Molecular Psychiatry (2004) 9, 174–183

www.nature.com/mp

ORIGINAL RESEARCH ARTICLE

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

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Stress insults intensify fear memory; however, the mechanism(s) facilitating this physiological response is still unclear. Here, we report the molecular, neurophysiological and behavioral findings attributing much of this effect to alternative splicing of the acetylcholinesterase (AChE) gene in hippocampal neurons. As a case study, we explored immobilization-stressed mice with intensified fear memory and enhanced long-term potentiation (LTP), in which alternative splicing was found to induce overproduction of neuronal 'readthrough' AChE-R (AChE-R). Selective downregulation of AChE-R mRNA and protein by antisense oligonucleotides abolished the stress-associated increase in AChE-R, the elevation of contextual fear and LTP in the hippocampal CA1 region. Reciprocally, we intrahippocampally injected a synthetic peptide representing the C-terminal sequence unique to AChE-R. The injected peptide, which has been earlier found to exhibit no enzymatic activity, was incorporated into cortical, hippocampal and basal nuclei neurons by endocytosis and retrograde transport and enhanced contextual fear. Compatible with this hypothesis, inherited AChE-R overexpression in transgenic mice resulted in perikaryal clusters enriched with PKCβII, accompanied by PKC-augmented LTP enhancement. Our findings demonstrate a primary role for stress-induced alternative splicing of the AChE gene to elevated contextual fear and synaptic plasticity, and attribute to the AChE-R splice variant a major role in this process.
Molecular Psychiatry (2004) **9**, 174–183. doi:10.1038/sj.mp.4001446
Published online 28 October 2003

Keywords: hippocampus; fear conditioning; synaptic plasticity; learning and memory; LTP

Introduction

Acute stress intensifies the evolutionarily advantageous memory of events that are potentially threatening to the organism.[1] 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,[2] which is also involved in forming episodic, spatial and contextual memories.[3–7] 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.[6,8–10] Stress-induced changes in hippocampal functioning require protein and RNA synthesis,[11] are associated with differential expression of immediate early genes (eg c-fos[12]) and involve alternative splicing of numerous transcripts, including potassium channels[13] and acetylcholinesterase (AChE).[14] Unlike the abundant AChE-S 'synaptic' variant, the stress-induced AChE-R possesses a hydrophilic C-terminus that is expected to be incapable of supporting membrane adherence. Neuronal AChE-R accumulation is accompanied by long-lasting hyperexcitation of glutamatergic activity[15] and prolonged conflict behavior.[16] 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,[17] stress-enhanced fear memory and facilitated long-term potentiation

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Received 04 July 2003; revised 25 August 2003; accepted 19 September 2003

(LTP) in the hippocampus.[18] Here, we report that, in the 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.

Materials and methods

Immunohistochemistry
Animals were deeply anesthetized and transcardially perfused at several time points (0, 1, 2, 3 or 24 h) after the end of the stress session. After the 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[19] included for detailed analysis were AP −1.34.[20]

Western blotting
Hippocampi were dissected out and immediately homogenized at 4°C with a plastic homogenizer, in a 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 *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, USA). The blot was probed using an anti-ARP antibody.[21] 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.[15] InvEN101 is the inverted sequence (negative control oligodeoxynucleotide). A measure of 5 μM oligodeoxynucleotides was 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).

Cannulation
Double guide cannulae (C235, Plastics One, Roanoke, VA, USA) were implanted into both lateral brain ventricles, using a stereotactic holder at Bregma AP 0 mm, lateral 1 mm and depth 3 mm. Alternatively, the cannulae were directed toward both dorsal hippocampi, AP −1.5 mm, lateral 1 mm and depth 2 mm.[19] 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.[18]

Peptide
Mouse (m)ARP (GRRMEWGEQGMHKAARVGRR-GERWGAKHRV) 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 four-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[22] was from Phoenix Pharmaceuticals (Belmont, CA, USA).

Fluorescent peptides 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. A time period of 5 h was allowed for endocytosis and peptide transport before the sacrifice period, followed by transcardial perfusion with 4% paraformaldehyde. Coronal brain sections (10 μm) were cut with a cryostat. The labeled peptides (NEN, Zaventhen, Belgium) were RITC-ARP (GRRMEWGEQGMHKAARVGRR-GERWGAKHRV) and FITC-ASP (DTLDEAERQWKA EFHRWSSYMVHWKNQFDHYSKQDRCSDL). Sections were inspected by means of confocal or fluorescent microscopy. Biotinylated ARP (NEN) was detected by rhodamine-labeled streptavidin (Sigma, St Louis, MO, USA), following 6 h incubation.

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

Immobilization stress
An acute immobilization stress of mice consisted of taping their limbs to a plastic surface for 1 h.[23]

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 a single footshock (US, 0.7 mA, 2 s, constant current). Under these conditions, the context served as the 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.[6] Freezing was recorded 24 h later in the same fear conditioning box for 180 s without tone presentation.[18]

Slice preparation and electrophysiology[18]
Hippocampal slices were obtained from 3–6 month-old male BALB/*c*, FVB/N or transgenic mice. Animals were killed 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_2PO_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 intertrain interval, pulse width doubled to 0.1 ms in the tetanus. The TBS consisted of 5 × 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, P8083), diluted 1 : 500. Secondary antibody solutions and preparation for microscopy were as detailed.[16] Slices were scanned by using a Bio-Rad MRC-1024 scanhead (Hemel Hempsted Herts.,UK) coupled to an inverted Zeiss Axiovert 135M microscope with a 40 × oil-immersion objective (NA 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 ± 16 nm) for detecting tetramethylrhodamine and a 525/40 filter for detecting fluorescein. The confocal iris was set to 3 mm. The 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, USA).

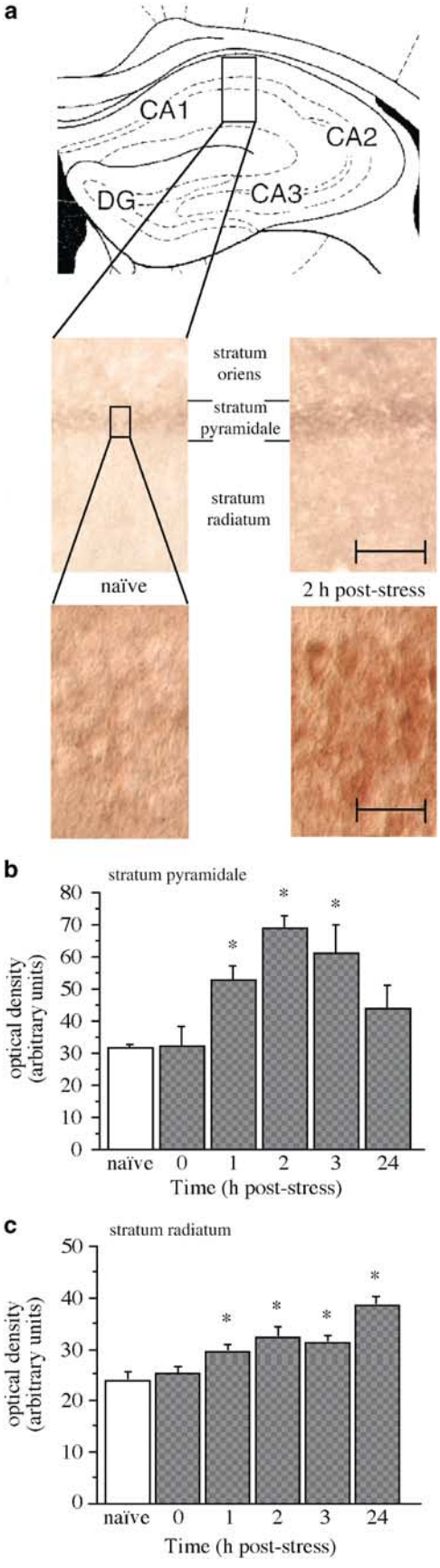
Statistics
Statistical comparisons were made by using unpaired Student's *t*-test and ANOVA. Significance was determined at the level of $P<0.05$ or 0.01.

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[21] revealed significantly increased labeling intensity of the stratum pyramidale and stratum radiatum 1, 2 and 3 h after 1 h immobilization, as compared to naïve mice (Figure 1a–c). This was compatible with the rapid post-stress increase of hippocampal AChE activities.[13] In the stratum pyramidale staining was maximal 2 h after immobilization and returned to baseline within 24 h (Figure 1b), demonstrating the transient nature of this response. In contrast, the labeling intensity of stratum radiatum was maximal at 24 h after immobilization (Figure 1c).

Enhanced memory of contextual fear depends on stress-induced AChE-R elevation
Following immobilization stress, RT–PCR analysis demonstrated a three-fold increase of AChE-R mRNA, using HPRT mRNA as control (Figure 2a). To study the implications of AChE-R mRNA upregulation for fear conditioning, we employed mEN101, an antisense oligonucleotide inducing murine AChE-R mRNA downregulation.[15] 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 (Figures 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 nonrelevant mRNA encoding the homologous protein butyrylcholinesterase (BuChE) (Figures 2a and b). Likewise, invEN101 or vehicle did not affect the level of the AChE-R mRNA or protein, as tested by RT-PCR and immunoblot analyses of hippocampal homogenates (Figures 2a and b and 3).

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.[18] Our index of memory was the conditioned freezing response, absence of all movement except for respiration and heartbeat. Freezing was shown in mice that returned to the training



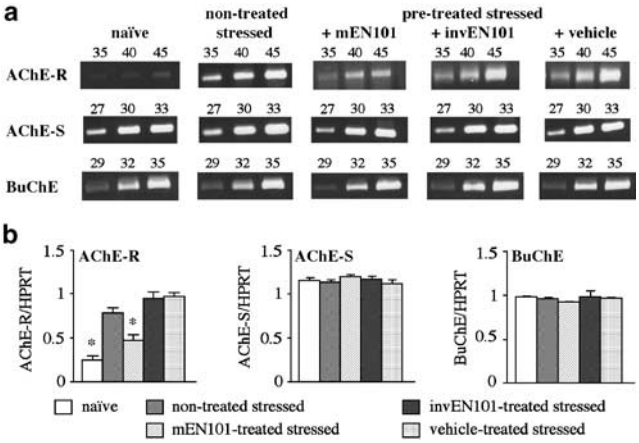


Figure 2 Antisense prevention of stress-induced AChE-R mRNA accumulation. RT-PCR analysis was performed on RNA extracted from the hippocampus of naïve or stressed mice, and from the hippocampus of mice that were injected 15 min before the stress session with the antisense agent (mEN101) that induces destruction of AChE-R mRNA, or with an inversely oriented sequence (invEN101) or vehicle. Each reaction mixture contained a set of primers specific for 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 the levels of AChE-R mRNA, AChE-S mRNA and BuChE mRNA 2 h after the end of stress exposure. The numbers above each band indicate the 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 coamplified HPRT product (mean ± SEM; 9–11 mRNA samples per group). Statistically significant differences: $^*P<0.01$ *vs* stressed animals.

context, in which they were previously exposed to a footshock.[24] Injection of mEN101 totally prevented the increased freezing response after stress, unlike invEN101 or vehicle injection that were ineffective (Figure 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

Figure 1 Acute immobilization stress induces AChE-R upregulation in hippocampal CA1 neurons. (a) Top, schematic representation of the analyzed hippocampal brain area. Middle, 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) is shown. Bottom, higher magnification images of the framed regions (scale bar=25 μm). Bars represent densitometric analysis (mean ± SEM; both hippocampi of n=5 per group) of AChE-R-positive cells in the stratum pyramidale (b) and stratum radiatum (c), as seen in the middle segment under (a). Statistically significant differences: $^*P<0.05$ *vs* naïves.

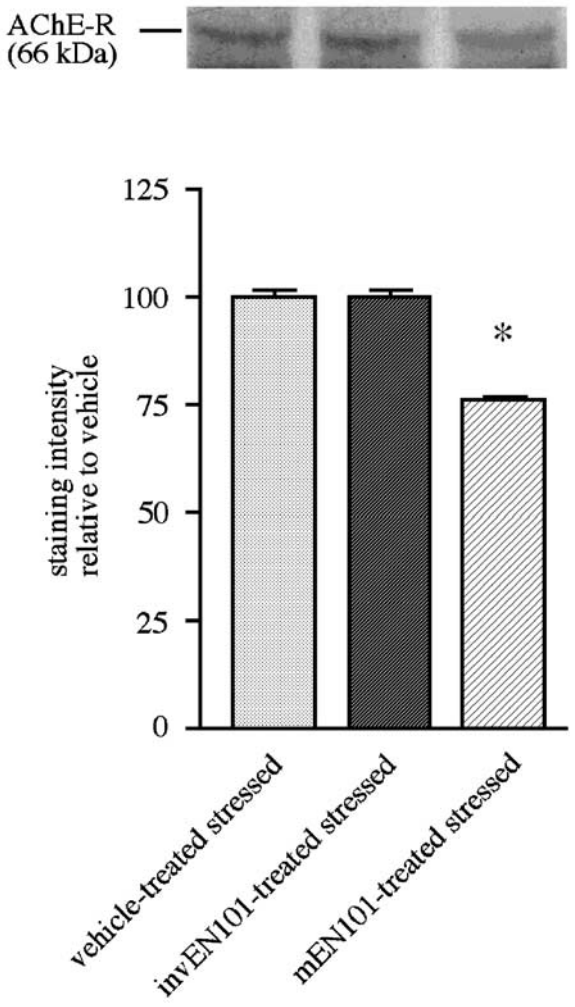


Figure 3 Downregulation of post-stress AChE-R protein levels by antisense treatment. A representative immunoblot reflecting AChE-R protein levels in hippocampi homogenates from stressed animals injected with mEN101, invEN101 or vehicle 15 min before stress exposure is shown. The hippocampi were removed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

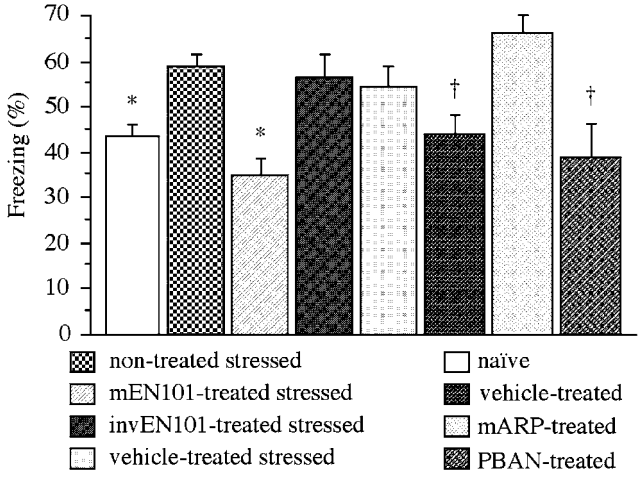


Figure 4 AChE-R elevation enhances contextual fear conditioning. Naïve (n=27), stressed (n=25) and mice injected i.c.v. 15 min before stress exposure with mEN101 (n=11), invEN101 (n=11) or vehicle (n=9) were trained 2 h after the end of the stress session or 2 h after intrahippocampal injection of vehicle (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.

progenitor cells.[22] 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 first 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 (Figure 5a and b). However, neurons in areas distant from the injection site, such as the parietal cortex and the basal forebrain, were stained as well, suggesting endocytosis and retrograde transport of ARP (Figure 5c–e). An alternative labeling group did not affect the neuronal accumulation of ARP (Figure 5f). Moreover, a negative control peptide representing the C-terminus of murine AChE-S (mASP) displayed a conspicuously different labeling pattern. While mARP was localized in the cytoplasm, mASP accumulated in the neuronal nuclei (Figure 5g). Thus, injected mARP demonstrated a potential capacity to transduce signals to the neuronal cytoplasmic element(s). At the behavioral level, intrahippocampal (i.h.) mARP injection (2 nM) without conditioning had no effect on the motor activity of mice 24 h later (data not shown), but 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,[22] also showed no effect on contextual fear conditioning (Figure 4).

Elevated fear response involves enhanced hippocampal LTP

To test if AChE-R mRNA and protein upregulation after acute stress enhance conditioned fear by altering synaptic plasticity, we measured hippocampal 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

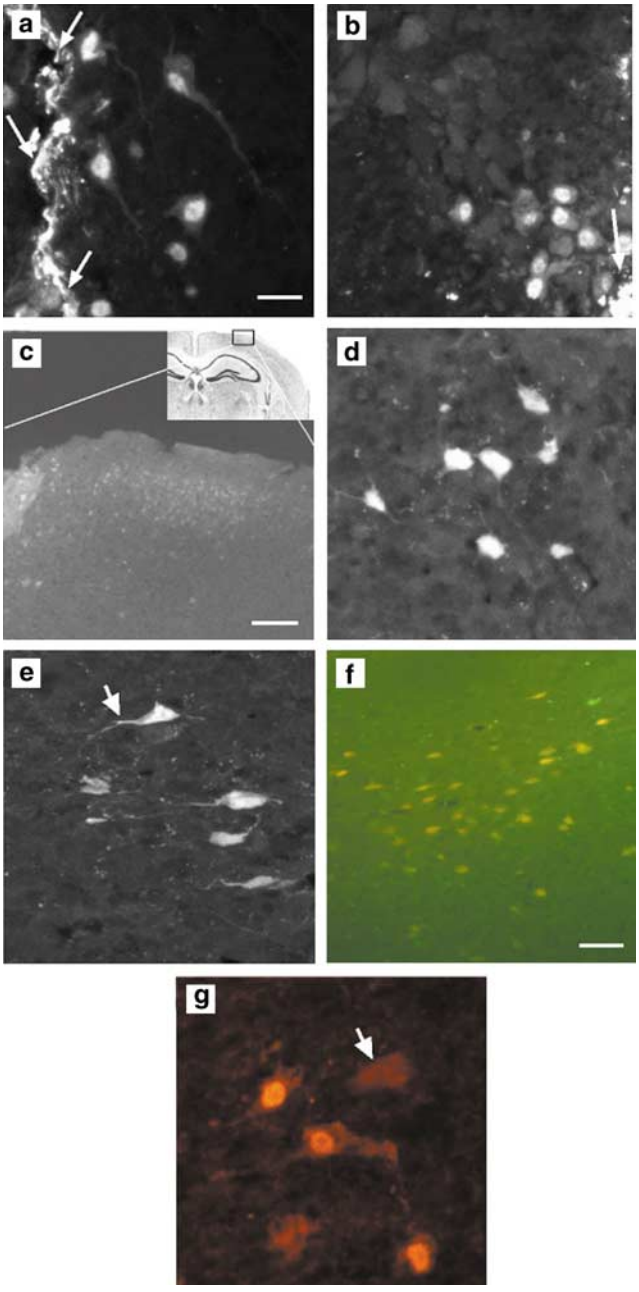


Figure 5 Neuronal endocytosis and retrograde transport of brain-microinjected synthetic mARP. Confocal section of cortex (a) and hippocampus (b) in which the injection needle is penetrated through (arrowheads). The nearby cells are stained for RITC-labeled ARP, probably via peptide diffusion. (c) Fluorescence microscopy of labeled areas distant from the injection site, for example, the parietal cortex. Confocal microscopy of stained cells from the basal forebrain; olfactory tubercle (d) and nucleus basalis of Meynert (e). Note the neural processes labeling with mARP (arrow). (f) Biotin and FITC-labeled mARP under simultaneous injection to the same animal (biotin is visualized with RITC-labeled streptavidin). In the nucleus basalis of Meynert, double-labeled cells appear yellow. (g) Simultaneous 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, b, d, e, g) 25 m; (c) 200 m; (f) 100 m.

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 (Figures 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.[14] This finding raised the question if persistent AChE-R upregulation 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.[25] 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.[16,21] LTP was stably elevated by 40% even 100 min after LTP induction when compared to LTP of the parent strain mice (Figure 6c).

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.[16] 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 (Figures 7a and b). Confocal microscopy revealed in CA1 hippocampal neurons from these transgenic mice enlarged intracellular clusters composed of both AChE-R and PKCβII (Figure 7c). This finding was compatible with the assumption that the elevated LTP maintenance in transgenic mice may require interaction between the C-terminal domain of AChE-R and PKCβII.

We therefore investigated by activating PKC with phorbol dibutyrate (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 potentials more dramatically in hippocampal slices from AChE-R transgenic mice than from control FVB/N mice (Figure 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[26]). 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 of the stratum pyramidale indicated maximal expression of the stress-associated AChE splice variant AChE-R 2 h after immobilization. Mice conditioned at this time point revealed the highest levels of contextual fear during the retention test. This fear enhancement was prevented by antisense destruction of AChE-R mRNA, attributing a central role in stress-mediated contextual fear conditioning to AChE-R. Interestingly, the amount of AChE-R protein returned to baseline levels in the stratum pyramidale within 24 h, whereas it was maximal at that time point in the stratum radiatum. This observation was consistent with the reported stress-induced translocation of AChE-R mRNA from the nucleus of hippocampal CA1 neurons into dendrites.[14] In both, acutely stressed animals 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.[18] 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 spliceosome proteins in fear memory consolidation is further indicated from the alternative pre-mRNA splicing at the ERK-MAP kinase pathway,[27] which is activated during fear conditioning.[28,29]

The physiological relevance of AChE-R overproduction may be multileveled, and should be discussed separately in the synaptic, intracellular and adaptive contexts. Following stress, AChE-R mRNA replaces AChE-S mRNA in neuronal processes in a manner associated with glutamatergic hyperexcitation.[14] AChE-R differs from the synaptic variant AChE-S in its C-terminal domain (ARP), which consists of 26 amino-acid residues as compared with 40 residues in AChE-S.[17] The unique ARP sequence, with no homologies in the database, is devoid of the cysteine residue that enables AChE-S to interact with the structural subunit PriMA,[30] and thus adhere to the synaptic membrane. Therefore, AChE-R would

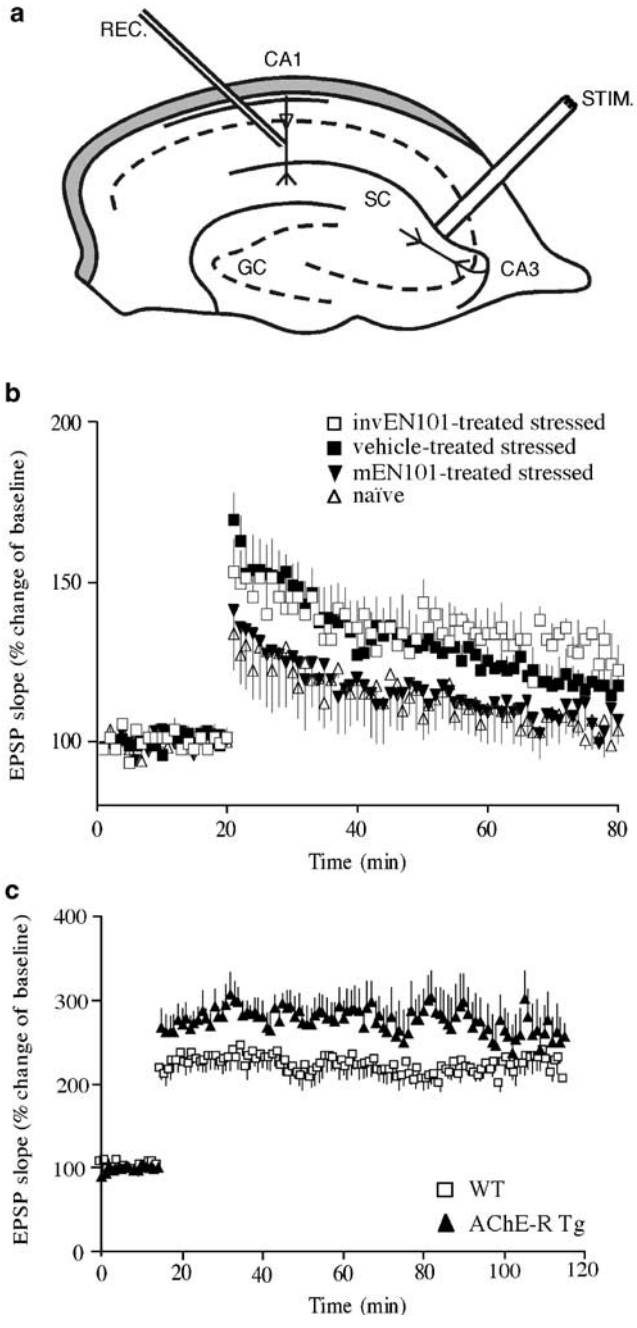


Figure 6 AChE-R facilitates LTP induction and maintenance in the mouse hippocampus. (a) Setup for hippocampal extracellular field recordings (REC) in the CA1 stratum radiatum upon stimulation (STIM) of the Schaffer collateral pathway (SC). (b) TBS-LTP elicited in slices from mice that were killed 2 h after 1 h immobilization (■) was significantly enhanced when compared to LTP induced in slices from naïve mice (△; seven slices, five mice; $P<0.001$). There was no statistical difference between stressed mice and mice that were preinjected with vehicle before stress exposure (six slices, five mice) (data not shown). The stress-induced enhancement was significantly attenuated when stressed mice were preinjected with mEN101 (▼; five slices, five mice; $P<0.001$). InvEN101 preinjection had no effect on stress-induced LTP facilitation (□; six slices, five mice). (c) Chronic effect of AChE-R overexpression. fEPSP changes from the baseline (%) of hippocampal slices from transgenic mice are shown (Tg; ▲; nine slices, six mice), reaching a value of $272\pm10\%$ potentiation extent, significantly higher than the $228\pm8\%$ potentiation extent in age- and strain-matched FVB/N control mice (□; 12 slices, nine mice, $P<0.05$).

primarily be expected to be secreted into the intercellular space, where it can rapidly hydrolyze the stress-elevated levels of acetylcholine.[13] However, the 534 residue core domain that is common to the two AChE variants shares sequence and function homologies with neuroligin, a postsynaptic cell adhesion molecule of excitatory synapses,[31,32] which triggers presynaptic development in contacting axons.[33] It is tempting to speculate that, under stress, excess AChE-R may compete with neuroligin, potentially impairing its interaction with β-neuroexin and modifying the subsequent activation of PSD95.[34]

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, which is devoid of catalytic activity, is sufficient for promoting fear memories. The enhanced enzymatic AChE activity found shortly after stress[13] thus appears to be mainly responsible for clearing the elevated levels of acetylcholine released after acute stress.[35] 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 nonenzymatic activity, possibly involving intracellular protein–protein interactions. On the other side, it is conceivable that corticosterone mediates alternative splicing of the ACHE gene, not only in response to stress but also in response to learning itself. There is much evidence that corticosterone is involved in various learning paradigms including spatial orientation in the Morris water maze or contextual fear conditioning (for a review, see De Kloet[36]).

Since the C-terminus of AChE-R interacts intracellularly with the scaffold protein RACK1 and through it with PKCβII,[16] 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,[37] and

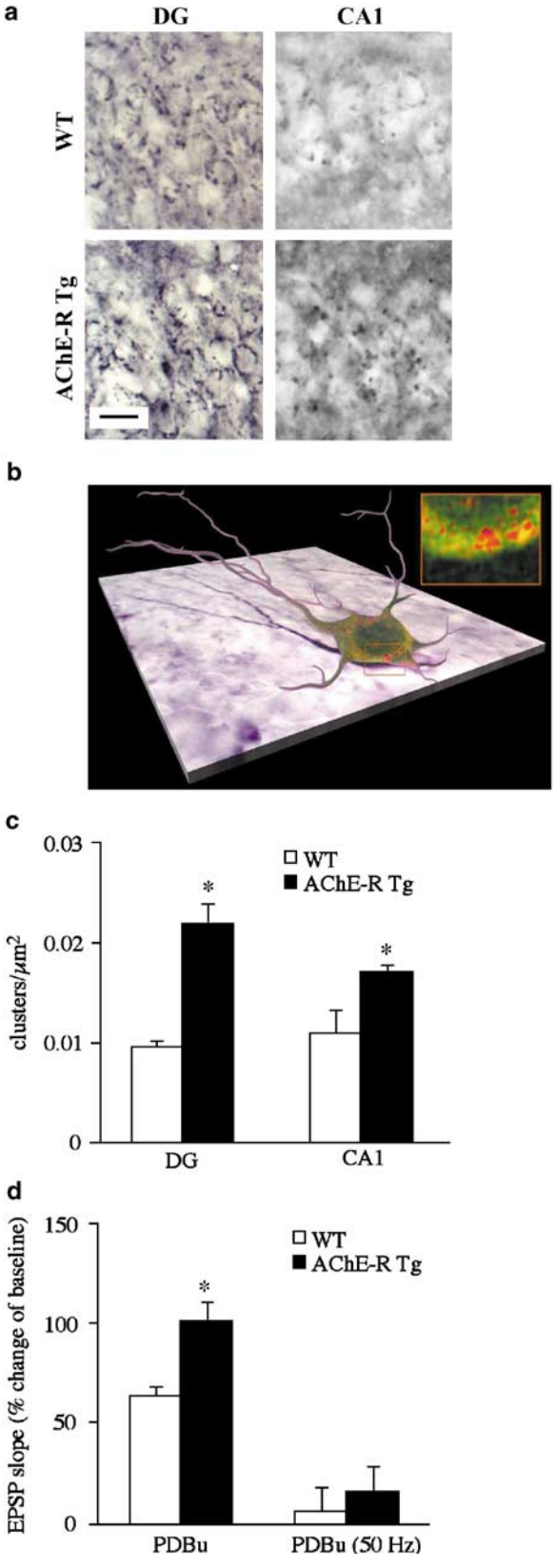


Figure 7 Activation of PKC induces LTP more profoundly under transgenic (Tg) AChE-R overexpression. (a) Facilitated PKC activation in the hippocampus of AChE-R transgenic mice. Typical pyramidal neurons in the hippocampal CA1 area and neurons in the granule cell layer of the dentate gyrus (DG) from WT and transgenic mice stained with a protein kinase C (PKC)βII antibody are shown. Scale bar, 25 μm. (b) Simulated immunomicrograph of a hippocampal neuron from a transgenic mouse overexpressing the stress-induced variant of acetylcholinesterase, AChE-R (green). The confocal images of clusters including AChE-R and PKCβII (red) are highlighted (inset). (c) Quantified labeling of the above sections. Sections from Tg (four sections, four mice) and WT (four sections, four mice) animals were quantified with significantly more clusters of PKCβII staining in transgenic neurons ($P<0.05$) in both DG and CA1 regions. Sections were 7 μm thick. (d) Summarized results of PDBu (5 μM) application for 20 min, which induced the facilitation of synaptic field potentials in AChE-R transgenic slices (nine slices, six mice), significantly higher than in WT (10 slices, six animals, $P<0.05$). Tetanic stimulation delivered after 20 min of PDBu wash did not induce further potentiation.

genomic disruption of mouse PKCβII causes inherited deficits in contextual fear responses.[38] However, PKCβII-deficient mice present apparently normal robust hippocampal LTP, albeit less sensitive to phorbol ester than that of control mice.[38] 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.[39–41] Thus, dysfunction of AChE-R or PKCβII or both are expected to be relevant to pathological conditions such as post-traumatic stress disorder (PTSD) and mood disorders.

Acknowledgements

We are grateful to Dr Michael Gait, Cambridge, UK, for advice and for critically reviewing this manuscript. This study was supported by the Max Planck Society, the Hebrew University of Jerusalem, the Israel Science Foundation (Grant no. 618/02-1 to HS), the US Army Medical Research and Materiel Command (DAMD 17-99-1-9547, to HS) and Ester Neurosciences, Ltd.

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