress (Durrington et al., 2001), to which AChE was shown to be particularly sensitive (Weiner et al., 1994).

At the expression level, the observed contribution of AChE regulation to the anxiety scores of otherwise healthy subjects may be relevant to the recently reported role of ACh in controlling the production of pro-inflammatory cytokines (Tracey, 2002). Increased serum AChE, and consequently decreased ACh, would alleviate the attenuation over release by macrophages of pro-inflammatory cytokines. Therefore, one's serum AChE levels may serve as an inverse predictor of ACh's power to suppress inflammatory responses. Anxiety-associated roles of such cytokines (Anisman and Merali, 2003) likely contribute to their reported effects in autoimmune and atherosclerosis diseases, and is compatible with the higher risk for inflammatory diseases with increasing age and BMI (Saito et al., 2003). Anxiety scores, in generally healthy subjects, may hence be relevant both for psychological and physiological symptoms. That they may be evaluated by genomic and biochemical measures reflecting the cholinergic balance in the circulation provides a previously unforeseen approach for studying and perhaps controlling human anxiety.

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Figures

A.

	PON1	5.5Mb	*ACHE*	
7q21.3-7q22				

-162 -126 -108   7704   16347   -18760   1682
A/G G/C C/T   T/A   A/G   T/A   C/T
          L55M   Q192R   GRE   P446

BCHE

3q26.1-q26

293
A/G
D70G
Atypical

B

Left panel — Trait anxiety (top 50% / lower 50% / P)

1. AChE P446 (1431 C/T)

PON-108 C/T

	CT	TT	CC	CT	TT	CC	P
CT	10	6	18	9	0	20	
TT	0	0	7	0	1	4	0.006*
CC	61	43	62	70	53	32	

PON-126 G/C

	GC	CC	GG	GC	CC	GG	P
CT	1	0	33	1	0	28	
TT	0	0	7	1	0	4	0.2
CC	5	1	160	17	1	137	

PON-162 G/A

	GA	AA	GG	GA	AA	GG	P
CT	8	21	5	13	10	6	0.03
TT	3	3	1	3	2	0	
CC	67	80	19	38	102	15	

PON 55 L/M (162 T/A)

	TA	AA	TT	TA	AA	TT	P
CT	17	3	14	6	1	22	0.2
TT	2	0	5	3	0	2	
CC	73	20	73	74	15	66	

PON 192 Q/R (575 A/G)

	AG	GG	AA	AG	GG	AA	P
CT	10	9	15	14	12	3	0.001*
TT	0	6	1	3	0	2	
CC	66	37	63	74	24	57	

3. PON -126 G/C

PON-162 G/A

	GA	AA	GG	GA	AA	GG	P
GC	5	1	0	13	1	5	
CC	0	0	1	0	0	1	0.006*
GG	73	103	24	41	113	15	

PON 55 L/M (162 T/A)

	TA	AA	TT	TA	AA	TT	P
GC	4	0	2	4	3	12	
CC	0	0	1	0	0	1	0.05
GG	88	23	89	79	13	77	

PON 192 Q/R (575 A/G)

	AG	GG	AA	AG	GG	AA	P
GC	3	2	1	10	5	4	
CC	0	1	0	0	0	1	0.03
GG	73	49	78	81	31	57	

Right panel — Trait anxiety (top 50% / lower 50% / P)

2. PON -108 C/T

PON-126 G/C

	GC	CC	GG	GC	CC	GG	P
CT	5	0	66	9	0	70	
TT	0	0	49	1	0	53	0.007*
CC	1	1	85	9	1	46	

PON-162 G/A

	GA	AA	GG	GA	AA	GG	P
CT	39	30	2	29	30	0	0.01
TT	0	49	0	0	53	1	
CC	49	25	23	25	11	20	

PON 55 L/M (162 T/A)

	TA	AA	TT	TA	AA	TT	P
CT	35	10	26	40	4	35	0.09
TT	28	10	11	28	8	18	
CC	29	3	55	15	4	37	

PON 192 Q/R (575 A/G)

	AG	GG	AA	AG	GG	AA	P
CT	28	10	33	38	14	27	0.02
TT	25	3	21	26	7	21	
CC	23	39	25	27	15	14	

4. PON -162 G/A

PON 55 L/M (162 T/A)

	TA	AA	TT	TA	AA	TT	P
GA	34	1	43	17	5	32	0.02
AA	52	21	31	61	9	44	
GG	6	1	18	5	2	14	

PON 192 Q/R (575 A/G)

	AG	GG	AA	AG	GG	AA	P
GA	36	20	22	30	10	14	
AA	38	17	49	53	21	40	0.02
GG	2	15	8	8	5	8	

5. PON 55 L/M (162 T/A)

PON 192 Q/R (575 A/G)

	AG	GG	AA	AG	GG	AA	P
TA	44	6	42	40	3	40	0.09
AA	4	1	18	2	1	13	
TT	28	45	19	49	32	9	

Fig. 1. The analysed genotypes and their frequencies. A. Shown are the chromosome positions and the polymorphic sites that were studied in the *PON1* (AF539592), *ACHE*

(AF002993) and *BCHE* (NM000055) genes. Nucleotide numbers begin at the translation start site at 0. B. Distributions and joint contributions of tested polymorphism pairs to trait anxiety scores.
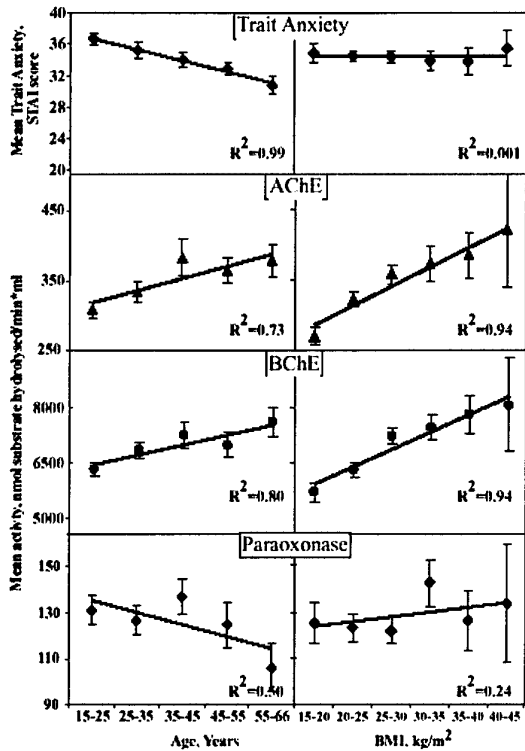

Fig. 2. Acquired changes in measured values. Graphs show mean ± SEM for each value (n=434). Note that trait anxiety scores decrease with age, whereas AChE and BChE activities are elevated with both age and BMI.
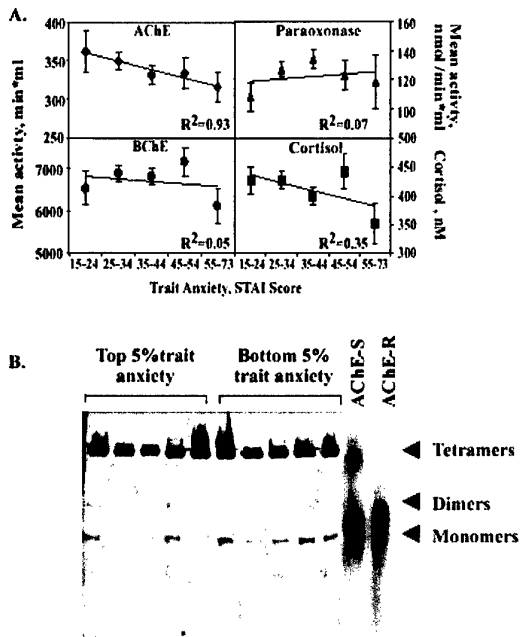

Fig. 3. Serum AChE activity inversely correlated with trait anxiety. A. Trait anxiety associations. Serum AChE activity, but not cortisol levels or BChE or PON activities, is inversely correlated with trait anxiety. Graphs show best fit lines through mean ± SEM values of each parameter. B. Over-represented AChE monomers in the serum of subjects with low anxiety scores. Non- denaturing gel electrophoresis of serum samples from subjects with the noted anxiety scores was followed by enzyme activity staining. Recombinant AChE-R and AChE-S were similarly separated (but signals were developed for a shorter time). Note that AChE-R remains monomeric, whereas AChE-S includes a significant tetrameric fraction.

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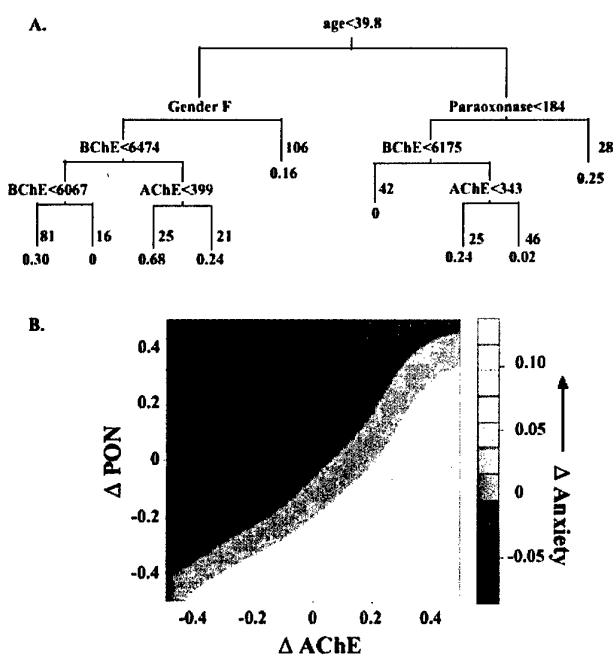
Fig. 4. Predictions of trait and state anxiety. A. A prediction tree for trait anxiety. Shown is a prediction tree based on age, gender, AChE, BChE and PON activities. The numbers at the terminal nodes represent the number of subjects and the probability of their belonging to the top 20% trait anxiety score group. B. Inter-related consequences of AChE and PON variations on state anxiety. The differences between the "observed" and "expected" state anxiety score values of each subject were plotted as a function of the corresponding differences in serum PON and AChE activities. Note increasing tendency for anxious feelings in those subjects with higher then expected serum AChE activities and the ameliorating effect on these values of higher than expected PON levels. Smoothness parameter equaled 0.3.

Table 1. PCR conditions for genotyping the different polymorphisms.

Gene (Accession No.)	Polymorphism	Primers	Product size	PCR conditions
ACHE (AF002993)	GRE T/A	(+)5'GTGAGAATGGCTG CTTCATA3' (-)5'CTCAGTTCTGGGAAATTCCTA3'	217bp	60°C, 37 cycles
	P446 C/T	(+)5'CGGGTCTACGCCTACGTCTTTGAACACCGTGCTTC3' (-)5'CCCGTCCTTTCTGTCTCGTGTG3'	284bp	60°C, 37 cycles
BCHE (AC009811)	Atypical (D70G)	(+)5'CTTGGTAGACTTCGATTCAAAAAGCCACAGTCT3'	187bp	55°C, 37 cycles
	A/G	(-)5'GAATCCATACATTTAGATATAAACAGTCTTCACTG3'		
PON1 (AF539592)	PON-108 C/T PON-126 G/C PON-162 G/A	(+)5'ACTGAATCT CTC TGAGACGCAAGGACC3' (-)5'ATAGACAAAGGGATCGATGGGCGCAGACA 3'	376bp	
	PON55 L/M	(+) 5'-GAAGAGTGATGTATAGCCCCAG-3' (-) 5'-ACACTCACAGAGCTAATGAAAGCC-3'	178bp	60°C, 37 cycles. 5% DMSO
	PON192 Q/R	(+)5'GGAATAGACAGTGAGGAATGCCAGT3' (-)5'CAGAGAGTTCACATACTTGCCATCGG3'	305bp	

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Anticholinesterase exposure leads to alterations in location and level of cortical activity

Numerous animal studies have suggested that chronic, sub-threshold exposure to OPs can result in neurodevelopmental and cognitive problems (Chakraborti et al., 1993; Eskenazi et al., 1999; Pascual-Marqui et al., 1994; Slotkin et al., 2001). In addition, other studies in rats treated with chronic, sub-threshold doses of OPs have reported alterations in nerve function and cholinergic receptor density (Averbook and Anderson 1983; (Stone et al., 2000). Several human studies have reported restlessness, forgetfulness and other neuropsychiatric symptoms in exposed populations (Levin and Rodnitzky 1976; Levin, Rodnitzky et al. 1976; Rodnitzky, Levin et al. 1978; Chuwers, Levy et al. 1989/90; (Stephens et al., 1995; Fiedler and Kipen, 1997; Ohayo-Mitoko et al., 2000), while other studies fail to recognize any significant difference between exposed and control groups (Brown and Brix 1998; Ray and Richards 2001). Although these accumulating data suggest alteration in the function of higher brain areas, which are essential to memory and other cognitive functions, no specific phenomenological or mechanistic data exist regarding such changes.

Here we combined methods to reveal physiological, molecular and behavioral changes in humans exposed chronically to sub-clinical doses of OPs. Quantitative electroencephalography (qEEG) using source localization technique showed a significant decreased activity in parahippocampal and anterior cingulate regions and increased activity in prefrontal and occipital regions.

Methods
The study was conducted using volunteers from a rural, agricultural community where organophosphate pesticides (Fenitrothion, Chlorpyrifos, Monocrotophos, Ethion and Azinphos-methyl) are routinely used. All participants were consented to participate in the and no history of acute intoxication was reported. Normal subjects were age- (± 2 years) and sex-matched and had no history of exposure to anticholinergic agents and no known neurological or psychiatric disease.

Human Electroencephalography
EEG was recorded from subjects using a 128 channel EEG unit (CEEGraph, Bio-logic, USA). Electrodes were applied using either individual Ag/AgCl electrodes in a 25 electrode montage (10/20 electrode system) or a 128 channel embedded electrode cap. Electrocardiogram and eye movement electrodes were used as aids for artifact identification. The sampling rate was 256 Hz and electrodes were referenced to a total electrode average. Data were stored digitally and analyzed off-line.

LORETA was used to locate the representative neurophysiological contributors to electrical scalp potentials (Pascual-Marqui et al., 1994; Pascual-Marqui et al., 1995). By minimizing the total squared Laplacian of source strength, LORETA computes the smoothest of all possible source configurations. LORETA is based on the single neurophysiological assumption that neighboring cortical areas are likely to be co-activated. Current density at each voxel in the brain grey matter is determined as the linear weighted sum of scalp electric potentials. Accordingly, LORETA analysis permits three-dimensional tomography of cortical brain electrical potentials while requiring only simple constraints (smoothness of the solution) and no predetermined knowledge about the putative number of discernible source regions. Based upon the digitized Talairach brain atlas (Talairach and Tournoux, 1988), LORETA estimates the absolute current density distribution of the source of any potential or an arbitrary chosen

frequency band on a grid of 2394 voxels restricted to the neocortical and hippocampal grey matter.

Data analysis and statistics:

EEG data were visually inspected, and 60-80 seconds of artifact-free, eyes-closed EEG data were extracted for quantitative analysis. Fast Fourier Transform (FFT) was applied to the EEG waveforms recorded from each electrode of each subject (Frei et al., 2001; Pascual-Marqui et al., 1999). The average discrete frequency was normalized to each subject's own total power of the 1.5-30 Hz frequency spectrum. Data are expressed throughout the text as mean ± standard error of the mean (S.E.M). Statistically significant differences between control and patient LORETA values were tested by using the post hoc t test, on a voxel-by-voxel basis (Nichols and Holmes, 2002), employing statistical non-parametric mapping techniques that correct for multiple testing.
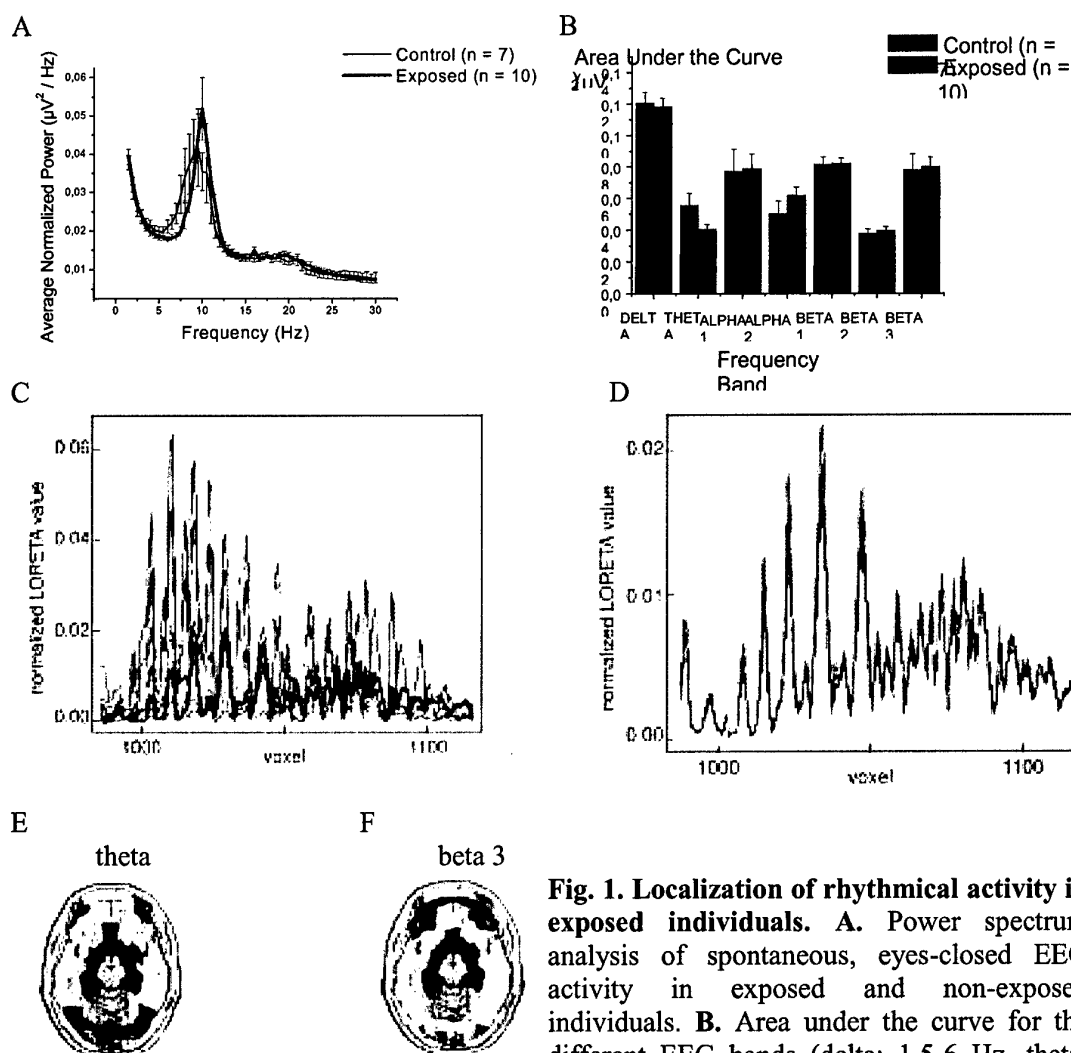
Fig. 1. Localization of rhythmical activity in exposed individuals. A. Power spectrum analysis of spontaneous, eyes-closed EEG activity in exposed and non-exposed individuals. B. Area under the curve for the different EEG bands (delta: 1.5-6 Hz, theta: 6.5-8 Hz, alpha1: 8.5-10 Hz, alpha2: 10.5-12 Hz, beta1: 12.5-18 Hz, beta2:18.5-21 Hz, and beta3: 21.5-30 Hz). Note the mild decrease in the theta range and increase in alpha 2 band. Despite similar power spectra between the two groups, LORETA localizes generators in different brain regions. C. Normalized LORETA values in voxels (990-1110). Each voxel

33

represents a distinct cortical region (~ 4 mm³). Note the difference in LORETA values between exposed individuals (n = 10) and the average non-exposed value (black line). **D.** Notice the low variability of LORETA values within the non-exposed group (n = 7). **E and F.** Statistical mapping showing cortical regions in which a significant (p < 0.01) statistical difference was found. Blue voxels: activity in non-exposed is greater than exposed. Red voxels: activity is greater in exposed. Note that in both the theta and beta 3 frequency ranges, activity in exposed individuals is lower in the hippocampus and para-hippocampal regions, and is greater in temporo-occipital cortex for the theta range and in the frontal regions in higher frequencies.

right, lateral   right, medial   left, lateral

non-exposed
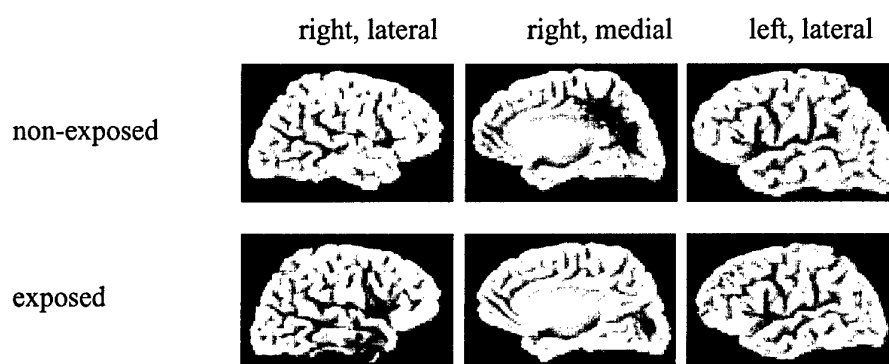
exposed

Fig. 2. Localization of theta generators in control and exposed individuals (*n* = 14 in each group). Note that while in non-exposed individuals activity is mostly localized in the medial cingulate gyrus, in the exposed group, activity is more superficial, mostly in the right temporal lobe.
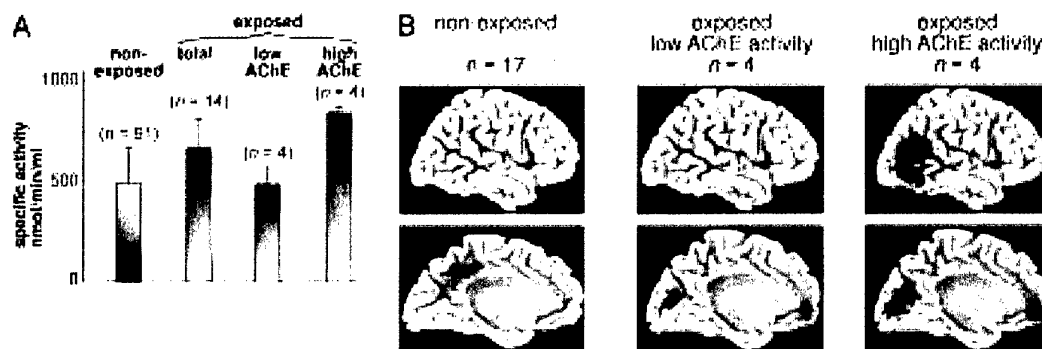
Fig. 3. Exosure to anti-AChE agents induces EEG abnormailities, more intense in subjects with elevated serum AChE levels. A. Subjects chronically exosed to pesticides display higher serum AChE levels than those not exposed. **B.** LORETA analysis maps the source of slow pathological theta activity (4-7 Hz) in those exposed individuals, subclassified in Fig. 3A, with the highest and lowest AChE levlels. Note that the 17 non-exposed individuals and in the exposed individuals with low AChE levels, theta activity is generated symmetrically in the medially located cingualte gyrus, whereas exposed individuals with high AChE levels, presented theta acativity abnormally located in peripheral cortical regions, mainly in the right temporal lobe.

Protection of mice against paraoxon and lipopolysaccharide intoxication by a mouse AChE-R-specific antisense reagent: preliminary experiments

Hypothesis: AChE-R accumulation, a known consequence of stress and anticholinesterase exposure, modulates organismal responses to such exposure, and affects body temperature and/or locomotor activities.

Test: Elevate AChE-R levels by injecting animals with mARP, the synthetic peptide with the C-terminal sequence of murine AChE-R; alternatively, prevent AChE-R accumulation post-insult by injecting mEN101, the antisense agent which induces AChE-R mRNA destruction.

Telemetric measures: Body temperature, motor activity pre- and post-treatment in transmitter-implanted animals.

Serum surrogate marker: AChE activity.

In preparation for monitoring body temperature (BT) and motor activity (MA), transmitters were implanted in the peritonea of mice (Cohen et al., 2002). After a 2-week recovery period, recording was begun. Two days after establishing baseline values, the mice were injected with saline (Fig. 1), 0.4 mg/kg paraoxon, *i.p.* (Fig. 2) or 2.5 mg/kg *E. coli* lipopolysaccharide (LPS), *i.p.* (Fig. 3). Daily throughout the experiment, the mice were injected with 0.5 mg/kg of mEN101, a 3'-terminally protected mouse AChE-R-specific antisense reagent, with 0.5 mg/kg of Inv-mEN101, a polynucleotide with the inverse sequence of mEN101, or with 0.25 mg/kg of mARP, a synthetic polypeptide with the sequence of the 26 C-terminal residues of mouse AChE-R (Grisaru et al., 2001). In these preliminary experiments, we present the effect on one of 3 or 4 mice, in each group, which showed the most extreme response in body temperature and/or locomotor activity.
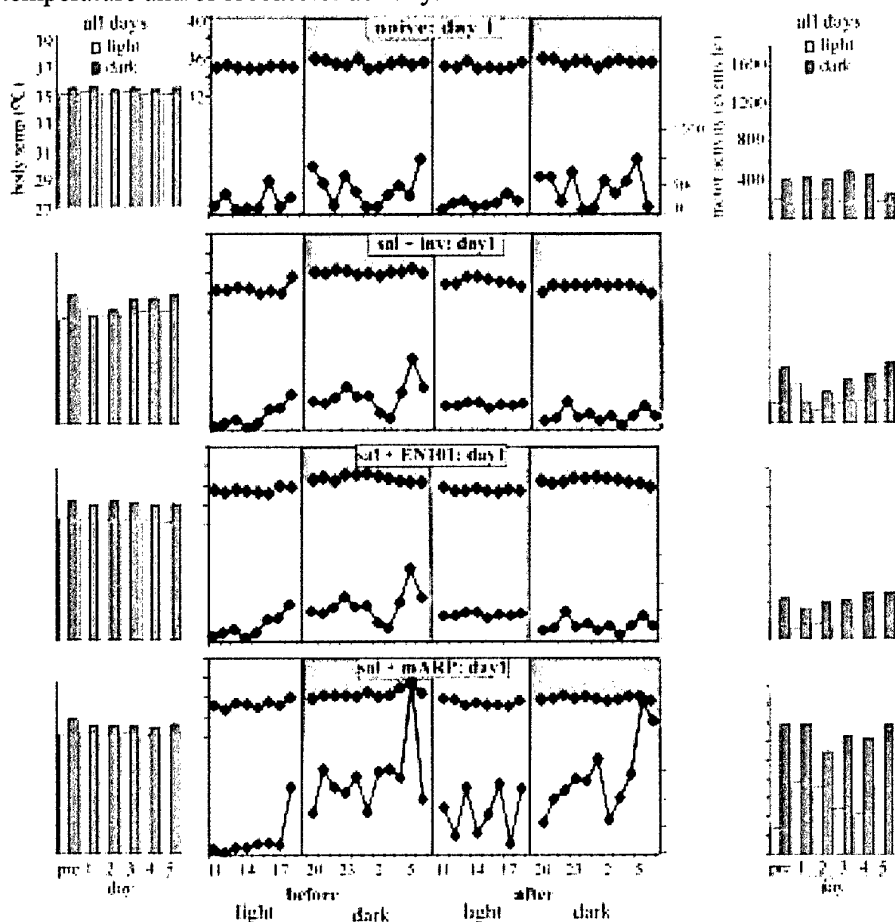
Fig. 1. Control tests. To the left, bar graphs indicate the avearge light- and dark-phase temperatures over the course of the experiment, both pre- and post-treatment. To the right, bar graphs indicate a measure of light- and dark-phase MA, over the post-treatment week, the number of events monitored by the implanted transmitter as it moves across a sensing platform under the mouse cage (Cohen et al., 2002). In the central panel, the BT and MA during the last pre-treatment day and the first post-treatment day are shown.

These control tests confirmed that injection of EN101 alone, or Inv-mEN101 alone, did not significantly change the body temperature or the MA value in the transmitter-implanted mice. In contrast, mARP increased locomotor activity during both light and dark phases of the post-treatment days.
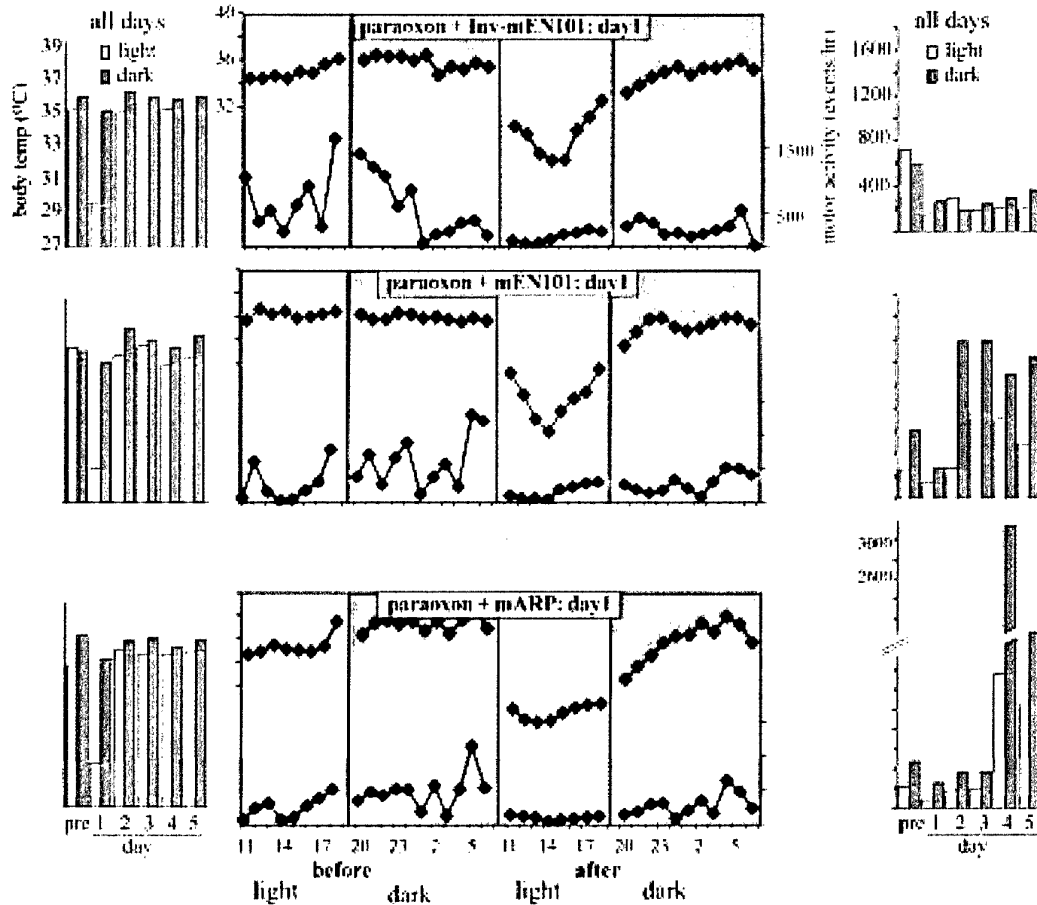
Fig. 2. AChE-R manipulations modify the consequences of paraoxon exposure. As in Fig. 1, the BT and MA are shown for the entire pre- and post-treatment periods. Paraoxon caused a reduction in light and dark-phase BT and MA in the first day post-treatment, followed by a partial recovery in BT after 6 hours.MA remained low throughout the 6 days post-exposure. Treatment with both paraoxon and mEN101 resulted in an unchanged response pattern during the first day (central panel) yet induced a delayed increase in MA (from day 2, on). mARP, unlike mEN101, prevented the decrease in BT during the first day post-treatment and elevated BT during the following dark phase (central panel). By days 4,5 post-treatment, mARP-injection also induced a dramatic increase in MA (right panel). In the long-term, therefore, both mEN101 and mARP dramatically increased light and dark-phase MA above normal values..
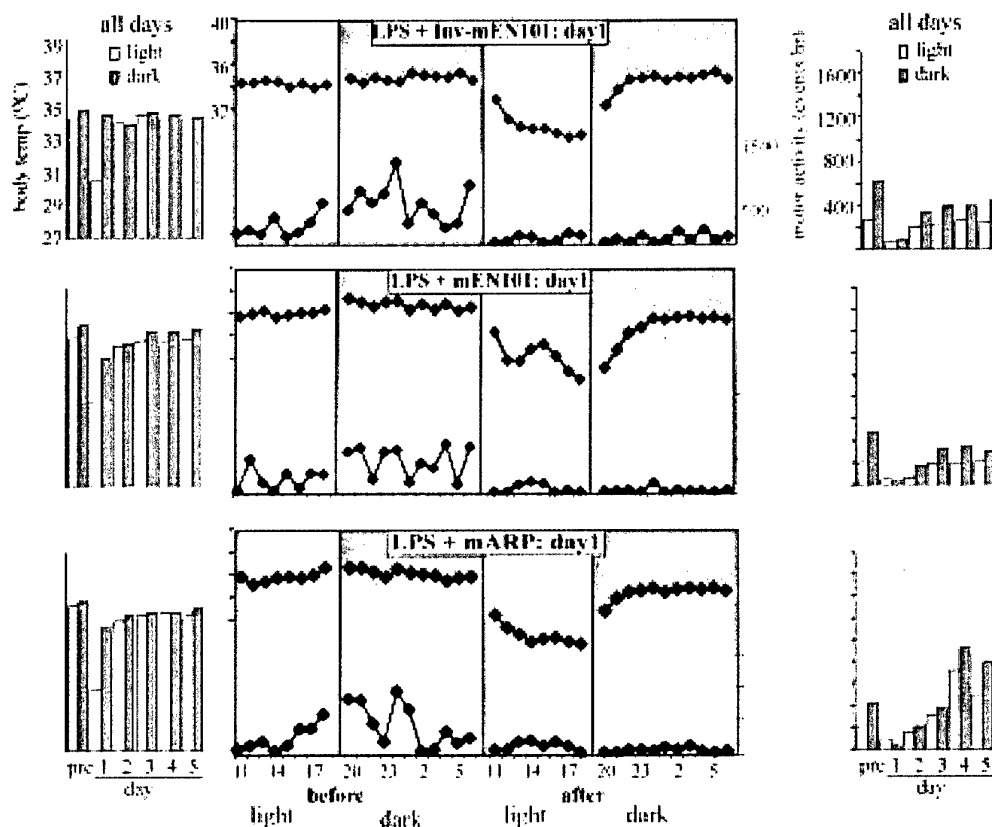
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Fig. 3. AChE-R manipulations affect LPS response. As in Fig. 1, BT and MA are shown for the entire pre- and post-treatment periods. LPS reduced both MA and BT, albeit less dramatically than paraoxon. mEN101, but not mARP facilitated the recovery of BT values. This recovery lasted for only two hours and was followed by a second decrease (central panel), perhaps reflecting the duration of the antisense effect. In the long term, mARP, but not mEN101, increased light an dark-phase MA above nomal values. This is compatible with the symptoms recorded for AChE-R overexpressing transgenic mice following a circadian shift (Cohen et al., 2002).
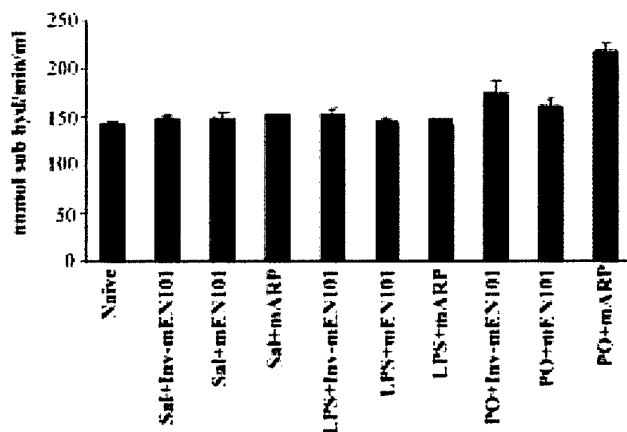
Fig. 4. Plasma AChE activities. AChE specific activities were measured in plasma of all animals in the presence of 50 µM ISO-OMPA (an inhibitor of BChE, the main ChE in the plasma). Paraoxon (PO) treatment caused an increase of plasma AChE activities which was even higher following treatment with mARP. Note that measurements reflect enzyme levels at day 7 post-treatment.

Two-hybrid approach to the intracellular function(s) of readthrough acetylcholinesterase

Acetylcholinesterase (AChE) performs both hydrolytic and morphogenic functions (Soreq and Seidman, 2001). Extensive research proves that some of these responses are at least in part specific to the distinct C- terminal sequences of the AChE variants which are produced by alternative splicing of the single *ACHE* gene (Taylor and Radic, 1994). These variants include the brain and muscle abundant "synaptic" major form, AChE-S, which is produced by splicing of exon 4 to exon 6. Expression of this AChE variant in cultured *Xenopus* motoneurons induces neurite growth (Sternfeld et al., 1998). Continuous transcription through intron 4 to exon 5 yields the second most common form, the "readthrough" form (AChE-R) which is expressed in embryonic and tumor cells (Karpel et al., 1994), in developing sperm (Mor et al., 2001) and is induced in neurons in response to psychological stress and AChE inhibition or following head trauma (Kaufer et al., 1998; Shohami et al., 2000). ARP, a synthetic peptide derived from the AChE-R C-terminus, but not ASP, the AChE-S C-terminal peptide, induces proliferation of human hematopoietic progenitors (Grisaru et al., 2001). In the third transcript exon 4 is spliced to exon 5. This variant, designated AChE-E (E, for erythrocytic) is linked to erythrocyte membranes through a phosphoinositide moiety (Futerman et al., 1985; Liao et al., 1992).

In addition to its conservative extracellular location, AChE appears in cytoplasmic and perinuclear sites where acetylcholine hydrolysis is unlikely. Examples include murine megakaryocytes (MK), where antisense inhibition of AChE gene expression suppresses differentiation (Patinkin et al., 1990; Soreq et al., 1994). Perinuclear AChE labeling was observed in small DAMI cells (a transformed human megakaryoblastic cell line) and large polyploid megakaryocytes (Lev-Lehman et al., 1997). Therefore, the AChE protein or fragments thereof may be expected to form intracellular protein interactions that can be variant-specific. This calls for delineating the putative partner(s) of the intracellular enzyme, identifying the molecular pathway(s) that might be induced by AChE's interactions with these partners and investigating the corresponding physiological processes.

To identify the protein partner(s) associated with cytoplasmic AChE-R we used the classical GAL4 based two-hybrid screening approach. Transcription factors, such as GAL4, consist of two discrete modular domains: the DNA- binding domain (DNA-BD) and the activation domain (AD). The two domains can function also when they are physically separated, provided that they are brought to close proximity by protein-protein interaction. The AChE-R derived "bait" peptide as bait. Of these, RACK1 appeared in six cDNA fragments of different lengths.

Structurally, RACK1 is a cytoplasmic G protein homolog, which serves as a protein kinase C (PKC) receptor anchoring it to the cell membrane after activation of its kinase was fused to the DNA-BD domain of GAL4 and was co-expressed in yeast cells with the protein products of human brain cDNA expressed as a fusion to the AD domain of GAL4. When the fusion proteins interact, the DNA-BD and AD are brought into close proximity. This reconstitutes GAL4 and activates transcription of a reporter gene (Fields and Sternglanz, 1994), enabling survival of the yeast cells co-expressing the bait and its protein partner.

Our basic assumption was that the C-terminal domain of AChE-R, which is unnecessary for acetylcholine hydrolysis (Sternfeld et al., 1998), may be involved with the predicted intracellular interactions. Therefore, we created a vector encoding for ARP_{51}, composed of the C-terminal 51-amino acid residues of human AChE-R. The last 26 amino acid residues of ARP_{51} are unique to the AChE-R variant. These residues extend from the common enzyme core and their structure was not resolved, however, the remaining conserved residues may

form a compact domain (Fig.1). By the use of this relatively small bait peptide instead of the full protein we wished to improve the specificity of the pursued interaction and increase its likelihood to be AChE-R-specific. Several potential partner proteins enabled survival of yeast clones in a screen which covered 240,000 independent clones from a human fetal brain library, with ARP$_{51}$ activity (Mochly-Rosen et al., 1991). RACK1 belongs to a superfamily of proteins that contain the WD repeat (Neer et al., 1994), a domain involved in protein-protein interactions and which is thus assumed to be an adaptor protein (Schechtman and Mochly-Rosen, 2001). Adaptor proteins play a crucial role by conferring specificity in action and regulation on PKCs which are biochemically similar kinases. This involves bringing and positioning the signaling enzyme in the appropriate location and close proximity with its substrate protein(s) (Jaken and Parker, 2000; Schechtman and Mochly-Rosen, 2001). Due to the conservation of the WD repeat family it is presumed that all proteins containing multiple repeats of this unit will form a propeller structure similar to the crystal structure solved for G protein β subunit homologous to RACK1 (Fig. 2) (Garcia-Higuera et al., 1996). In this propeller structure every repeat forms one blade of the propeller, creating a densely packed globular structure which is expected to interact simultaneously with more then one protein through its multiple blade domains (Schechtman and Mochly-Rosen, 2001).

The binding partners reported for RACK1 include cellular signaling proteins like phospholipase C γ (Disatnik et al., 1994) and the PDE4D5 isoform of cAMP-specific phosphodiesterase (Yarwood et al., 1999). Others display pleckstrin homology domains, such as the Ras GTPase-activating protein p120GAP, the β-adrenergic receptor, β-spectrin and Dynamin-1 (Rodriguez et al., 1999). Synapse-specific functions for RACK1 partners are indicated from its C2 region homologous proteins, e.g. P65 synaptic vesicle specific protein (Mochly-Rosen et al., 1995). Other RACK1 partners are involved in adhesion, like PTPmu (Hellberg et al., 2002), Integrinβ1 (Liliental and Chang, 1998), Insulin-Like Growth Factor I (IGF-I) Receptor (Hermanto et al., 2002), the P0 (MPZ) myelin protein (Xu et al., 2001) along with other proto-oncogenic, viral and bacterial proteins. Interaction between RACK1 and AChE-R can hence be the potential link between AChE and other signaling molecules through which it exerts its non-catalytic intracellular functions. AChE-R-RACK1 interactions are currently being investigated, with the hope that these interactions will shed light on the molecular mechanisms underlying AChE-R's involvement in acute and chronic stress responses, normal and tumorogenic development and other non-catalytic functions.

Figure 1: Partial structure of the bait peptide. Shown is part of the *Electrophorus electricus* AChE crystal structure (http://www.rcsb.org/pdb/ ,accession No. 1C2B). Hydrophobic amino acids are indicated in red, basic - in blue, polar - in pink. Outlined is the part of the conserved domain, homologous to that included in the bait (amino acids 509-535 in the core domain of *Electrophorus electricus* AChE, which corresponds to amino acids 548-574 in the human AChE used in the bait). The C-terminus is not shown since its structure has not been solved. Note that the outlined part extends from the protein and is free to interact with other proteins.
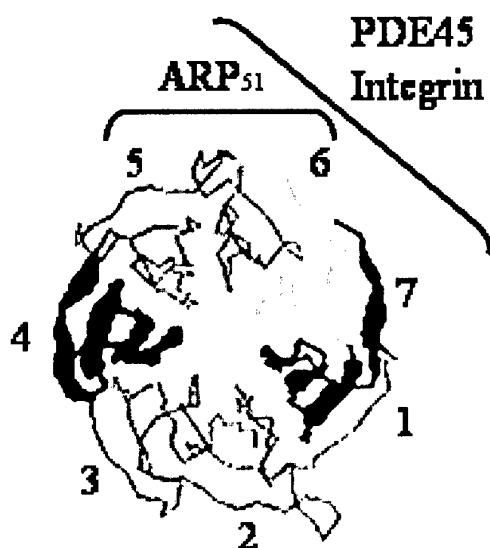
Figure 2: Scaffold protein interactions. Shown is the schematic structure of the G protein ß subunit (http://www.rcsb.org/pdb/, accession No.1GP2). Repeat boundaries are numbered (1-7), a red bracket notes the putative ARP51 binding site (repeats 5 and 6), and a blue bracket notes the putative binding site for PDE45 and integrin (repeats 5-7). The overlap between the two sites may indicate that these proteins compete on RACK1 interaction.

Conditional suppression of AChE *in vitro* and *in vivo*

Accumulating evidence suggests non-catalytic functions ascribed to acetylcholinesterase (AChE), apart from its known catalytic function in hydrolyzing acetylcholine. Most of the available evidence is circumstantial, for example, the expression of AChE in non-neural tissues such as cells of the hematopoietic and osteogenic lineages (Karpel et al., 1994), and developing oocytes and sperm (Karpel et al., 1996; Taylor and Radic, 1994) where its catalytic function is hard to explain. More "incriminating" evidence comes from the incompatibility of the occurrence of AChE and other cholinergic proteins such as choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine (Kaufer et al., 1998; Liao et al., 1992). Moreover, non-neuronal brain cells such as meninges (Lev-Lehman et al., 1997), blood vessel endothelium (Soreq et al., 1994) and glia (Karpel et al., 1994) also express AChE, and a variety of non-neuronal tumors, among them leukemias (Lapidot-Lifson et al., 1989), ovarian carcinoma (Zakut et al., 1990) meningioma, astrocytoma and glioblastoma (Gurwitz et al., 1984; Razon et al., 1984) express high levels of AChE. This ubiquitous distribution of AChE, which could not be entirely explained by its cholinergic functions, reinforced the hypothesis that this protein is involved in novel functions such as cell growth and adhesion, differentiation and responses to various insults including stress (Soreq and Seidman, 2001).

Extensive research proves that some of these responses are at least in part specific to the distinct C- terminal variants of AChE. Distinct morphogenic activities for two of these variants were observed in transfected cells (Karpel et al., 1996) and in *Xenopus* motoneurons (Sternfeld et al., 1998). The AChE C- terminal variants are produced by alternative splicing of the single ACHE gene (Taylor and Radic, 1994). They include the brain and muscle abundant major form AChE-S (S, for synaptic), which is produced by splicing of exon 4 to exon 6. Expression of this AChE variant in cultured *Xenopus* motoneurons induces neurite growth. Continuous transcription through intron 4 to exon 5 yields the second most common form, the "readthrough" form (AChE-R) which is expressed in embryonic and tumor cells (Karpel et al., 1994; Karpel et al., 1996), and is induced in response to psychological stress and AChE inhibition (Karpel et al., 1996). In the third transcript exon 4 is spliced to exon 5. This variant designated AChE-E (E, for erythrocytic) is linked to erythrocyte membranes through a phosphoinositide moiety (Kerem et al., 1993; Liao et al., 1992).

Several lines of experimental evidence indicate that AChE-S and AChE-R give rise through proteolytic cleavage to independent and biologically active C-terminal peptides designated ASP (AChE synaptic peptide) and ARP (AChE readthrough peptide) respectively. Antibodies selective to ARP identify serum accumulation of the peptide after forced swim stress. Nanomolar concentrations of synthetic ARP stimulated hematopoietic stem cells proliferation and differentiation. Furthermore, ARP increased the effects of forced swimming on hematopoietic expansion while antisense oligonucleotides targeted to AChE-R mRNA antagonized this effect (Grisaru et al., 2001).

In addition to its conservative extracellular location, AChE appears in cytoplasmic and nuclear sites where acetylcholine hydrolysis is unlikely. This calls for delineating the molecular pathway(s) for its non-catalytic activities and for developing approaches for manipulating these pathways to reveal their biological significance.

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There are pros and cons for the two major approaches for studying gene function in mammalian cells which are overexpression of a transfected genes or targeted genomic disruption. The main drawback of genomic disruption is its irreversibility which is particularly problematic regarding to neuronal proteins, considering their terminal differentiation. The principal substitute for appropriate neuronal models until now has been pharmacological manipulation with a growing arsenal of agonists and antagonists. Unfortunately, these display complex pharmacokinetics, are notoriously non-specific, and often cannot distinguish between specific receptor subtypes or isoforms.

Because AChE gene expression is rapidly subjected to both aging and stress-induced changes and because of AChE's developmental importance (Behra et al., 2002 1439; Xie et al., 1999) we wished to conditionally and transiently manipulate AChE gene expression. Previously, we had found domains on the AChE mRNA that are particularly vulnerable to antisense oligonucleotides (AS-ONsGrifman et al., 1998)and to ribozyme (RZ) degradation *in vitro* (Birikh et al., 1997). In this study we investigated the same RZ and AS targeted to these sites on AChE mRNA endogenously expressed under the control of the inducible tetracycline-dependent system (Gossen et al., 1995; Schultze et al., 1996) in cultured cells and in transgenic mice.

The use of AS and RZs targeted to specific mRNAs offers a promising approach to overcoming the problem of specificity in the nervous system (reviewed by Soreq and Seidman, 2000). Cloned into a plasmid vector under a tetracycline-controlled promoter, these sequences were stably transfected into PC12-TetON cells. This yielded over 50% reduction in basal AChE levels upon addition of the inducer. We further created transgenic mice which carry these vectors and tested their susceptibility to conditional regulation of AChE gene expression *in vivo*. AChE activity was constitutively decreased in the cortex and intestine of the AS but not RZ expressing TetON mice, reflecting leakage of the tetracycline control. Doxycycline (Dox, a tetracycline derivative) treatment further reduced AChE activity in the brain, intestine and plasma of the AS-TetON mice; moreover, administration of bacterial lipopolysaccharide (LPS) as inflammation stressor failed to induce AChE-R accumulation in the serum of AS-TetON mice, which also displayed reduced plasma corticosterone levels as compared to mice without dox treatment. These mice may prove invaluable for studying the molecular mechanisms through which AChE variants confer their C-terminal specific morphogenic functions in acute and chronic stress responses in mammalian development and tumorogenesis, and in future studies of the levels and/or properties of the AChE protein partners to be identified in the *in vivo* conditional suppression models.

Materials and Methods
Plasmid constructs Two duplex-forming oligonucleotides 5'ATTCATGTAGTG AAGAGCGAAAGCGAAAGCTCCAGCTGGATCGGTCCCT and 5'CTAGAGGGAC CGATCCAGCTGGAGCTTTCGCTTTCGCTCTTCAGCTACAGT were annealed creating EcoRI and XbaI sticky ends that were cloned into EcoRI-XbaI sites of pTRE plasmid (Clontech, Palo Alto, CA) yielding the pTRE-RZ construct. AS expressing plasmid was obtained by recloning EcoRI-fragment from pCR-AS plasmid (Grifman et al., 1998) containing nt 1728-1832 of mouse AChE (GeneBank accession No.13928663) into EcoRI site of pTRE plasmid. Clones containing the AS orientation of the insert were designated pTRE-AS, whereas clones containing

the sense orientation were designated pTRE-Sense and used to create a substrate for in vitro RZ cleavage.

In vitro transcription Templates for in vitro transcription were synthesized using an upstream primer containing the T7 promoter: TreT7 5' <u>TGTAATACGACTCACTATAGGG</u>ACCGATC CAGCCTCCGC (T7 promoter underlined) and two downstream primers Tre520 5'TGCCAAACTCATCAATGTACTTTATC and Tre715 5'TCAGCCATACCACATTTGTAGA GGT. Amplification of pTRE-RZ with TreT7-Tre520 and TreT7-Tre715 primers gave a 130 bp and 325 bp products respectively. PCR products were purified (High-Pure PCR Product Purification Kit, Roche, Basel, Switzerland) and concentration determined. In vitro transcription of the RZs was carried out in a 100μl reaction mixture using T7 RNA Polymerase (Roche) and 200 ng template 3h, 37°C. To radioactively label the substrate it was *in vitro* transcribed in the presence of α^{32}P-UTP (0.7 μCi/μl). Following in vitro transcription, template DNA was digested with DNaseI (15 min 37°C) and purified by Nucleotide removal kit (Qiagen, Germantown, MD) and RNA concentrations were determined.

In vitro RZ cleavage Cleavage reactions were carried out under single turnover conditions (30 nM substrate, 50 nM RZ in 50 mM Tris-HCl pH7.5, 10 mM MgCl$_2$, at 37°C, 6 h). Following incubation loading buffer was added (8 M urea in TBE pH 8, 0.02% bromphenol blue) and samples were separated by denaturing PAGE. Signals were detected and quantified with the Fuji BAS 2000 Bio-Imaging Analyzer.

Transient transfections Were carried out using Lipofectamine Plus (Life Technologies, Paisley, UK). Dox (10 μg/mL, Sigma, St. Louis, MO) was added to the medium 24 h after transfection, cells were lysed 48 h after dox addition.

Stable transfections To obtain stably transfected cell lines, semi-confluent PC12-tetON cells (Clontech) were co-transfected with pTRE-Rz (or pTRE-AS) and pTK-Hyg (Clontech) at a ratio of 10:1 using Lipofectamine Plus (Life Technologies). Three days after transfection, hygromicin (Sigma) was added to the medium to the final concentration of 200μg/ml. The cells were grown in presence of hygromycin until the formation of hygromicin-resistant colonies. The colonies were trypsinized and seeded in a 24 well dish, one colony per well.

AChE activity measurement Cells were lysed in 0.1 M phosphate buffer pH 7.4, 1% Triton X-100 and centrifuged 12,000 x g, 4 °C, 30 min, clear supernatant was transferred to a fresh tube and protein content was determined. AChE activity was measured by adaptation of Ellman's reaction (Ellman et al., 1961) to a 96-well plate as described (Kaufer et al., 1998). Hydrolysis rates were measured following 20 min incubation with $5 \cdot 10^{-5}$M tetraisopropyl pyrophosphoramide (iso-OMPA, Sigma), a specific BChE inhibitor. The obtained activity was normalized to the total amount of protein.

Neurite measurements Neurite length and width measurements were done using the Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD).

Transgenic mice Transgenic mice were generated at the Hebrew University's Transgenic Unit, by injection of the *XhoI-HindIII* DNA fragment from TRE-AS, TRE-RZ plasmids and from the previously described and the *ScaI-XhoI* fragment of pTet-ON (Clontech) into the pronuclei of fertilized eggs of CBA/6 inbred mice according to standard procedures (Hogan et al., 1986). In this manner 2 lines of transgenic mice were generated per construct (6 lines altogether). Mice were screened for presence of the transgene by PCR. Two pairs of primers were used TetON+ 5'ACAGCGCATTAGAGCTGCTTAATGA3' and TetON- 5'GAGTGCATATAACGCGTTC TCTAGT 3', and another pair to amplify the TRE response gene TRE474+ 5'CCTCTAGAG GATCCAGACATGATAA 3' and TRE715- 5'TCAGCCATACCACATTTG TAGAGGT3'.

Laboratory animal experiments were approved by the Hebrew University's Committee for Animal Experimentation.

Dox administration Dox hydrochloride (2 mg/ml, Sigma) dissolved in 5% sucrose supplied as drinking water, which was exchanged every 3 days for 6 consecutive days.

LPS administration Bacterial LPS (*E. coli* O55, Disco labs, Detroit, MI). Was administered intraperitoneally (i.p., 50 µg/kg) dissolved in saline.

RT-PCR RNA was extracted using EZ-RNA total RNA isolation kit (Biological Industries, Beit Haemek, Israel). Reverse transcription and PCR amplification were both performed using the OneStep RT-PCR Kit (Qiagen) with the specific primers mentioned previously.

Protein extraction The dissected brain tissue samples were homogenized in 1:9 wt/ vol LSD buffer (0.01 M sodium phosphate pH 7.4, 1% Triton X-100). Tissue samples from intestine were homogenized in 1:9 wt/vol solution D (0.05M Tris pH 7.4, 1 M NaCl, 2 mM EDTA, 1% Triton) in a glass-teflon homogenizer to ensure complete disruption of tissues. Complete Protease Inhibitor Cocktail Tablets (Hoffmann-La Roche, Basel, Switzerland) were added to the homogenates.

Non-Denaturing Gel Electrophoresis Serum was electrophoresed (10 µl of 1:10 dilution per lane) in 7% non-denaturing polyacrylamide gels; catalytically active AChE was stained histochemically (Karnovsky and Roots, 1964).

Denaturing Gel Electrophoresis Samples were separated on 7% or gradient 4-20% polyacrylamide gels (Bio-Rad, Hercules, CA), blocked (1 h, 3% nonfat dried milk, 2% BSA, 0.2% Tween-20 in TBS), and blotted.

Immunodetection Immunodetection was done with rabbit anti-N terminus AChE antibodies (N-19, Santa Cruz Biotechnology, CA), dilution 1:500; and polyclonal rabbit anti-ARP antibodies (Sternfeld et al., 2000), diluted 1:500. Secondary antibodies were horseradish-peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse (1:10,000, Jackson ImmunoResearch Laboratories, West Grove, PA). Chemiluminescent detection was with the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Life Sciences, Uppsala), as instructed.

Quantification of corticosterone levels Corticosterone levels were measured from plasma samples using the Coat-A-Count® Rat Corticosterone kit (Diagnostic Products Corporation, Los Angeles, CA) according to provided instructions.

Results

The design and optimization of exogenously expressed AS and RZ sequences

In order to conditionally and transiently manipulate AChE gene expression we designed endogenously expressed AS and RZ constructs and cloned them under the control of the tetON inducible promoter system. We have previously identified several sites on the AChE mRNA which are efficiently cleaved by RZs *in vitro* (Birikh et al., 1997). The most efficient was the target site on exon 6 of the AChE mRNA (Fig. 1A). We attempted to check whether this site would be as efficient *in vivo*. The synthetic RZ sequence used in the *in vitro* experiments had to be modified for *in vivo* use: an endogenous RZ has to be imbedded in a longer RNA molecule, though the length of the annealing arms remained 7 nt long as in the *in vitro* RZ. Addition of expression vector encoded flanking 3' and 5' regions is also necessary for efficient expression and RNA stability. Since others have shown that intramolecular base pairing can destroy the RZ structure, a stable stem-loop structure-forming sequence was inserted in the center of the expressed part of the RNA gene. The RZ gene was positioned within the loop to allow the structure of the RZ to form independently of the structure of the flanking regions (Lieber and

Strauss, 1995). The resulting 51 bp long duplex coding for the catalytic domain and the stem determinant was synthesized and cloned into pTRE plasmid (Clontech) behind the minimal CMV promoter.

In the overall RZ transcript structure, the catalytic core is embraced within a stem-loop structure which is followed by the vector encoded extended 3' fragment (Fig. 1B). In order to verify that the extended 3' fragment does not interfere with the correct folding of the RZ we compared *in vitro* the catalytic activity of a full length RZ transcript and a shortened transcript devoid of the 3' region. To *in vitro* transcribe the truncated 95 nt RZ and the full length 290 nt RZ two PCR fragments with the T7 promoter introduced in the upstream primer were generated these fragments served as templates for transcription. As a substrate for these RZs another *in vitro* transcribed 150 nt fragment of AChE mRNA was generated (Fig.1C). No difference was observed in cleavage efficiency of these two RZs, indicating that the 3'-flanking region does not interfere with the RZ activity (Fig. 1D, E). The AS sequence, a DNA fragment encoding a long chain of AS RNA (100 nt), was also targeted to exon 6 of AChE mRNA (Fig. 1B), which has been shown to be vulnerable to AS-mediated inhibition of AChE expression in PC12 cells (Grifman et al., 1998) was also cloned into the pTRE plasmid (Clontech).

Transient transfection of Tet-off CHO cells with the RZ and AS expressing constructs The TetON and TetOff Systems are based on regulatory elements derived from the *E. coli* tetracycline-resistance operon: the Tet repressor protein (TetR) and the Tet operator DNA sequence (*tet*O). Regulator (tet) and response plasmids (TRE) deliver these elements into the cells where they are integrated into the host genome to establish a double-stable cell line. Once established, this cell line responds to tetracycline or its derivatives, such as dox in a dose-dependent manner, allowing the controlled expression of the target gene.

The pTetOff regulator plasmid expresses a fusion protein known as the tetracycline-controlled trans-activator (tTA), which is composed of TetR and the VP16 activation domain (AD). tTA activates transcription in the absence of dox. The TetOn System uses the pTetOn regulator plasmid, which expresses the reverse tet controlled transactivator (rtTA), and activates transcription in the presence of dox. pTRE is the response plasmid used by both systems. It encodes the tetracycline response element (TRE), which contains seven repeats of the *tet*O sequence, and the target gene. tTA or rtTA bind to the TRE, activating the transcription of the target gene.

To test the tetracycline responsiveness of the resulting plasmids we first checked RZ and AS activity in a transient transfection of CHO-TetOff cells (Clontech) which are stably transfected with a pTetOff plasmid. These cells do not express endogenous AChE, therefore, we co-transfected each of the RZ/AS or empty vehicle with an AChE-S expression construct (Ben Aziz Aloya et al., 1993) at a ratio of 10:1 respectively. AChE activity was determined three days post-transfection (Fig. 2A). In the absence of dox (transcription is activated) both RZs and AS showed about 30% and 50% inhibition of the AChE activity respectively. The empty vehicle had no effect on AChE activity in these cells. In the presence of dox (10 µg/ml), AS still inhibited the AChE expression to the same extent, probably due to leakiness of the system. Co-transfection of the RZs/AS or empty vehicles with an AChE-R construct (Seidman et al., 1995) gave similar results with the AS construct but the RZ construct was not effective (Fig. 2B).

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Stable transfection of PC12-Tet-ON cells with the RZ and AS expressing constructs To establish cell lines of neuronal origin with inducible inhibition of AChE expression. We stably transfected the PC12-TetON cell line (Clontech) with the AS and RZ expressing constructs. In order to find the colonies which are the most responsive to doxycycline, we screened 50 colonies from each transfection. Two RZ and two AS lines with highest response to dox were selected for further investigation. However, when we compared these cells to the non-transfected PC12-TetON cells, the overall AChE level was greatly reduced, indicating that the RZ/AS expression control was leaky (Fig. 3A). Interestingly, the overall inhibition of AChE expression by the RZ was greater, than that of the AS. To test which of the AChE isoforms were suppressed, proteins from the AS expressing cell lines were western-blotted with antibodies targeted to the AChE N-terminal this antibody detects all isoforms, or to the C-terminal peptide of the stress-associated AChE-R isoform. The overall amount of the AChE protein was reduced as expected (Fig. 3B). This reduction occurs even before dox addition as we saw in the previous experiment due to the leakiness of the system. Dox addition further reduced AChE levels. AChE-R specific immunolabeling was decreased in the AS transfected cells indicating suppression of this transcript. These results are in agreement with Grifman et al which showed by RT-PCR a reduction of ACHE-S and a complete suppression of the AChE-R transcript (Grifman et al., 1998).

AChE suppression is associated with changes in NGF-induced neurite outgrowth
Since previous studies attribute a morphogenic role of AChE in neuritogenesis in various systems from different organismal origin (Grifman et al., 1998; Koenigsberger et al., 1997; Layer and Willbold, 1995; Sharma et al., 2001; Small et al., 1995) We examined the effect of AChE suppression on neurite extension following NGF-mediated differentiation in the AS and RZ expressing cell lines. Population analysis of NGF-induced neurites in parent PC12 cell line following dox administration showed longer (Fig. 4A, Student T-test, $P < 0.01$) and narrower (B, $P < 0.01$) neurites. AS primarily reduced NGF-induced process elongation (C, $P < 0.01$) without changing their width (D). The morphology of the non-differentiated cells remained typical for PC12 cells (Fig. 4, insets). In contrast, RZ expression, reduced process length (E, $P < 0.05$) and decreased process width (F, $P < 0.01$) upon NGF treatment yielding a fibroblast like shape of the cells (inset), as reported by Grifman et al. (Grifman et al., 1998).

Inducible in vivo suppression of AChE expression To further explore AChE suppression effects *in vivo* transgenic mice expressing either TetOn or the AS/RZ constructs were generated. Mating of heterozygous mice from the two lines created binary transgenic animals expressing both transgenes. Mice of the parent CBA/6 strain served as controls. Every mouse was checked for the presence of both transgenes by PCR. Animals were offered dox in their drinking water (2 mg/ml) for 6 consecutive days and transgene expression in various tissues was confirmed by RT-PCR. Since Grifman et al. (Grifman et al., 1998) which used the same AS-ON as was used to generate our mice showed the this ON reduced the amount of AChE-S transcript and a completely suppressed the expression of the AChE-R transcript we aimed to verify if this is the situation *in vivo* as well. We therefore stressed the mice by LPS injection (50 µg/Kg) in order to induce endotoxic stress and to elevate AChE levels. Mice were sacrificed 24 hr post-treatment, plasma samples were removed and AChE activity in various tissues was measured. Plasma samples displayed significant suppression (Student's t test, $P < 0.05$) of AChE activities following dox treatment of AS mice, as compared to similarly treated mice of the parent strain (Fig. 5A).

Intestinal homogenates presented lower AChE activity, even in untreated Tet-AS mice, as compared to controls (which were insensitive to dox treatment, data not shown). Dox treatment, further, reduced intestinal enzyme activity, suggesting both leakiness and tetracycline sensitivity of the suppression activity (Fig. 5B). A similar pattern was observed in cortical homogenates, with yet higher significance (Fig. 5C). In contrast, there was no apparent change in hippocampal homogenates (Fig. 5D). AChE activity was also measured in tissue homogenates from TetON/RZ mice, in a similar fashion. In all tissues tested there were no significant differences in AChE activity between the transgenic animals who had received doxycycline and the control groups (data not shown).

AS expression selectively suppress the AChE-R variant production in vivo In order to determine which of the AChE variants is more sensitive to the AS suppression *in vivo* the above plasma samples were subjected to non-denaturing PAGE and blotting. Parallel gels were subjected to blotting and immunolabeling with anti-AChE-R antibodies or to cytochemical staining of AChE activity (Fig. 6). Plasma samples from AChE-R transgenic mice with constitutive AChE-R overexpression served as controls (Sternfeld et al., 1998). Of the three alternatively spliced *ACHE* products the primary AChE-S variant forms tetramers, the erythrocytic AChE-E protein appears as glycophosphoinositide-bound dimers and the stress-induced AChE-R variant remains monomeric. In the Non-denaturing gel the monomeric AChE-R (Fig. 6, black arrows) migrates considerably faster than the multimeric forms.

Other clusters of cholinesterase activity were not detected by the anti-AChE-R antibody, perhaps indicating that they consist of AChE-E dimmer complexes, including the erythrocytic variant with other plasma proteins (Fig.6, white arrows). The cholinesterase activity at the top of the gel probably belongs to AChE-S tetramers. Control (wt) mice showed no AChE-R bands in activity gels under normal conditions and intensified labeling following stress. Intriguingly, the TetON/AS mice showed no rapidly migrating immunopositive bands with anti-AChE-R antibodies (perhaps reflecting leakage of the TetON system which induces constitutive suppression of serum AChE-R levels). Moreover, they totally failed to induce AChE-R accumulation following LPS administration (Fig. 6). Immunoblot analysis also showed accumulation of AChE-R in the plasma of a wt mouse that underwent stress, an effect that has been demonstrated in previous studies (Kaufer et al., 1998). Immunoblots of the non-stressed transgenic mice (AS) showed slightly weaker signals than those of the non-stressed control mice (wt). However, the greatest difference was between the stressed transgenic mice (AS + stress) and the stressed control mice (wt + stress). In striking contrast to the stressed wt mice, stressed transgenic mice showed no increase in AChE-R expression on either of the gels. This indicates that the AChE-AS gene was indeed expressed in the transgenic mouse, and it's suppression of AChE activity all but eliminated the stress-induced overexpression of AChE-R. Previous studies have shown that the AChE-R isoform is much more susceptible to suppression by AS than AChE-S (Grifman et al., 1998), so it is not surprising that the upper bands are not suppressed as effectively as the AChE-R. Corticosterone levels in plasma of transgenic TetON/AS mice that were subjected to endotoxic stress were significantly lower in mice that were administered doxycycline prior to stress than in those that were not administered doxycycline. This difference may be attributed to the expression of the AS-AChE transgene in the animals that had received dox prior to stress induction.

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Discussion

We attempted to create *in vitro* and *in vivo* model systems in PC12 cells and transgenic mice with inducible suppression of specific AChE isoforms. For this purpose we combined AS-ON and RZ techniques with the inducible tetracycline controlled system. The main drawback of the original tetracycline system (TetOff), is that the animal model must be continuously fed with tetracycline or its derivatives in order for gene of interest to remain silent. In the improved TetOn system the "reverse" tTA (rtTA), activates transcription in the presence, rather than in the absence, of tetracycline. In this system the expression level of the target gene in the presence of a dox was reported to be induced to a level of up to 1000-fold over the background. (Gossen et al., 1995) The use of RZ under the control of tetracycline system was reported previously (Thomas et al., 2001; Thybusch-Bernhardt et al., 2001; Wirth et al., 2002, and others] All but one of these reports used the TetOff system and reached 40-70% inhibition of the target gene with a reasonable amount of background inhibition. However, in Thomas et al, where the TetOn system was used, extensive leakiness of expression was observed. In our study two independent stably transfected cell lines showed more than 80% inhibition in the induced state and only a slightly lower inhibition in the non-induced state, indicating that this RZ efficiently operates even at low expression levels. This basal leakiness of the system is especially significant in the case of the RZ or AS expression, because although on the one hand they might efficiently work at very low concentrations, on the other hand once the concentration is well above the K_m its reaches saturation it terms of activity. Adding to this the feedback response, which is reported to be activated following AChE inhibition (Kaufer et al., 1998), and the RZ might be even more efficient at lower concentrations. The above considerations might explain the seemingly contradictory results obtained with the transient and stable transfection. Under transient transfection conditions, where the expression level of the RZ and AS should be higher, the percentage of inhibition is lower. This might well be, because the RZ level reached the saturation point, whereas it must cleave a much higher level of the AChE mRNA. However, modulation of its activity using tetracycline-controlled expression system proved to be difficult, apparently due to the catalytic nature of the RZ, complex expression control of the AChE gene and leakiness of the TetOn system.

The physiological effectiveness of the AS and RZ agents was tested by measuring the length and width of NGF induced neurite outgrowth. The AS sequence predictably suppressed neurite extension, but the RZ suppressed both length and width, compatible with its enhanced effectiveness. Intriguingly *in vivo*, the AS and not the RZ agent appeared effective.

AS efficiency was further confirmed *in vivo* by the results of the AChE activity assay (Fig. 5), which unambiguously demonstrated the ability of the TetON/AS transgenes to effectively lower the levels of AChE in the transgenic animal's cortex, hippocampus (although not significantly) and intestine, and to prevent the plasma AChE-R accumulation under endotoxin stress (Fig. 6A). The results in plasma samples are somewhat puzzling, it would seem that transgenic mice that did not receive dox have higher AChE activity than the wt controls. While this may be an artifact, it may also confirm the existence of feedback regulation of AChE-R in the blood. Taken together, these results make clear that oral administration of dox indeed induced AS-AChE expression in the TetON/AS mice.

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The finding that AS-AChE expression causes a reduction of up to 50% in corticosterone levels in the plasma of the TetON/AS transgenic mice that received dox is further evidence of the involvement of AChE (very possibly AChE-R) in stress response pathways (Fig. 6B). While AChE gene expression is known to be regulated by corticosteroids via a Glucocorticoid Response Element (GRE) upstream of the *AChE* gene (Meshorer et al., 2002), it is not clear as yet why the levels of corticosterone in the animals expressing AS-AChE are lower than those of controls. However, we do know that corticosteroids are largely produced in the adrenal, which is subject to cholinergic innervation (Sapolsky, 1996a; Sapolsky, 1996b). Thus, it appears from our current study that AChE-R and corticosteroids are subject to reciprocal regulation supported by reports the anti-cholinesterases, which induce AChE-R overproduction, also induce elevations in cortisol levels (Kaufer et al., 1998).

These findings do much to emphasize the important role that TetON/AS mice could play in future research of the stress response. In addition, the difference in susceptibility to antisense suppression between the different AChE isoforms suggests that the TetON/AS transgenic mouse lines will prove invaluable to future studies of the non-catalytic functions carried out by the various AChE splice variants.
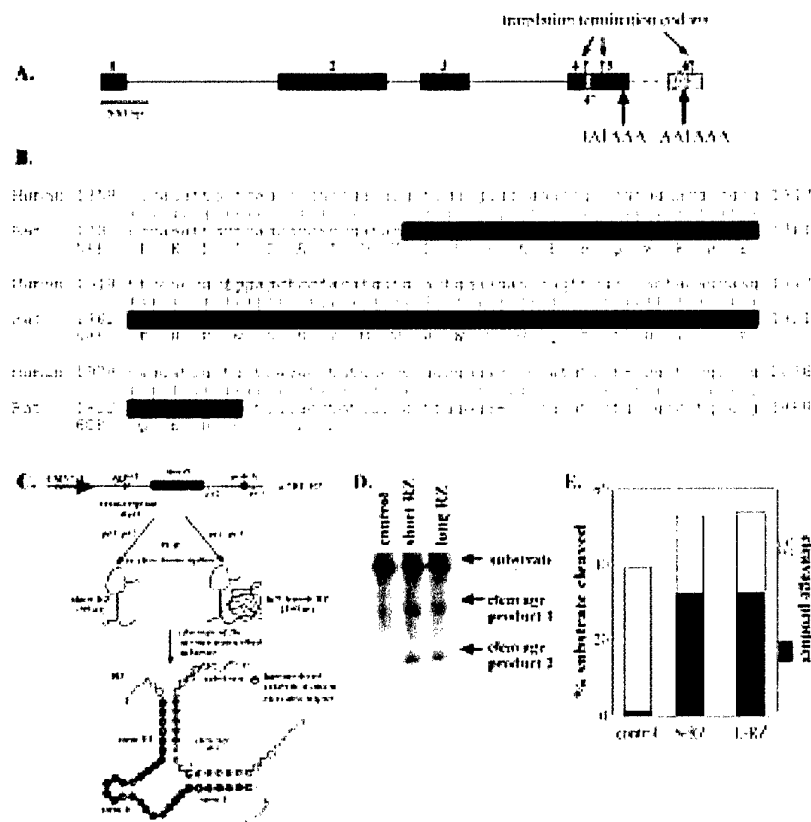
Fig. 1. Designing and optimizing the AS and RZ sequences. A. Schematic presentation of AChE mRNA splicing isoforms. The target site for the RZ and AS-ON is indicated as concentric

circles. B. Aligned sequences of the target region from human and rat AChE-S mRNA. Note that the RZ binding site (indicated in yellow) is conserved between these species. The murine AS-ON sequence has only two mismatches with rat mRNA on the span of a 100 nt (red). C. Experimental paradigm of testing of the interference of the vector encoded 3'-flanking sequence of the RZ with its activity *in vitro*. D. Autoradiograph of the RZ cleavage reactions. E. Comparison of cleavage efficiencies of the short and long RZs.
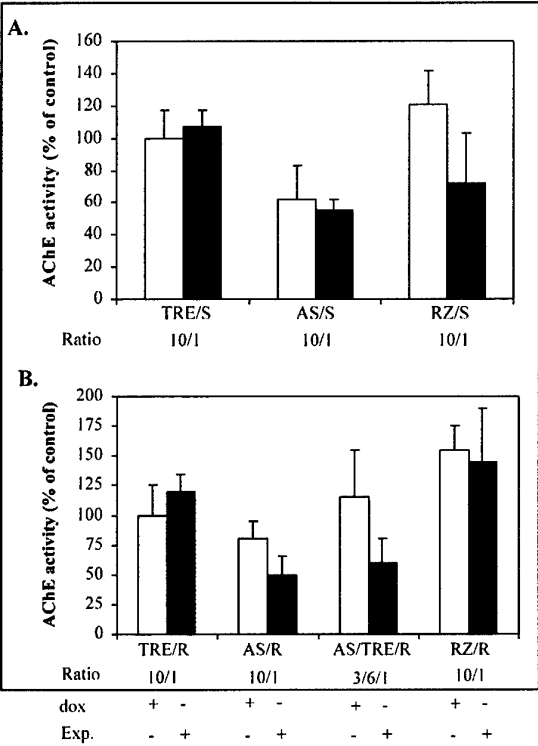
Fig. 2. Inhibition of the recombinant AChE expression by RZ and AS in CHO tet-off cells. AS and RZ efficacy was evaluated in transiently transfected CHO-Tet-Off cells. Ratios of the substrate encoding plasmid pAChE-S (S) or pAChE-R (R) and the RZ/AS/vehicle (TRE) are indicated.

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A.
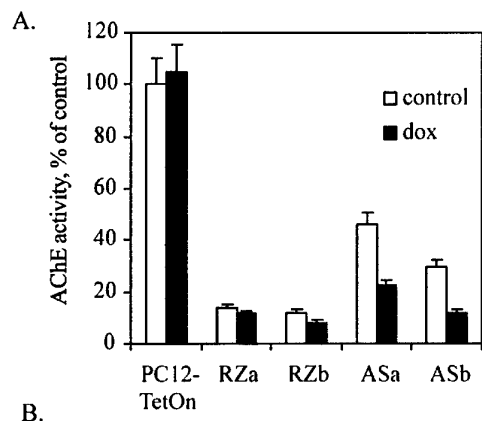
Fig. 3. Stable transfection of PC12-Tet-ON cells with the RZ and AS expressing constructs. A. Inhibition of endogenous AChE expression in stably transfected PC12-Tet-on cell lines with AS/RZ constructs. Two independent cell lines for each construct are shown. The AChE activity is presented as percent of PC12 control. Note that significant dox-induced inhibition is observed in addition to basal dox independent inhibition. B. Immunoblot analysis. Cell lines were grown in the presence (+dox) or absence (-dox) of dox.

B.
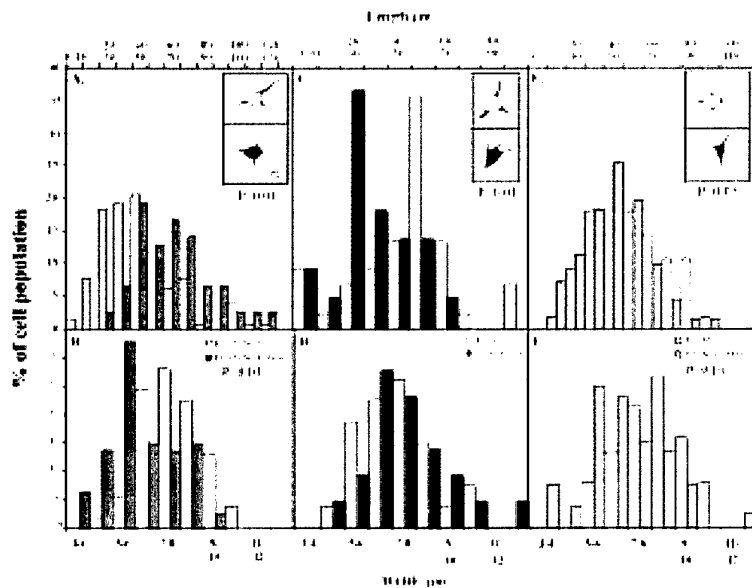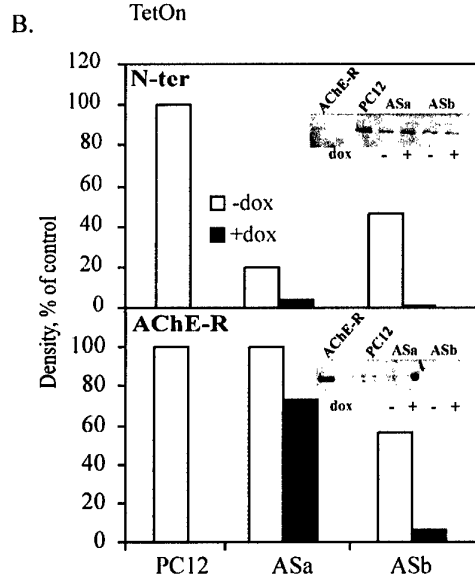
Fig. 4. NGF-induced differentiation of parent and AS/RZ cell lines. A. Three days after NGF treatment, transfected cell lines extended abnormally-shaped processes in response to NGF. Lower panels: population analysis. AS expression primarily reduced NGF-induced process elongation (C, $P < 0.01$) but did not change process width (D). In contrast, RZ expression reduced process length (E, $P < 0.05$) and decreased process width (F, $P < 0.01$).
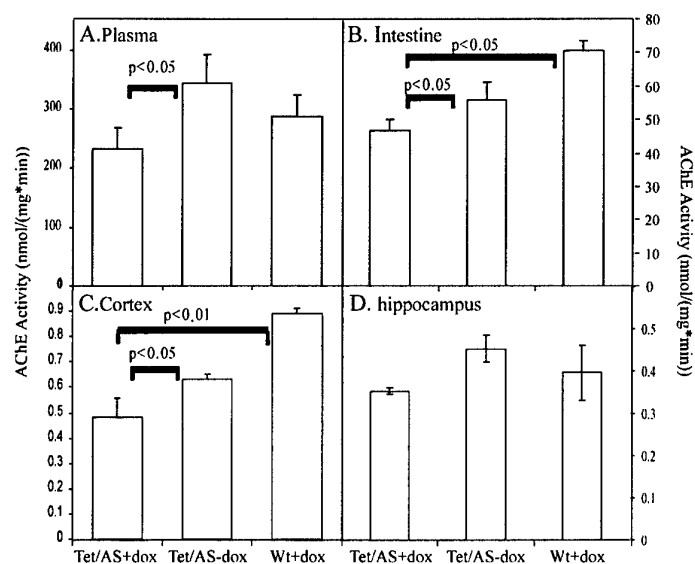
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Fig. 5. Inducible in vivo suppression of AChE activity AChE activity in (a) plasma (b), intestine (c) cortex, and (d) hippocampus from transgenic TetON/AS mice who underwent activation of the AS gene by doxycycline administration (Tet/AS+dox), TetON/AS mice that were not administered doxycycline (Tet/AS-dox), and control mice that were administered doxycycline (w.t.+dox).
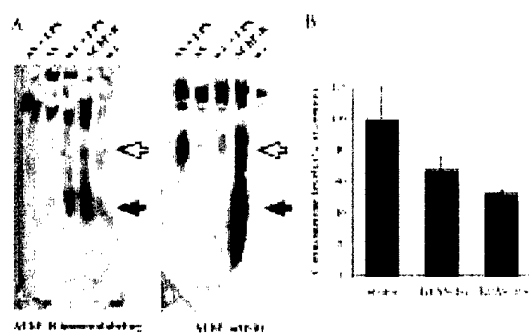

Fig. 6. AS expression selectively suppresses the AChE-R variant production in vivo.
A. a. Immunolabeling with anti-AChE-R antibody. b. cytochemical staining for AChE activity (left to right) Tet-On/AS mouse after doxycycline administration and endotoxic stress induction (AS + stress), Tet-On/AS mouse after doxyclycine administration without stress induction (AS), wild type CB6 mouse after endotoxic stress induction (wt + stress), mouse from a separate transgenic line which over-expresses AChE-R (AChE-R), control; − wild type mouse that was not stressed (wt).White arrows indicate presumed position of AChE-R. B. Corticosterone levels in stressed AS and control mice. Note that the corticosterone levels are much lower in the AS mice that received doxycycline.

A rare *ACHE* – *PON1* haplotype increases the risk of insecticide-induced Parkinson's disease

Parkinson's disease (PD) is a multi factorial, late onset neurodegenerative disease that occurs with an incidence of 1% in subjects of age 65 years and older (Anca et al., 2002; Lang and Lozano, 1998a; Lang and Lozano, 1998b). Biochemically debilitating polymorphisms in the Paraoxonase (*PON1*) gene, encoding the paraoxon-hydrolyzing enzyme paraoxonase, PON (Brophy et al., 2001); (Mackness et al., 1998) were associated with increased risk of PD (Akhmedova et al., 2001; Kondo and Yamamoto, 1998), as was chronic exposure to commonly used agricultural insecticides (e.g. organophosphates such as paraoxon, the major metabolite of parathion. Other inherited increases in organophosphate sensitivity include an activating deletion in the acetylcholinesterase gene, *ACHE* (Shapira et al., 2000), located 5.5 Mb upstream to *PON1* on Chr 7q21-22 (Getman et al., 1992; Shapira et al., 2000). Also, the AChE protein was reported to enhance the electrophysiological activity of substantia nigra dopaminergic neurons, which die prematurely in PD patients (Holmes et al., 1997).

Here we identify a rare haplotype in the human *ACHE/PON1* locus, including biochemically effective polymorphisms in both genes. In PD patients under chronic insecticide exposure, haplotypes of the *ACHE* polymorphism that induces anti-AChE hypersensitivity and *PON1* mutations that reduce PON activity were strongly over-represented, whereas in urban patients with no history of exposure, these haplotypes were under-represented. Our data indicate that *ACHE/PON1* polymorphisms jointly affect the insecticide-induced risk of PD.

We carried out nucleotide polymorphisms (SNP) analysis on two 7 Kb regions in the *PON1* (Brophy et al., 2001) and *ACHE* (Shapira et al., 2000) genes of 39 PD patients who live in a rural area that is under routine exposure to insecticides, especially parathion (Herishanu et al., 1989; Herishanu et al., 2001), 59 patients with PD from an urban area with no history of exposure and 454 unrelated disease-free subjects. Seven polymorphic sites and the *ACHE* promoter deletion were tested. SNP analysis revealed a total of 38 haplotypes in the Israeli population, out of the possible 338. Nineteen of these were represented in the PD patients, reflecting relatively high diversity in the haplotype composition of patients as compared with controls. The *ACHE/PON1* haplotypes can be divided into two subfamilies, designated H1 and H2 that differ in at least 5 positions, including the activating 4-nucleotide deletion in the *ACHE* promoter and the biochemically debilitating polymorphism in the *PON1* promoter (Fig. 1). The haplotype composition of insecticide-exposed and non-exposed PD samples did not differ substantially; however, the rare H2x haplotype was strongly over-represented in the exposed PD samples and under-represented in the non-exposed PD samples as compared with no-disease controls (Fig. 2).
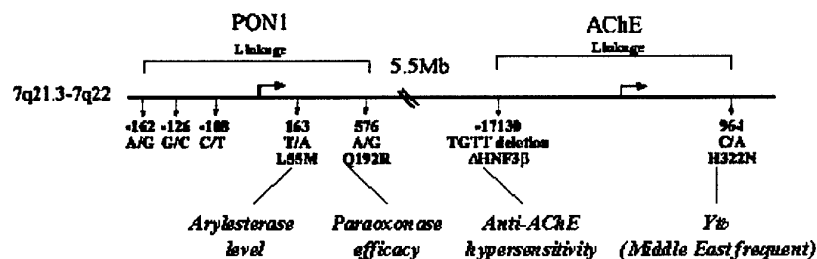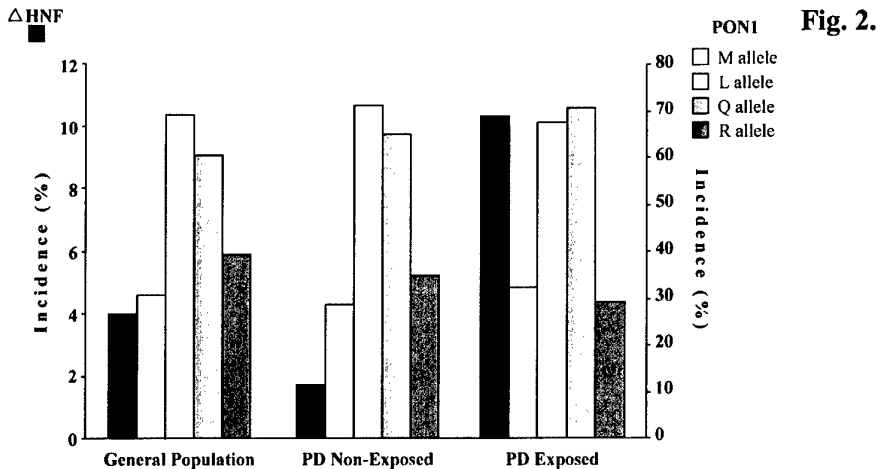

Fig. 1.

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Fig. 2.

Inherited AChE overproduction was predicted to compromise the capacity of carriers to respond to anticholinesterase exposure or stressful insults by secondary overproduction of catalytically active AChE (Shapira et al., 2000). Likewise, reduced PON activities could be expected to subject carriers' AChE to additional risk because of their insufficient capacity to hydrolyze irreversible organophosphate AChE inhibitors (Costa et al., 2003). To test this hypothesis, we measured serum AChE and PON activities in exposed and non-exposed PD patients and controls with or without the potentially predisposing haplotypes (Fig. 3). Significant differences were found in these enzyme activities, with both serum AChE (but not the homologous enzyme butyrylcholinesterase, BChE) and PON activity (but not protein levels reflected in arylesterase activity) being considerably lower in PD patients ($P = 10^{-17;}$ = 10^{-11}, respectively). In PD subjects, the AChE activity is greatly and significantly ($P =$) decreased in the promoter polymorphism carriers (Fig. 4). Measuring the arylesterase activity of paraoxonase provided information on the amount of the corresponding protein; this additional test demonstrated that the over-represented haplotype in exposed PD patients have normal or higher amounts but impaired activity of PON, which supports the notion that inadequately protected AChE in the circulation of these patients increases their risk of dopaminergic hyperactivation, leading to PD.
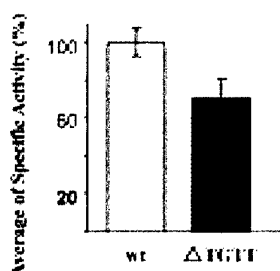

Fig. 3.

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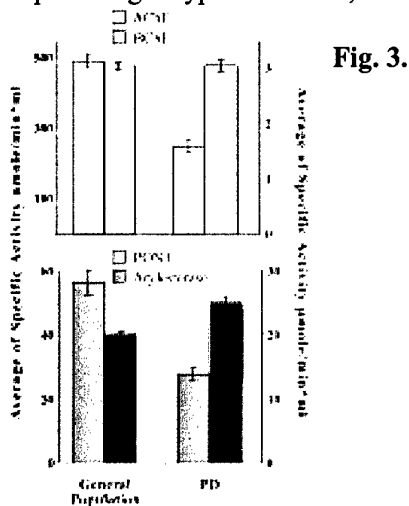
Fig. 4. AChE specific activity in PD subjects. The average serum values of carriers and non-carriers of the promoter polymorphism are expressed as a percent of the non-carrier average.

Several studies have indicated potential association of both AChE and PON with the environmentally-induced risk for PD. Agricultural workers, under chronic exposure to anticholinesterases, suffer increased risk of developing PD (Gorell et al., 1998; Semchuk et al., 1992). Carriers of the M allele of PON1, with deficient capacity to degrade paraoxon, present similar predisposition (Akhmedova et al., 2001). However, to the best of our knowledge, our study is the first to address the possibility that these 2 genes exert inter-related effects on the risk for neurodegeneration under the combined load of inherited *ACHE/PON1* allelic variants with decreased capacity to confront an environmental challenge. This assumption, further, is compatible with recent reports that associate AChE overproduction with apoptotic cell death (Zhang et al., 2002). In addition to the risk of PD, the M allele of PON1 was reported to increase the risk of early onset cardiovascular disease, where cholinergic neurotransmission should play an active role.

ACHE and _PON1_ genotypes and their relation to acetylcholinesterase and paraoxonase activity levels in humans

Introduction
Inhibitors of acetylcholinesterase (AChE) have many faces. They are extensively used as agricultural and household insecticides, and they are among most feared weapons of mass destruction as nerve gases; they also serve as drugs to treat several diseases, including Alzheimer disease and myasthenia gravis. Most of these inhibitors are organophosphates (OPs) or carbamate esters. Both classes are pseudosubstrates, which form a covalent complex with AChE in what would be the first phase of a normal enzymatic reaction, but rather than being quickly cleaved to a hydrolysis product of the pseudosubstrate and to the regenerated enzyme, the complex is quite stable. Even if it slowly hydrolyzes to regenerate AChE, the biological effect is to inhibit AChE for long periods of time. AChE inhibitors have been intensively studied and developed for many years, and have been responsible for new insights into the biological roles of acetylcholinesterase and related enzymes.

In mammals there are several levels of protection against environmental cholinesterase inhibitors. In blood, OP inhibitors they can be hydrolyzed by paraoxonase (PON); both OPs and carbamates inactivate butrylcholinesterase (BChE) and AChE. BChE is a broad substrate specificity esterase with no certain biological function. It is a major cholinesterase in plasma and it is thought to act as a "scavenger", hydrolyzing numerous natural and artificial substrates, and thus protecting AChE.

By hydrolyzing OPs, PON provides significant protection of AChE from OP intoxication. However, whether this is the "true" biological role of PON is uncertain: PON is a high-density lipoprotein (HDL)-associated plasma enzyme capable of protecting low-density lipoprotein (LDL) from oxidation, thus affording protection against cardiovascular diseases. In fact, this protection is affected by the polymorphisms in the PON gene (_PON1_) (Hong et al., 2001), which suggests that PON serves such a biological role.

Our group has previously reported _ACHE_ distal promoter polymorphism, associated with constitutive over-expression and hypersensitivity to cholinesterase inhibitors, such as pyridostigmine (Shapira et al., 2000). This 4-base deletion, which abolishes a HNF3β transctiption factor binding site, is almost 10-fold more frequent in the Israeli population than in the United States.

Genetic studies of the _PON1_ gene revealed many polymorphisms in the coding and regulatory region, which affect enzyme levels and its substrate specificity. Interestingly, the human _PON1_ gene maps to the q21-22 band of the chromosome 7 long arm, only 5.5 Mb from the _ACHE_ gene (Fig. 1), which raises the question whether it is coincidence or whether the proximity may serve a biological function; for instance, one can conceive of some kind of general regulation of the locus. We initiated this study to assess potential relations between PON1 and AChE activities in the sera, due perhaps to strains on these enzymes from exposure to OP pesticides, which are widely used in Israel. Furthermore, in light of the locally high frequency of the AChE promoter polymorphism, it was also interesting to note how _PON1_ polymorphisms affect both PON and

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AChE activities. As BChE also affects AChE activity, it, and one of the frequent polymorphisms of the *BCHE* gene, were also monitored.
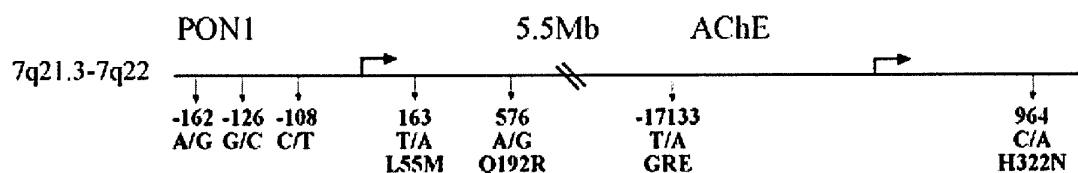

Fig. 1. The *PON1-ACHE* locus

Results and Discussion

Cholinesterase inhibitors can enter the body via inhalational, gastrointestinal or dermal routes and then is brought to target sites (brain, muscle) via the bloodstream. En route, OPs can be either hydrolyzed by PON1, which is present in blood, or inhibit BChE and AChE by covalent binding, in which case they are also inactivated. AChE inhibition leads to increased acetylcholine levels, which up-regulates AChE mRNA and finally AChE itself (Kaufer et al., 1998). This feedback loop is not functional in ΔHNF3 mutation carriers and stressed individuals, who have already elevated AChE levels and cannot increase its expression.

We began with genetic studies of *PON1* and *ACHE* by investigation of the genetic background of our study group. Genetic analysis of 3 promoter region and 2 coding region polymorphisms of *PON1* revealed that allele frequencies in Israeli population are generally similar to that reported for Caucasians, and different from Japanese as expected (Table 1). -108C/T is the most significant polymorphism in the regulatory region with a contribution of 22.4% to the variation in *PON1* expression, apparently by abolishing a potential SP1 transcription factor binding site. The -108C allele is associated with high PON levels and -108T allele is associated with low PON levels (Brophy et al., 2001b).

Table 1. Allele frequencies of the *PON1* and *ACHE* polymorphisms, compared to the previously reported frequencies.

position, allele		Israel; this study	USA; Brophy et al., 2001	Europe; Leviev & James, 2000	Japan; Suehiro et al., 2000
PON1					
-162					
	A	0.18	0.23	ND	0.10
	G	0.82	0.77	ND	0.90
-126					
	G	0.97	ND	ND	0.91
	C	0.03	ND	ND	0.09
-108					
	C	0.42	0.50	0.46	0.48
	T	0.58	0.50	0.54	0.52
162					
	T (55L)	0.64	0.64	0.65	0.94

A (55M)	0.36	0.36	0.35	0.06
575				
A (192Q)	0.65	0.73	0.69	0.40
G (192R)	0.35	0.27	0.31	0.60
ACHE				
-17116 ΔHNF3β	0.016	0.003[a]	ND	ND
H322N	0.044 n=79	0.05[b]	ND	ND

[a]from Shapira et al., 2000)
[b]from Bartels et al., 1993)

The polymorphism at position -162G/C has a minor effect, contributing to 2.4% of variation (by changing a potential NF-1 binding site) and -126C/G has no apparent effect on *PON1* expression (Brophy et al., 2001a; Brophy et al., 2001b; Costa et al., 2003; Suehiro et al., 2000). In the *PON1* coding region, we genotyped the substitutions of L55M (CTG to ATG), reducing PON mRNA and protein levels (Garin et al., 1997) and Q192R (CAA to CGA), which affects the catalytic efficiency and substrate preferences of PON (Davies et al., 1996). The 192R variant hydrolyzes paraoxon more efficiently than the 192Q variant, while diazoxon, sarin, and soman are better substrates for 192Q than for192R.

In our study, we only found 6 out of 9e possible genotypes, in agreement with reported linkage of 55L allele with 192R allele (Table 2). In our sample, the linkage of 192R to 55L was complete: 192R and 55M never appeared together. The most abundant genotype was MLRQ - heterozygous for both polymorphisms.

Table 2. Coding region *PON1* genotype distribution and corresponding enzyme activity levels for each genotype.

PON1 genotype	frequency, per cent	average activities, nmol/min/ml	
		paraoxonase	arylesterase
MMQQ	15	17.0	13.0
MMQR	0	--	--
MMRR	0	--	--
MLQQ	19	29.2	20.1
MLQR	23	71.7	19.0
MLRR	0	--	--
LLQQ	11	47.8	28.7
LLQR	18	70.8	21.3
LLRR	14	99.9	21.3

Allele 192R is known to be much more effective in paraoxon hydrolysis than 192Q. However it provides no substantial protection against paraoxon since its catalytic efficiency, although 7-fold greater than that of 192Q, is still too low. The experiments in *PON1* knockout mice showed that neither of the recombinant proteins, derived from two alleles could protect mice from paraoxon toxicity (Li et al., 2000).

In our data it is clear that each R allele increases the activity, with the highest activity associated with the RR genotype. As for the 55 position, it seems that the 55L allele is associated with high, and 55M with low, activity. It was reported that 55M allele is in linkage disequilibrium with -108T, which is associated with low PON activity, and that the 55 polymorphism effect results only from linkage with 108, and has no influence by itself (Brophy et al., 2001b).

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We investigated whether there is a connection between AChE and PON activities, and discovered that there is an inverse relationship between them, namely those individuals with low PON activity have higher AChE activity than those with high PON activity (Fig 2). No relationship was detected between BChE and PON. Given the ability of PON to hydrolyze OPs, it is tempting to speculate that people with high PON activity are protected against low-dose subacute exposure which is relevant to anyone consuming fresh agricultural production. In those with low PON levels, insecticides persist to inhibit AChE, leading to the feedback response and increased AChE levels.
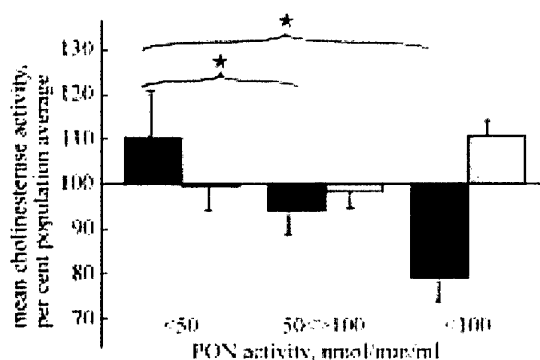

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This relationship may reflect the role of PON as a protection against the low, residual anticholinesterases, to which we are all exposed, e.g. pesticide residues in foods. The AChE of individuals with low PON levels up-regulate their AChE due to the severity of the exposure, while those having high PON levels are protected, hence their AChE remains low,

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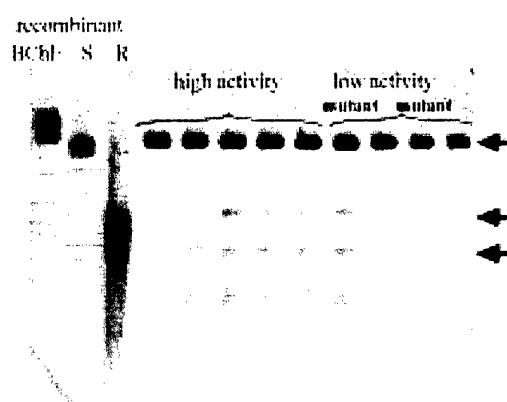
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The great inter-species variability of PON, and its high variability in humans (Fig. 4) are probably indications that PON is not a vital enzyme. Moreover, additional missense and even nonsense mutations have been reported recently without any noticeable effect on health. However, in modern times with our increasingly unnatural way of life and exposure to man-made toxic chemical its protective role may become significant.
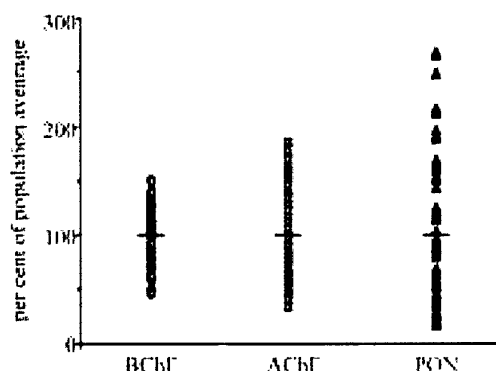
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Serum AChE may reflect brain AChE levels, and that its overproduction and/or lower turnover accompanies various neurological symptoms (Fig. 5).
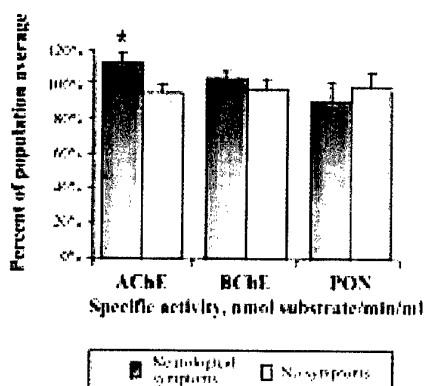

Fig. 5. Enzyme activities in sick vs. healthy subjects. AChE, BChE and PON specific activities ± standard error of the mean (SEM), normalized as percentage of population average, in non-exposed individuals who reported subjective neurologically-related symptoms (e.g. headache, anxiety or impaired memory and/or concentration ability) and in apparently healthy individuals. AChE activity, but none of the other enzymes, displayed significantly higher levels in individuals with neurological symptoms than in healthy subjects. Asterisk indicates $p < 0.05$.

The effect of PON on AChE may be both through destruction of OP anticholinesterases and through the action of PON to decrease free oxygen radicals (Fig. 6).
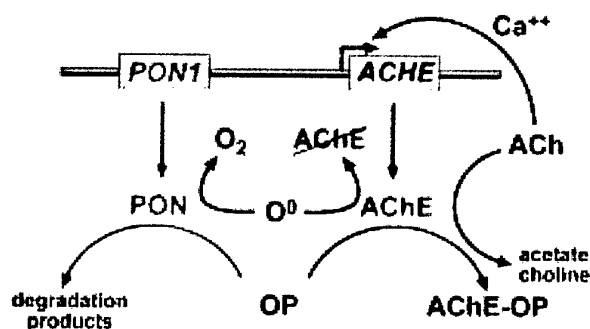

Fig. 6. A hypothesis. AChE overproduction is noted as Δ (delta activity). PON may regulate this reponse by hydrolyzing organophosphates (OP) that would otherwise inhibit AChE, or by (indirectly) interfering with the generation of free oxygen radicals (O^0), which inactivate AChE.

Materials and Methods

Subjects

The study was approved by the Helsinki Committee for human studies of the Israeli Army Medical Corps. A total of 91 generally healthy individuals, mostly men (average age 34.2 ± 8.9 years) from reserve units of Israeli Defense Forces, were available for this study. After they gave informed consent for the study, around 9 ml of blood were drawn to vacutainers, containing citrate. The blood was centrifuged at 4000 rpm at 4° C for 15 min in an Eppendorf centrifuge and the plasma was obtained. Whole blood and plasma were kept at -70° C until used. The subjects filled a questionnaire assessing general health status, medicine intake and demographic parameters, including known exposure to agricultural pesticides.

Genotyping

Genomic DNA was prepared from blood cells using the Gentra Whole Blood DNA Extraction Kit. Genotyping involved PCR amplification of the corresponding gene regions, using Taq

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polymerase (Sigma Chemical Co., St. Louis, MO) followed by agarose gel electrophoresis and Exo-Sap enzymatic purification (USB, Cleveland, OH) of the PCR product. Standard automated sequencing utilized the BigDye Terminator cycle sequencing chemistry, ABI 3700 DNA Analyzer and Data collection and Sequence Analysis software (Applied Biosystems, Foster City, CA). The reactions employed are detailed under Table 3. PON192Q/R polymorphisms were detected using the single nucleotide primer extension method (SNaPshot ddNTP Primer Extension kit, Applied Biosystems).

Table 3: PCR conditions for genotyping the different polymorphisms.

gene (accession no.)	polymorphism	primers	product size	PCR conditions
ACHE (AF002993)	ΔHNF3β T/A	(+)5'GTGAGAATGGCTG CTTCATA3' (-)5'CTCAGTTCTGGGAAATTCCTA3'	217bp	60°C, 37 cycles
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PON1 (AF539592)	PON-108 C/T PON-126 G/C PON-162 G/A	(+)5'ACTGAATCT CTC TGAGACGCAAGGACC3' (-)5'ATAGACAAAGGGATCGATGGGCGCAGACA3'	376bp	60°C, 37 cycles, 5% DMSO
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	PON192 Q/R A/G	(+)5'GGAATAGACAGTGAGGAATGCCAGT3' (-)5'CAGAGAGTTCACATACTTGCCATCGG3'	305bp	

Following PCR amplification and purification the SNaPshot reaction was performed using probe 5'-GGCAGAAACTGG CTCTGAAGAC-3' for the PON55 and 5'-GATCACTAT TTTCTTGACCCCTACTTAC –3' for PON 192. Following extension and calf intestine phosphatase treatment (Amersham Biosciences, Freiburg Germany), the products were electrophoresed on a 3700 ABI analyzer and the results analyzed with Genescan software.

Enzyme activity measurements

Plasma PON1 activity was determined by an adaptation of the spectrophotometric method (Furlong et al., 1989) to a microtiter plate assay. 10 μl of diluted (1:10) serum were placed in microtiter plate wells in duplicates; initiation of the reaction was by adding 190 μl of the substrate, 1.2 mM paraoxon, in 0.26 mM Tris-HCl, pH 8.5, 25 mM $CaCl_2$ and 0.5 M NaCl. Readings at 405 nm were repeated at 2-min intervals for 10 min. Non-enzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. Enzyme activity was calculated using the molar extinction coefficient for *p*-nitrophenol, 17,100 $M^{-1}cm^{-1}$.

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(ATCh, Sigma) or butyrylthiocholine (BTCh, Sigma) hydrolysis rates were measured following 20 min incubation with $5 \cdot 10^{-5}$ M tetraisopropyl pyrophosphoramide (Sigma), a specific BChE inhibitor, or 10^{-5} M 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (Sigma), a specific AChE inhibitor. Readings at 405 nm were repeated at 2-min intervals for 20 min. Non-enzymatic hydrolysis of substrate was subtracted from the total rate of hydrolysis. Enzyme activity was calculated using the molar extinction coefficient for 5-thio-2-nitrobenzoate, 13,600 $M^{-1}cm^{-1}$.

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Statistical significance for the differences between AChE, BChE and paraoxonase activities were calculated using Student's t-test.

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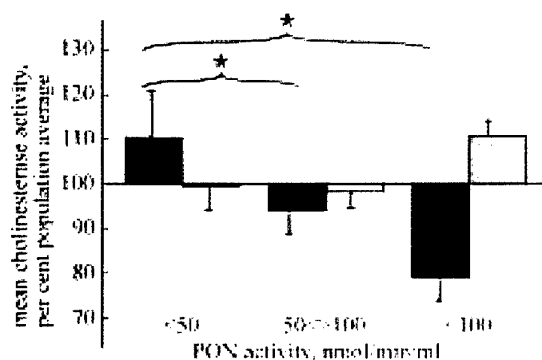

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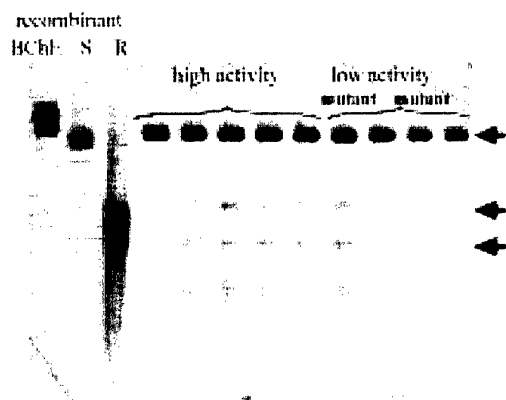

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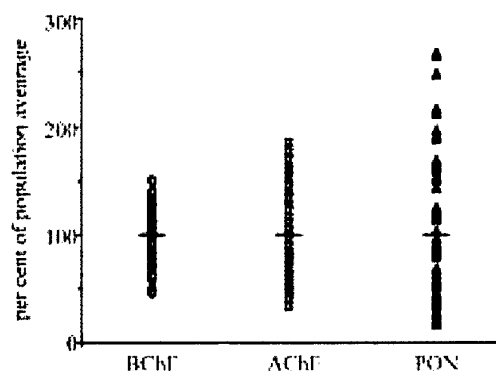

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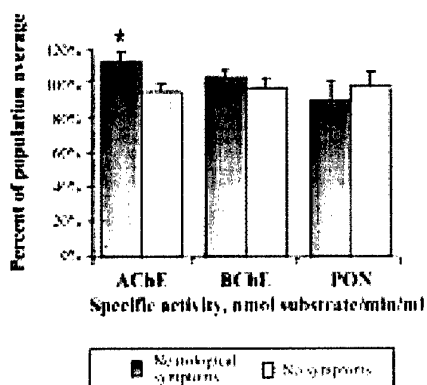

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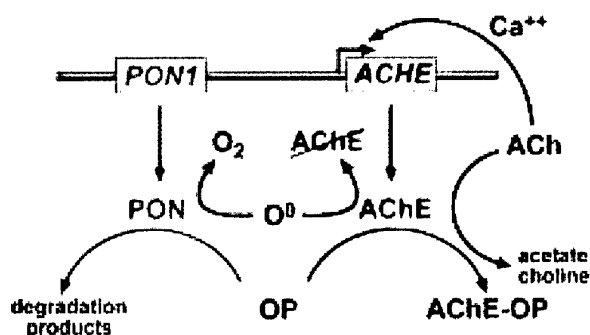

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Major lectures by H. Soreq:

July 2002: Genomics meeting of the German-Israel Foundation, Heidelberg, Germany, "Inherited impairments in cholinergic homeostasis are subjected to stress-induced intensification and antisense suppression"

October 2002: Meeting of the Federation of European Biochemical Societies, Istanbul, Turkey, "The Genomic Basis of Mammalian Stress Responses"

November 2002: VII International Cholinesterase Meeting, Pucon, Chile, "AChE as a window to stress responses"

February 2003: Research Advisory Committee on Gulf War Veterans Illnesses of the Dept. of Veterans' Affairs, Washington DC, USA,"EN101" and "Defining organismal cholinergic responses to CBW agents"

April 2003: 7th International Geneva/Springfield Alzheimer Meeting, Geneva, Switzerland, "Antisense diversion of molecular genetic impairments in cholinergic balance"

April 2003: Defense Advanced Research Projects Agency meeting, Galveston, TX, USA, "Human acetylcholinesterase isoforms from transgenic plants"

April 2003: Arizona State University, Tucson, AZ, "The cholinergic link to mammalian stress responses"

May 2003: 6th International AD/PD Meeting, Seville, Spain, "Genotype correlates underlying the cholinergic links to Parkinson's disease"

June 2003: European Molecular Biology Organization meeting on Molecular Medicine, Dubrovnik, Croatia, "Retrieval of cholinergic balance by antisense oligonucleotides: From animal models to clinical trials"

June 2003: Paul Martini Siftung meeting on therapeutic oligonucleotides, Berlin, Germany, "The antisense nucleotide EN101 retrieves cholinergic homeostasis: from animal models to clinical trials"

June 2003: 8th Symposium on Catecholamines and other Neurotransmitters in Stress, Smolenice, Slovakia, "Deciphering the Cholinergic Component of Stress-Associated Syndromes"

CONCLUSIONS

A major part of our effort was devoted to searching for protein partner(s) that interact with the AChE variants, and identifying potential phenotype(s) that may be associated with such interactions. The yeast two-hybrid screen that was performed yielded a number of candidate partners, of which we have so far focused on the scaffold protein RACK1, which, together with AChE-R, forms multiprotein complexes with PKCβII. Formation of such complexes was shown to be associated with intensified fear-induced conflict behavior, directing our attention to fear-associated phenotype(s) as reflecting imbalanced AChE expression (in terms of expression efficacy and/or splice variation).

Another focus of this research project related to the possibility to retrieve cholinergic balance by administering EN101, an antisense oligonucleotide which selectively induces destruction of the stress-induced AChE-R mRNA variant. EN101 was found to effectively suppress the behavioral impairments associated with neuronal AChE-R over-production in transgenic mice. It has further been shown to retrieve normal muscle action potentials in rats with experimental autoimmune myasthenia gravis; indeed, it successfully retrieves normal functioning in ongoing clinical trials with humans suffering from autoimmune myasthenia gravis. EN101 may, thus, become a clinically important deliverable of this research project.

At the level of basic research, several deliverables have been developed. These include a mouse model with inherited, inducible antisense suppression of AChE-R overproduction under external stressors (still under characterization); a phage display-selected monoclonal antibody directed toward the C-terminal peptide that is unique to the synaptic variant, AChE-S; and a procedure for comparing an individual's serum AChE activity with the value predicted for that individual's demographic criteria (age, gender, ethnicity and body mass index). And last, but not least, a thorough characterization was performed of the neuromuscular consequence of chronic AChE excess.

In summary, much of the research effort involved the post-transcriptional regulation of the *ACHE* gene and more specifically, alternative splicing regulation of AChE pre-m RNA. At the biomedical research level, this study has emphasized the importance of alternative splicing regulation for maintaining the cholinergic balance in mammals, as indicated by a number of adverse responses that result from impairments in this process. At the clinical level, we indicated putative contributions of this process toward diverse symptoms, including but not limited to, anxiety and neurodegeneration.

Findings under this research project pave the way to future developments, especially with regard to the implication in humans of changes in *ACHE* gene expression and the phenotype(s) that such changes may reflect. Particularly important is the association observed between state and trait anxiety on the one hand and with paraoxonase (PON) activities and genomic polymorphisms, on the other hand. Preliminary findings on the over-representation of interrelated *ACHE/PON1* polymorphisms in Parkinson's disease patients that had been chronically exposed to agricultural anticholinesterase insecticides should be pursued further in a large population, and the methods for determining the relevant criteria and predicting the involved risk should be worked out.

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The following abstract reports preliminary results of clinical trials of EN101, the antisense reagent that was developed under USAMRMC support. Although this work was not directly supported under DAMD-99-1-9547, the information will be of interest to the agency.

Objective: An open label, phase Ib trial of a novel antisense oligonucleotide EN101 (Ester Neurosciences, Ltd.) in patients with MG.

Background: EN101 is a 20-mere oligonucleotide with a sequence complementary to a coding region in the human acetylcholinesterase (AChE) gene, enabling it to prevent AChE translation. EN101 powerfully suppresses AChE production in vitro and reverses symptoms and signs in rats with experimental autoimmune MG (Brenner et al., FASAB J. 2003, In press).

Methods: 16 patients with stable generalised MG requiring constant AChE inhibitors (pyridostigmine) for daily function were recruited, after approval by human ethics committees. Patients were hospitalised and pyridostigmine was discontinued for 12-18 hours before EN101 testing. Assessment of MG status was performed at entry, after pyridostigmine stoppage and regularly after EN101 using the Quantitative MG score (QMG). Escalating oral doses of EN101 (10 - 150 µg/kg) were given in the first day, followed by a daily dose of 500 µg/kg for 3 days. This was followed by a washout period until reinstitution of pyridostigmine became necessary. Patients were monitored for 1 month thereafter.

Results: In 15/16 patients, the initial deterioration in MG status after stopping pyridostigmine was followed by a clear symptomatic improvement due to EN101. Analysis of the mean daily QMG scores showed a continuous decreased in each of the study days (decrease in QMG means improvement in disease status). The baseline (entry) mean total score was 13.2. The score decreased for days 2 through 6 in the amounts of 3.0, 4.8, 5.7, 5.5 and 6.0 ($p<0.001$). The mean percent improvements of total QMG for these days ranged from 27.8% to 53.4% ($p<0.01$). All the individual test item scores, except vital capacity and left arm out stretched time, had statistically significant change from entry for days 2 - 6 ($p<0.05$). Improved QMG scores following the final dose of EN101 were sustained for up to 72 hours. No serious adverse effects were observed. Vital signs, clinical chemistry, hematology, urinalysis, ECG, physical examination remained unchanged throughout the experimental period and in the following month after discharge. Cholinergic side effects were not reported.

Conclusions: EN101 appears to be powerfully effective in reversing symptoms in patients with stable MG. EN101 has potential advantages over conventional cholinesterase inhibitors with respect to dosing, specificity, side-effect profile, duration of efficacy and treatment regimen.