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(54) Titre : DESOXYOLIGONUCLEOTIDES ANTISENS ACHE UTILISES COMME AGENTS ANTI-INFLAMMATOIRES
(54) Title: ACHE ANTISENSE DEOXYOLIGONUCLEOTIDE AS AN ANTI-INFLAMMATORY AGENT

(57) Abrégé/Abstract:
The present invention provides a novel use for AChE antisense oligonucleotides as anti-inflammatory agents, wherein said oligonucleotides are preferably as denoted by SEQ. ID. NO:1, SEQ. ID. NO:2 and SEQ. ID. NO:7. Described are pharmaceutical compositions for the treatment of inflammatory conditions, as well as methods of treatment thereof, comprising as active agent said AChE antisense oligonucleotides.

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(54) Title: AChE ANTISENSE DEOXYOLIGONUCLEOTIDE AS AN ANTI-INFLAMMATORY AGENT

(57) Abstract: The present invention provides a novel use for AChE antisense oligonucleotides as anti-inflammatory agents, wherein said oligonucleotides are preferably as denoted by SEQ. ID. NO:1, SEQ. ID. NO:2 and SEQ. ID. NO:7. Described are pharmaceutical compositions for the treatment of inflammatory conditions, as well as methods of treatment thereof, comprising as active agent said AChE antisense oligonucleotides.

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AChE ANTISENSE DEOXYOLIGONUCLEOTIDE AS AN ANTI-INFLAMMATORY AGENT

Field of the Invention

The present invention relates to the field of anti-inflammatory agents. More specifically, the present invention provides a novel use for an antisense oligonucleotide targeted to the coding domain of the acetylcholinesterase (AChE) nucleotide sequence, as an anti-inflammatory agent, particularly for the treatment and/or prevention of inflammation in the joints, central nervous system, gastrointestinal tract, endocardium, pericardium, lung, eyes, skin and urogenital system.

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Background of the Invention

All publications mentioned throughout this application are fully incorporated herein by reference, including all references cited therein.

Inflammation plays a crucial role in defense against pathogen invaders as well as in healing and recovery processes following various types of injury. However, the magnitude and duration of inflammatory responses have to be tightly regulated, because excessive inflammatory reactions can be detrimental, leading to autoimmune diseases, neurodegeneration, sepsis, trauma and other pathological conditions. It has long been recognized that

regulation of inflammatory reactions is mediated both by immune responses (particularly the secretion of anti-inflammatory cytokines) and by neuroendocrine factors, particularly the activation of the pituitary-adrenal axis and the secretion of glucocorticoids. Recently it became evident that neural mechanisms are also involved in limiting inflammatory responses. In particular, it was found that cholinergic neurons inhibit acute inflammation, providing a rapid, localized, and adaptive anti-inflammatory reflex system (Tracy, 2002). In the periphery, acetylcholine (ACh) is mainly released by the efferent vagus nerve. It significantly attenuates the production of the pro-inflammatory cytokines TNFα, interleukin-1β (IL-1β), IL-6 and IL-18, but not the anti-inflammatory cytokine IL-10 [Tracey, K.J. (2002) *Nature* **420**, 853-859]. Reciprocally, IL-1 causes AChE over-production both in PC12 cells and in the rat cortex [Li, Y. *et al.* (2000) *J. Neurosci.* **20**, 149-155], suggesting a closed loop whereby ACh suppresses IL-1, ablating the induction of AChE production.

Within the mammalian spinal cord, several subsets of interneurons function in concert to translate converging cortical inputs into synchronized motoneuron activities [Noga, B.R. *et al.* (1995) *J. Neurosci.* **15**, 2203-2217; Phelps, P.E. *et al.* (1990) *J. Comp. Neurol.* **291**, 9-26; Sherriff, F.E. & Henderson, Z. (1994) *Brain Res.* **634**, 150-154; Perlmutter, S.I. *et al.* (1998) *J. Neurophysiol.* **80**, 2475-2494; Prut, Y. & Fetz, E.E. (1999) *Nature* **401**, 590-594]. Allostatic breakdown of this intricately controlled pathway may occur under various stressors, including glycinergic (strychnine) or cholinergic agents (succinylcholine), or under myasthenic crisis or post-anesthesia effects [Becker, C.M. *et al.* (1992) *Neuron* **8**, 283-289; Millard, C.B. & Broomfield, C.A. (1995) *J. Neurochem.* **64**, 1909-1918; Subramony, S.H. *et al.* (1986) *Muscle Nerve* **9**, 64-68; Krasowski, M.D. *et al.* (1997) *Can. J. Anaesth.* **44**, 525-534]. These and other acute stressors may induce massive tremor and spastic paralysis, reflecting failure of the quality control processes which presumably act to sustain cholinergic homeostasis in spinal cord motoneurons. In addition to these modulations in cholinergic neurotransmission, both injury and chemical stressors induce up-regulation of

pro-inflammatory cytokines in the spinal cord (e.g. IL-1β following experimental spinal injury) or organophosphate inhibitors of acetylcholinesterase (AChE) [Wang, C.X. *et al.* (1997) *Brain Res* **759**, 190-196; Svensson, I. *et al.* (2001) *Neurotoxicology* **22**, 355-362; Dyer, S.M. *et al.* (2001) *Toxicology* **169**, 177-185]. The cholinergic control over peripheral release of pro-inflammatory cytokines [Bernik, T.R. *et al.* (2002) *J. Exp. Med.* **195**, 781-788; Borovikova, L.V. *et al.* (2000) *Nature* **405**, 458-462; Tracey, K.J. *et al.* (2001) *Faseb J.* **15**, 1575-1576] thus provoked the question whether cholinergic allostasis serves to control pro-inflammatory responses also in central nervous system (CNS) neurons.

Because spinal cord motoneurons respond to ACh, the presumed quality control process should exert regulatory effects upon cholinergic neurotransmission. As it needs to function rapidly, it likely involves short-lived molecules. Furthermore, in order to be broad-ranged, the proposed mechanism is likely to be induced under widely diverse stressors. The normally rare, stress-induced acetylcholinesterase variant AChE-R meets all of the requirements from an inducer of such response(s). AChE-R is overproduced under psychological, chemical and physical stresses [reviewed by Soreq, H. & Seidman, S. (2001) *Nat. Rev. Neurosci.* **2**, 294-302]. A parallel stress response involves down-regulation of choline acetyltransferase (ChAT) [Kaufer, D. *et al.* (1998) *Nature* **393**, 373-377] and the genomically linked vesicular acetylcholine transporter (VAChT) [Weihe, E. *et al.* (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3547-3552], together limiting the production and vesicle packaging of acetylcholine while expediting its degradation. This yields down-regulation of the cholinergic hyperexcitation that is associated with many stresses. At a longer range, this stress response is associated with hypersensitivity to both agonists and antagonists of cholinergic neurotransmission [Meshorer, E. *et al.* (2002) *Science* **295**, 508-512] and abnormal locomotor activities that can be ablated under antisense destruction of AChE-R mRNA [Cohen, O. *et al.* (2002) *Mol. Psychiatry* **7**, 874-885]. Finely-tuned control over AChE-R levels thus

emerged as a key component of stress management by spinal cord motoneurons. AChE-R over-expression, which suppresses ACh levels, further lead to increased IL-1 production. Should this be the case, antisense suppression of AChE-R production [Brenner, T. *et al.* (2003) *Faseb J.* **17(2)**, 214-22] would increase ACh levels and reduce the levels of pro-inflammatory cytokines in CNS neurons.

In counterpart, parallel inflammatory responses and production of cytokines, particularly within the brain, has raised the suggestion that illness-associated alterations in memory functioning caused by medical conditions like Alzheimer's disease [Arendt, T. (2001) *Neuroscience* **102**:723-65], multiple sclerosis [Thornton, A.E. *et al.* (2002) *J. Int. Neuropsychol. Soc.* 8:395-409], acquired immunodeficiency syndrome [Navia, B.A. *et al.* (1986) *Ann. Neurol.* **19**:517-24] and infectious diseases [Capuron, L. *et al.* (1999) *Psychol. Med.* **29**:291-7], are at least partly mediated by immune activation [Rachal Pugh C., *et al.* (2001) *Neurosci. Biobehav. Rev.* **25**:29-41; Maier S. F. and Watkins L. R. (1998) *Psychol. Rev.* **105**:83-107; Yirmiya R. (1997) *Current Opinion in Psychiatry*, **10**: 470-476; Yirmiya, R. *et al.* (2002) *Neurobiology of Learning and Memory*, **78**: 379-389]. Cytokine-induced memory impairments in humans, including cancer and hepatitis-C patients [Capuron L. *et al.* (2001) *Psychosom. Med.* **63**:376-86; Meyers C. A. (1999) *Adv. Exp. Med. Biol.* **461**:75-81], as well as in experimental animals [Gibertini M. (1996) *Adv. Exp. Med. Biol.* **402**:207-17; Oitzl M. S. *et al.* (1993) *Brain Res.* **613**:160-3], support this notion. Thus, like many other stressful stimuli, which are known to affect learning and memory processes [Kim J. J. and Diamond D. M. (2002) *Nat. Rev. Neurosci.* **3**:453-62], inflammation can cause marked alterations in memory functioning. Administration of endotoxin (lipopolysaccharide), a complex glycolipid found in the outer membrane of all gram-negative bacteria, serves to assess the cognitive consequences of the acute host response to infection in humans. Endotoxin administration induces fever, malaise and increased production and secretion of cytokines, particularly TNF-α, IL-6, IL-1 and IL-1ra and cortisol

[for review see Burrell R. (1994) *Circ. Shock* **43**:137-53], as well as proteases [Fahmi H. and Chaby R. (1994) *Immunol. Invest.* **23**:243-58]. In healthy humans, endotoxin-induced cytokine secretion is correlated with impairments in verbal and non-verbal declarative memory functions [Reichenberg A. *et al.* (2001) *Arch. Gen. Psychiatry* **58**:445-52].

Memory deficits and profound neurobehavioral and neuroendocrine symptoms were also reported to be correlated with endotoxin-induced secretion of cytokines in experimental animals [Hauss-Wegrzyniak B. *et al.* (2000) *Neuroreport* **11**:1759-63; Pugh C. R. *et al.* (1998) *Brain Behav. Immun.* **12**:212-29; Shaw K. N. *et al.* (2001) *Behav. Brain Res.* **124**:47-54]. While these findings suggest that cytokines are involved in mediating the effects of endotoxin on memory, little is known about the neurotransmission pathways associated with these cytokine activities. The inventors initiated a search into the possibility that cholinergic processes are relevant to endotoxin responses because in the central nervous system (CNS), cholinergic responses are notably involved in several important aspects of cognitive functioning, including attention, learning and memory (for reviews see Levin E. D. and Simon B. B. (1998) *Psychopharmacology (Berl)* **138**:217-30; Segal M. and Auerbach J. M. (1997) *Life Sci.* **60**:1085-91]. Moreover, endotoxin decreases brain choline acetyltransferase activity [Willard L. B. *et al.* (1999) *Neuroscience* 88:193-200], similar to the effects of psychological stress [Kaufer (1998) *id ibid.*]. In the periphery, endogenous or exogenous acetylcholine (ACh) attenuates the release of pro-inflammatory cytokines from endotoxin-stimulated human macrophages [Borovikova (2000) *id ibid.*; Bernik (2002) *id ibid.*; Tracey (2001) *id ibid.*]. The ACh hydrolyzing enzyme acetylcholinesterase (AChE) was considered as potentially being of particular relevance to these processes because AChE controls ACh levels and since AChE inhibitors improve cognitive functions in both clinical and experimental paradigms [Palmer A. M. (2002) *Trends Pharmacol. Sci.* **23**:426-33; Weinstock M. (1995) *Neurodegeneration* **4**:349-56]. Moreover, AChE over-expression is triggered by acute and chronic stressful

insults [Meshorer (2002) *id ibid.*] and induces progressive memory impairments, as was demonstrated in transgenic mice [Beeri R. *et al.* (1995) *Curr. Biol.* **5**:1063-71].

Stress-induced transcriptional activation of AChE gene expression is associated with accumulation of the normally rare "readthrough" AChE-R splice variant [Soreq and Seidman (2001) *id ibid.*]. In the short range, the AChE-R excess reduces the stress-induced cholinergic hyperexcitation [Kaufer (1998) *id ibid.*]; in the long range, it induces hypersensitivity to cholinergic agonists and antagonists [Meshorer (2002) *id ibid.*]. Mice that overexpress both AChE-S and AChE-R present progressive dendritic and spine loss [Beeri R. *et al.* (1997) *J. Neurochem.* **69**:2441-51], as well as altered anxiety responses [Erb C. *et al.* (2001) *J. Neurochem.* **77**:638-46]. Furthermore, these mice display early-onset deficits in social recognition and exaggerated responsiveness to stressful insults. These can be briefly ameliorated by conventional anticholinesterase treatment or for longer periods by an antisense oligonucleotide capable of specifically inducing the destruction of AChE-R mRNA [Cohen (2002) *id ibid.*], suggesting that AChE-R is the primary cause. Thus, AChE-R production may lead to both positive and negative effects on cognition.

The role of cholinergic mechanisms in learning and memory, the involvement of AChE-R in stress responses, the suppression by ACh of pro-inflammatory cytokines production and the effects of endotoxin on memory functions suggested involvement of AChE-R in mediating endotoxin-induced memory alterations. Stressful insults induce AChE-R production in the periphery as well (e.g., in the small intestines), and failure to induce this production in response to aversive stimuli results in hypersensitivity to relatively mild stressors [Shapira M. *et al.* (2000) *Hum. Mol. Genet.* **9**:1273-1281]. This raised the possibility that peripheral AChE modulations may serve as a surrogate marker of endotoxin-induced changes in cognition as well. However, in plasma,

proteolytic cleavage of AChE-R leads to the appearance in the serum of a short immunopositive C- terminal peptide which facilitates the hematopoietic stress responses [Grisaru, D. *et al.* (2001) *Mol. Med.* 7, 93-105]. Hence, the inventors investigated the effects of endotoxin administration on both AChE activity and AChE-R cleavage in healthy human volunteers and explored potential correlations between these parameters, the secretion of cytokines or cortisol, and changes with time in memory functions. In addition to declarative memory, which involves consciously accessible records of facts and events through concerted functioning of hippocampal and prefrontal structures [Kim and Diamond (2002) *id ibid.*], the inventors assessed the effects of endotoxin and its interactions with AChE cleavage on working memory, which involves temporary storage and manipulation of information necessary for cognitive functioning [Baddeley A. (1992) *Science* **255**:556-9], and has been shown to involve prefrontal cholinergic mechanisms [Furey M. L. *et al.* (2000) *Science* **290**:2315-9].

The prospect of therapeutic agents of exquisite specificity and action at very low concentration has stimulated the development of antisense oligonucleotides (AS-ON) targeted against a variety of mRNAs. Major problems remain access to the RNA processing machinery of the cell, potential differences between specific cell types and the mode of chemical protection employed. When the cell of interest is within the CNS, the problem of access is compounded by the presence of the blood-brain barrier [Tavitian, B. *et al.* (1998) *Nat. Med.* **4**, 467-471]. Nevertheless, some attempts have been successful even in primates [Kasuya, E. *et al.* (1998) *Regul. Pept.* **75-76**, 319-325; Mizuno, M. *et al.* (2000) *Endocrinology* **141**, 1772-1779]. The inventors have previously demonstrated antisense suppression of the stress-induced AChE-R mRNA, enabling retrieval of normal cellular and physiological functions following stress-induced changes in cultured rat and human cells [Galyam, N. *et al.* (2001) *Antisense Nucleic Acid Drug Dev.* **11**, 51-57; Grisaru, D. *et al.* (2001) *id ibid.*] and in live mice [Cohen *et al.* (2002) *id ibid.*; Shohami,

E. *et al.* (2000) *J. Mol. Med.* **78**, 228-236] and rats [Brenner, T. *et al.* (2003) *id ibid.*]. While the tested consequences in all of these studies were limited to direct measurement of the target protein and mRNA, the working hypothesis predicted additional, anti-inflammatory effects for antisense retrieval of cholinergic balance. Here, the inventors report the outcome of experiments aimed at addressing the stress-induced overproduction and selective AS-ON retrieval of normal AChE-R levels under injection stress in cynomolgus monkeys. The findings demonstrate differential susceptibility of specific neuron types to AS-ON responses, as well as concomitant suppression of IL-1β and IL-6 following the retrieval of cholinergic balance in spinal cord neurons.

The present inventors have previously found that antisense oligonucleotides against the common coding region of AChE are useful for suppressing AChE-R production [see WO 98/26062]. In particular, the inventors have shown the use of an antisense oligonucleotide against the AChE sequence for the treatment of myasthenia gravis [WO 03/002739 and US 10/402,016].

Based on the inventors' herein described results, the present invention provides a novel use for an antisense oligonucleotide directed against the AChE mRNA sequence, as a new anti-inflammatory agent.

Other purposes and advantages of the invention will become apparent as the description proceeds.

Summary of the Invention

In a first aspect, the present invention refers to the use of an inhibitor of AChE expression, as an anti-inflammatory agent. Preferably, said inhibitor of AChE expression is an antisense oligonucleotide directed against AChE, having any one of the following sequences:

5' CTGCCACGTTCTCCTGCACC 3' (SEQ. ID. NO:1);

5'-CTGCAATATTTTCTTGCACC-3' (SEQ. ID. NO:2); and

5' CTGCCACGTTCTCCTGCA*C*C* 3' (SEQ. ID. NO:7), wherein the three 3' terminal residues are modified with 2-O-methyl groups (*).

In another aspect, the invention provides the use of an inhibitor of AChE as defined herein, as a suppressor of pro-inflammatory cytokines release. Preferably, said inhibitor of AChE is the antisense oligonucleotide denoted by SEQ. ID. NO. 1.

In a further aspect, the present invention intends to provide a pharmaceutical composition for the treatment of conditions triggering an inflammatory response, comprising as active agent the above-defined inhibitor of AChE expression. Optionally, the composition further comprises additives, carriers and/or diluents. Preferably, said inhibitor of AChE expression is an antisense oligonucleotide directed against AChE. More preferably, said antisense nucleotide has the sequence as denoted in SEQ. ID. NO:1.

In a yet further aspect, the present invention provides a pharmaceutical composition for the treatment and/or prevention of inflammation in the joints, central nervous system, gastrointestinal tract, endocardium, pericardium, lung, eyes, skin and urogenital system, comprising as active agent the inhibitor of AChE expression as defined above, optionally further comprising any one of additives, carriers and/or diluents. Preferably, said inhibitor of AChE expression is an antisense oligonucleotide. More preferably, said oligonucleotide has the sequence as denoted in SEQ. ID. NO:1.

The inhibitor of AChE expression, as defined herein, is to be used in the preparation of the pharmaceutical composition of the invention.

Finally, the invention teaches a method of treatment of conditions triggering an inflammatory response, wherein said method comprises administering an effective amount of an inhibitor of AChE expression, as defined herein, or a composition comprising as active agent an inhibitor of AChE expression, prepared as described in the description.

Preferably, said inhibitor of AChE expression to be used in the method of the invention is an antisense oligonucleotide, which, more preferably, has the sequence denoted by SEQ. ID. NO:1.

Brief Description of the Figures

Figure 1A-F: Reduced VAChT accumulation in cholinergic terminals and partition cells of treated monkeys.

Fig. 1A: Confocal microscopy projections of spinal cord motoneurons (cell diameter = 40 μm), immunolabeled (white) with anti-VAChT antibody. The total volume and average number per cell of labeled terminals were measured using Image-Pro Plus software, and the result of each treatment (1, 2, 3 and 4) plotted in the graphs shown in Figs. 1B and 1C.

Fig. 1B: Average value of volume and average number per cell of labeled terminals, including all motoneurons detected in a section.

Fig. 1C: Population distribution of volume and average number per cell of labeled terminals, including all motoneurons detected in a section.

Fig. 1D: Average values of Figs. 1B, 1C analyses (± Standard Evaluation of the Mean, SEM). Significant reductions are marked by asterisks ($p < 0.01$, Student's t test).

Fig. 1E: Immunolabeling with anti-ChAT antibody in partition cells from naïve spinal cord, localized in close proximity to the central canal (arrows). Hematoxylin was used for background staining.

Fig. 1F: Higher magnification of ChAT positive partition cells in naïve monkeys (1) or following oral (p.o.) administration of 150 μg/Kg/day (2) or 500

μg/Kg/day (3) and *i.v.* administration of 500 μg/Kg/day hEN101 (4). Note dose-independent handling – induced reductions in both terminals volume and density.

Abbreviations: n., naïve; Term., terminal; vol., volume; Part. Ce., Partition cell; Cent. Can., Central canal.

Figure 2A-J: Selective AChE-R mRNA suppression by hEN101 in monkey spinal cord neurons.

Fig. 2A: Scheme of the human *ACHE* gene coding exons and two of its alternative transcripts, the synaptic AChE-S (S) and the stress-associated AChE-R (R) mRNA. The S transcript includes exons 2, 3, 4 and 6, whereas the R transcript contains exons 2, 3, 4, 5 and pseudointron 4'. These distinctions served to prepare transcript-specific probes, indicated by an asterisk.

Fig. 2B. Sampling site on the dissected monkey lumbar spinal cord is indicated by an arrow.

Fig. 2C-J. Tissue sections from lumbar spinal cords were prepared following 7-day treatment with the noted doses of hEN101 by *p.o.* or *i.v.* administration. Shown is *in situ* hybridization used to compare neuronal labeling pattern with the noted probes. Nuclei were visualized by DAPI staining (white). There was no difference between tested sections in total cell numbers and/or general histology. Note that AChE-S mRNA labeling displayed significant changes following treatment only in neuronal process sections (2F, 2H and 2J as compared to 2D), whereas neuronal AChE-R mRNA labeling was notably reduced in cell bodies.

Fig. 2C: No treatment, staining specific for AChE-R mRNA.

Fig. 2D: No treatment, staining specific for AChE-S mRNA.

Fig. 2E: Treatment with 150 μg/kg/day of EN101, p.o., staining specific for AChE-R mRNA.

Fig. 2F: Treatment with 150 μg/kg/day of EN101, p.o., staining specific for AChE-S mRNA.

Fig. 2G: Treatment with 500 μg/kg/day of EN101, p.o., staining specific for AChE-R mRNA.

Fig. 2H: Treatment with 500 μg/kg/day of EN101, p.o., staining specific for AChE-S mRNA.

Fig. 2I: Treatment with 500 μg/kg/day of EN101, i.v., staining specific for AChE-R mRNA.

Fig. 2J: Treatment with 500 μg/kg/day of EN101, i.v., staining specific for AChE-S mRNA.

Figure 3A-C: Cell size-dependent efficacy of neuronal AChE-R mRNA suppression.

Fig. 3A: Scheme of the lumbar spinal cord and its three compartments: the ventral and dorsal horns separated by the intermediate zone and the central canal.

Fig. 3B: Histological staining (Hematoxylin and eosin) of a representative field in the intermediate zone of the lumbar spinal cord. Three cells are marked according to their perikaryon diameters: 10-20 μm (arrowhead, the majority of those cells is located in the dorsal horn), 20-40 μm (asterisk) and = 40 μm (arrow).

Fig. 3C: Shown are fractions of AChE-R positive neurons from the three size groups under the different treatment regimens. Insets: representative neurons from the different size groups, taken from the *p.o.* 150 μg/Kg/day regimen. Columns show average AChE-R positive cells in each size group ± SEM representing repeated analyses of the entire lumbar spinal cord gray matter in multiple sections. Stars note significant differences ($p< 0.05$, Wilcoxon test).

Abbreviations: Cent. Can., central canal; D. h., dorsal horn; I. z., Intermediate zone; V. h., ventral horn; pos. ce., positive cells; si. gr., size group; Ce. Bo. Diam., cell body diameter.

Figure 4A-C: Suppression of stress-induced neuronal pro-inflammatory cytokines under antisense intervention with AChE-R expression.

Fig. 4A: Shown are fractions of IL-1β positive spinal cord neurons of medium and large sizes under the different treatment regimens (columns ± SEM representing repeated analyses of the ventral horn and intermediate zone of lumbar spinal cord gray matter in multiple sections). Insets: representative medium and large size positive neurons, taken from the *p.o.* 500 μg/Kg/day regimen. *: $p \leq 0.05$, **: $p = 0.067$.

Fig. 4B: Graph showing the correlation between the average fractions of AChE-R and IL-1β positive medium-sized cells (20-40 μm) in the different hEN101 treatments. Large cells (> 40 μm) did not display such correlation ($R^2 = 0.1778$).

Fig. 4C: Fractions of IL-6 positive spinal cord neurons were evaluated essentially as under 4A. Note decreases in both IL-1β and IL-6 in spinal cord neurons of monkeys treated with 500 μg/Kg/day EN101.

Abbreviations: pos. ce., positive cells.

Figure 5A-D: Changes over time in the human plasma levels of AChE activity and in AChE-R cleavage.

Fig. 5A: Hydrolytic activities. Shown are plasma AChE activities (mean ± SEM) for ten volunteers injected twice, with endotoxin or saline (placebo) at the noted intervals after injection. Pre-injection (baseline) AChE level was considered as 100% for each individual. Asterisks denote statistical difference ($p<0.05$).

Fig. 5B. Immunoblot. Shown are consecutive results for one individual. Plasma samples underwent electrophoresis by SDS-PAGE, and the blot immunoreacted with anti-AChE-R antibodies. Note the 6.5kDa AChE-R cleavage product. Left lanes indicate the response to a placebo injection; right lanes demonstrate elevated AChE-R cleavage in response to endotoxin.

Fig. 5C: Densitometric intensities. Shown are average values (mean ± SEM) of the rapidly migrating AChE-R cleavage product in plasma of the endotoxin and placebo treated individuals as % of baseline (described in A).
Note: Elevated AChE-R cleavage in endotoxin-treated subjects co-appeared with decreased AChE activity.
Fig. 5D: Association analysis. Highly significant negative association (correlation coefficient, r=-0.65) emerged between the increases in AChE-R cleavage and the decrease in AChE activity under endotoxin during the last testing period (t=9 hr). Each dot represents a single individual.
Abbreviations: Act., activity; bas., baseline; H. p. inj., Hours post-injection; T. p. inj., Time post-injection; Plac., placebo; Endot., endotoxin; Cleav. Prod., cleavage product.

Figure 6: Mass spectroscopy of gel-eluted band.
Shown is the outcome of electron spray mass spectrometry analysis of the gel-eluted rapidly migrating band that immunoreacted with anti-AChE-R antibodies. Note that the main peptide displayed a molecular mass of 3613-3615. Calculation of predicted masses positioned the presumed proteolytic cleavage site 36 residues from the C-terminus of AChE-R, between asparagine and arginine residues in the sequence presented, with the presumed cleavage site arrowed and the diversion site starred.
Abbreviations: Rel. abund., relative abundance.

Figure 7A-C: AChE-R is expressed in human vascular endothelial cells from various tissues.
Fig. 7A: AChE-R mRNA. Shown are the results of *in situ* hybridization using a 5'-biotinylated cRNA probe selective for the AChE-R mRNA variant on sections of human vascular endothelial cells affected by an inflammatory process (skin hypersensitivity vasculitis; labeling is seen as pink color, red arrow).

Fig. 7B: AChE-R protein. Shown is an immunomicrograph of human kidney vascular endothelial cells from a patient with vasculitis, labeled with antibodies targeted at the AChE-R C-terminal peptide (red arrow).

Fig. 7C: Image analysis. Shown are average AChE-R mRNA and AChE-R protein labeling intensities (black and white columns, respectively), in kidney, skin and muscle vascular endothelial cells (mean values ±SEM) as the percentage of red pixels, falling within a defined intensity range.

Abbreviations: prot., protein; int., intensity; k. rej., kidney rejection; k. vas., kidney vasculitis; nonspec., non-specific; n. end., normal endothelium; m., muscle; hyp. vasc., hypersensitivity vasculitis.

Figure 8A-C: Bidirectional associations between AChE-R cleavage and the changes in cortisol and cytokines.

Shown are average ±SEM changes with time (left) in the plasma levels of cortisol, TNF-α and IL-6 of the 10 patients treated with endotoxin or placebo, and the associations (right) at the noted time points between these changes and the changes in AChE-R cleavage (measured by densitometric quantification of the C-terminus AChE-R cleavage product).

Fig. 8A: cortisol.

Fig. 8B: TNF-α.

Fig. 8C: IL-6.

Abbreviations: r, correlation coefficient; t, time after injection; Plac., placebo; end., endotoxin; H. p. inj., hours post-injection; cleav. prod., cleavage product.

Figure 9: Endotoxin impairs declarative memory.

Shown are average ±SEM values for the performance in the immediate story recall test of the endotoxin and placebo treated individuals at the noted time following treatment as well as the associations of the changes in these values at 9 hr post-injection with the changes in AChE-R cleavage (b) and AChE activity (c).

Abbreviations: I.s.r., immediate story recall; plac., placebo; endot., endotoxin; H.p.inj., hours post-injection; cleav. prod., cleavage product; act., activity.

Figure 10: Endotoxin-induced improvement in working memory.
Shown are the performance values (average +SEM) in the span background test for the endotoxin and placebo treated individuals (a) and the association of the changes in this performance at 3 hr post-injection with the changes in AChE-R cleavage (b).
Abbreviations: r, correlation coefficient; t, time after injection; S.b., Span backward; plac., placebo; endot., endotoxin; H.p.inj., hours post-injection; cleav. prod., cleavage product; act., activity.

Figure 11A-C: Scheme - Endotoxin induces interrelated cytokine-cholinergic effects on memory.
Shown are the cellular and biochemical events that were explored in this study and which explain the changes in memory processes and the dynamic modifications in these changes during the post-treatment observation period. The thickness of arrows reflects the relative intensity of the relevant processes.
Fig. 11A: At 1 hr post-treatment: Endotoxin induces the release of cytokines, cortisol and proteases. Cytokines elevation associates with impaired declarative memory, which is a medial temporal lobe - associated phenomenon. Cortisol induces AChE-R production, which elevates the immunopositive AChE-R amounts in plasma. Vesicular ACh is released into the synaptic cleft, where it affects neuronal electrophysiology and may improve working memory, which is a neocortex - associated property. In the periphery, ACh begins to suppress cytokines production in macrophages (circular arrow).
Fig. 11B: At 3 hr post–treatment: Proteases release a C-terminal fragment of 36 amino acids in length from AChE-R and initiate further destruction, followed by decreases in AChE activity. Endotoxin is already gone, and ACh effectively suppresses cytokines production; Increased ACh levels (reflecting enhanced secretion and the decrease in AChE's hydrolytic activity) are

probably associated with activated working memory, whereas the elevation in AChE-R cleavage product is associated with a lower working memory improvement.

Fig. 11C: At 9 hr post-treatment: Cortisol is gone as well. However, the persistent, although slow decrease in AChE activity is associated both with the impaired declarative memory and, probably through ACh increases, with the activated working memory. The steady increase in AChE-R cleavage product is now associated both with a greater impairment in declarative memory and with lower improvement in working memory.

Abbreviations: inc. lev., increased level; dec. lev., decreased level; cleav. Prod., cleavage product.

Figure 12A-B: Transgenic mice display higher body temperature than wild-type mice.

Fig. 12A: Graph showing the temperature of each mouse over time, squares represent transgenic mice, circles, control.

Fig. 12B: Graph showing the average temperature of each group (transgenic or control) over time, diamonds represent transgenic mice, squares, control.

Abbreviations: An. T., Anal temperature; Aver. An. T., Average Anal temperature; T. p. anest., time post-anesthesia.

Figure 13A-C: Effects of Tacrine on LPS-induced IL-1 secretion in the hippocampus and IL-1 and TNF-α secretion in the serum.

Fig. 13A: Graph showing the levels of IL-1β in the hippocampus.

Fig. 13B: Graph showing the levels of IL-1β in the serum.

Fig. 13C: Graph showing the levels of TNF-α in the serum.

Abbreviations: prot., protein; ser., serum; sal., saline.

Figure 14A-C: Effects of Rivastigmine on LPS-induced IL-1 secretion in the hippocampus and IL-1 and TNF-α secretion in the serum.

Fig. 14A: Graph showing the levels of IL-1β in the hippocampus.

Fig. 14B: Graph showing the levels of IL-1β in the serum.

Fig. 14C: Graph showing the levels of TNF-α in the serum.

Abbreviations: prot., protein; ser., serum; sal., saline.

Figure 15A-H: Effects of surgery stress on emotional and cognitive parameters.

Fig. 15A: Graph showing the effect of surgery stress on anxiety.

Fig. 15B: Graph showing the effect of surgery stress on depression.

Fig. 15C: Graph showing the effect of surgery stress on fatigue.

Fig. 15D: Graph showing the effect of surgery stress on pain.

Fig. 15E: Graph showing the effect of surgery stress on word list recall.

Fig. 15F: Graph showing the effect of surgery stress on word list recognition.

Fig. 15G: Graph showing the effect of surgery stress on story recall.

Fig. 15H: Graph showing the effect of surgery stress on figure recall.

Abbreviations: Cont., control; str., stress; T., time; Anx., anxiety; Dep., depression; Fat., fatigue; P., pain; W.L.R., word list recall; W. L. Recog., word list recognition; S. R., story recall; Fig. R., figure recall.

Figure 16A-C: Effect of surgery stress on cytokine levels.

Fig. 16A: Graph showing the effect of surgery stress on IL-1 and IL-6 levels.

Fig. 16B: Correlation between IL-1 and depression.

Fig. 16C: Correlation between cytokines and cognitive parameters.

Figure 17A-C: Reduction of AChE gene expression upon EN301 treatment.

Fig. 17A: Analysis of RT-PCR reaction (AChE exon 2 product after 31 PCR cycles). From left to right: lane 1, marker; lanes 2-8, samples from EN301-treated mice; lanes 9-14, samples from PBS-treated mice.

Fig. 17B: Histogram representing quantitative analysis of the results obtained in the PCR reaction using primers targeting the common sequence in exon 2 of murine AChE cDNA.

Fig. 17C: Histogram representing quantitative analysis of the results obtained in the PCR reaction using primers targeting the sequence in exon 6 unique to the AChE-S variant.
Abbreviations: c.d., common domain; Arb. U., arbitrary units; sal., saline.

Detailed Description of the Invention

For the purposes of clarity, the following abbreviations and terms are defined herein:

- AChE: acetylcholinesterase
- AChE-R: acetylcholinesterase, "readthrough" variant or isoform, its mRNA includes pseudo-intron I4
- AChE-S: acetylcholinesterase, synaptic variant or isoform
- AS-ON: antisense oligonucleotide
- CNS: central nervous system
- EN101: may also be referred as AS3, antisense oligonucleotide targeted against human, rat or mouse (hEN101, rEN101 or mEN101, respectively) AChE mRNA
- EN301: may also be referred as mEN101, antisense oligonucleotide targeted against mouse AChE mRNA
- i.v.: intravenous
- i.p.: intraperitoneal
- o.g.: oral gavage
- p.o.: per os

Antisense oligonucleotide: A nucleotide comprising essentially a reverse complementary sequence to a sequence of AChE mRNA. The nucleotide is preferably an oligodeoxynucleotide, but also ribonucleotides or nucleotide analogues, or mixtures thereof, are contemplated by the invention. The antisense oligonucleotide may be modified in order to enhance the nuclease

resistance thereof, to improve its membrane crossing capability, or both. The antisense oligonucleotide may be linear, or may comprise a secondary structure. It may also comprise enzymatic activity, such as ribozyme activity.

To reveal if cholinergic allostasis and CNS inflammatory processes are inter-related, the inventors studied spinal cord neurons from *Cynomolgus* monkeys following one week daily treatment with hEN101 (SEQ. ID. NO:1), a 2'-oxymethylated antisense oligonucleotide inducing AChE-R mRNA destruction. hEN101 prevented the stress-induced increases in plasma AChE activities and selectively suppressed neuronal AChE-R mRNA and interleukins -1β and -6 levels in a dose- and cell size-dependent manner. In contrast, VAChT and ChAT levels were reduced dose-independently in all of the handling-stressed monkeys, demonstrating distinct regulation for the corresponding genes. These findings allude to a causal association between cholinergic allostasis and inflammatory responses in the primate CNS and suggest antisense intervention with AChE-R accumulation for the management of both these impairments.

In a first aspect, the present invention refers to the use of an inhibitor of AChE expression, as an anti-inflammatory agent.

As herein defined, said inhibitor of AChE expression is any agent which is capable of blocking or hindering the expression of the AChE gene, particularly by interacting with its mRNA. Thus, said inhibitor may be an AChE-specific ribozyme, a double-stranded nucleotide sequence used for RNA interference of the AChE gene, or an antisense oligonucleotide directed against AChE. Antisense nucleotides are preferably nuclease resistant.

Preferably, said inhibitor of AChE expression selectively inhibits the AChE-R mRNA, consequently selectively inhibiting the expression of the AChE-R isoform. In this regard, any agent capable of inhibiting the soluble AChE-R

isoform may also be an anti-inflammatory agent. Therefore, a putative molecule that could block AChE-R expression and/or function would be an anti-inflammatory agent.

As shown in Example 1, BuChE levels in the plasma of treated monkeys were not significantly altered, supporting the notion of a selective antisense effect over AChE alone. Both plasma AChE activity and neuronal AChE mRNA labeling increased in monkeys treated with 150 μg/Kg hEN101, potentially reflecting increased production at the tested daily time (Table 1 and data not shown). Alternatively, or in addition, the mild stress associated with the insertion of cannula for *p.o.* administration of hEN101 could be the cause. Plasma AChE increases in the absence of hEN101 would likely be even higher, as is indicated from the suppression of plasma AChE activity in monkeys treated similarly with the higher dose of 500 μg/Kg hEN101. An apparent 3 hr delay was observed in the drug-induced decreases of plasma AChE under this low hEN101 dose, possibly reflecting prevention by antisense agents of the synthesis of their target protein(s). This further indicates a short half life for primate AChE-R mRNA *in vivo*, compatible with previous findings by the inventors and others [Brenner *et al.* (2003) *id ibid.*; Chan, R.Y. *et al.*(1998) *J. Biol. Chem.* **273**, 9727-9733].

The fraction of AChE-R mRNA positive neurons, the intensity of AChE-R mRNA labeling and the fraction of cells with AChE-R mRNA labeled processes were all reduced under antisense treatment (Figures 2A-2J and 3A-3C). Neuronal susceptibility of AChE-R overproduction to antisense suppression appeared inversely proportional to cell body size, possibly reflecting distinct membrane and/or metabolic properties, different cell volumes or a combined contribution of these properties. In addition, antisense-independent reductions in VAChT and ChAT likely indicated a slowdown of vesicle recycling [Soreq, H. *et al.* (1990) *Proc Natl Acad Sci U.S.A.* 87: 9688-9692], potentially modulating the pace of cholinergic neurotransmission. Under naïve conditions, AChE-S

mRNA appeared in processes of many more spinal cord neurons than AChE-R mRNA, creating a pattern reminiscent of VAChT labeling in the rat spinal cord ventral horn [Weihe *et al.* (1996) *id ibid.*]. Expectedly, hEN101 treatment was highly efficient with neuronal AChE-R mRNA and much less effective with AChE-S mRNA. However, the reduced intensity of neuronal AChE-S mRNA labeling likely reflected limited reduction in neuronal AChE-S mRNA levels as well. Under hEN101 treatment, AChE-S mRNA in processes was reduced, suggesting common tendency for reduced dendrite translocation of the rodent and primate AChE-S mRNA transcript under stress [Meshorer *et al.* (2002) *id ibid.*]. This difference further strengthened the notion that the naïve monkey was indeed under no stress, an important fact in a study with strictly limited number of animals. The reduced AChE-S mRNA in neuronal processes of the treated monkeys may be treatment- and/or drug-induced. Following 7 days treatment, a shift from the primary AChE-S mRNA transcript to the stress-induced antisense-suppressible AChE-R mRNA may be visualized in the neuronal processes (Fig. 2A-2J).

Preferably, said inhibitor of AChE expression is an antisense oligonucleotide directed against AChE, having any one of the following sequences:

5' CTGCCACGTTCTCCTGCACC 3' (SEQ. ID. NO:1); and
5' CTGCCACGTTCTCCTGCA*C*C* 3' (SEQ. ID. NO:7), wherein the three 3' terminal residues are modified with 2-O-methyl groups (*).

The antisense oligonucleotides denoted by SEQ. ID. NO:1 or SEQ. ID. NO:7 are also referred to herein as EN101, or hEN101.

The antisense oligonucleotides directed against AChE have been described in the past by the present inventors [WO 03/002739], and were shown to have a potent effect in the treatment of the neuromuscular pathology myasthenia gravis [applicant's co-pending US 10/402,016]. In the inventors' herein

described results, as shown in Example 5 and Figure 4, the antisense oligonucleotide directed against AChE was able to reduce the release of IL-1β, which is a pro-inflammatory cytokine.

As shown in Example 1, AChE-R mRNA levels in motoneurons were minimally affected, However, elimination of AChE-R production in spinal cord smaller neurons potentially increased ACh signaling within the treated tissue, in spite of the stress-induced reduction in VAChT and ChAT [Kaufer *et al.* (1998) *id ibid.*]. This attributes to AChE-R the primary role of regulating ACh levels in the CNS. Findings of others show large variability in the electrophysiological activity patterns of spinal cord interneurons [Perlmutter (1996) *id ibid.*] as well as pre-movement instructed delay activity in them [Prut and Fetz (1999) *id ibid.*]. The inventors observed the largest variability in AChE-R levels within small cells, probably interneurons, suggesting that these modulations may contribute towards the wide electrophysiological variability between these neurons. Under normal conditions, AChE-R expression in small cholinergic neurons, localized to the dorsal horn of the spinal cord, may thus contribute to the control of motoneuron activities (e.g. motor reflexes). C-terminal structures, which affect the cholinergic input to motoneurons, were considered to originate in proximity to the motoneurons themselves [Hellstrom (1999) *id ibid.*]. This study attributes this origin to AChE mRNA positive interneurons and small cholinergic neurons located in the ventral horn and intermediate zone of the lumbar spinal cord. The numbers of VAChT-labeled C-terminals surrounding motoneuron cell bodies decreased in all of the handled animals. This observation attributes this decrease to the handling stress, compatible with the stress-induced decreases in ChAT and VAChT mRNA in hippocampal neurons [Kaufer *et al.* (1998) *id ibid.*].

Additional antisense oligonucleotides directed against AChE have also been described, and potentially have the same anti-inflammatory effect as hEN101, as demonstrated in Example 16 for mEN101. These are antisense

oligonucleotides derived from the mouse and the rat AChE homologous sequences, which have the following sequences:

mEN101 5'-CTGCAATATTTTCTTGCACC-3'
(SEQ. ID. NO:2) [Grifman and Soreq, (1997) *Antisense Nucleic Acid Drug Dev.* 7(4):351-9]
Also referred herein as EN301.

rEN101 5'-CTGCCATATTTTCTTGTACC-3'
(SEQ. ID. NO:3)

hEN103 5'-GGGAGAGGAGGAGGAAGAGG-3'
(SEQ. ID. NO:4)
[Grisaru, D. *et al.* (1999) *Mol. Cell Biol.* **19**(1):788-95]

Example 16 demonstrates how administration of mEN101 (EN301) was able to reduce the levels of AChE-R in the brain. This could be done directly, upon crossing the blood-brain-barrier, or indirectly, by reducing the levels of peripheral AChE, increasing the levels of ACh, which would then suppress the production of pro-inflammatory cytokines by macrophages.

Thus, in another aspect, the invention provides the use of an inhibitor of AChE as defined herein, as a suppressor of pro-inflammatory cytokines release. Preferably, said inhibitor of AChE is the antisense oligonucleotide denoted by any one of SEQ. ID. NO:1, SEQ. ID. NO:2 and SEQ. ID. NO:7. Most preferably, said inhibitor of AChE is the antisense oligonucleotide denoted by SEQ. ID. NO:1 or SEQ. ID. NO:7.

Known pro-inflammatory cytokines are IL-1β, TNFα, IL-6, IL-8, IL-12 and IL-18, amongst others. Preferably, IL-1β is the pro-inflammatory cytokine to be

suppressed by the antisense oligonucleotide denoted by any one of SEQ. ID. NO:1, SEQ. ID. NO:2 and SEQ. ID. NO:7.

Pro-inflammatory cytokine release may be triggered by factors of acquired, chemical or genetic origin. Amongst others, these may be stress, bacterial infection, drugs, irradiation, exposure to AChE inhibitors, stroke, auto-immune diseases, multiple chemical sensitivity, or any cumulative age-dependent damages.

Known conditions which trigger pro-inflammatory cytokine release are bacterial infection, drugs, irradiation, exposure to AChE inhibitors, stroke, auto-immune diseases, multiple chemical sensitivity, or any cumulative age-dependent damages.

Stress-induced spinal IL-1β over-production and spinal IL-1β suppression following AS-ON inhibition of AChE-R, support the notion of cholinergic regulation of anti-inflammatory response in the CNS. According to this scheme, "stressed" neurons produce high levels of AChE-R, reducing ACh and allowing uninterrupted production of IL-1β in CNS neurons that do not express IL-1β under normal conditions. Antisense suppression of the stress-induced AChE-R would increase ACh levels, which can then suppress IL-1β production in CNS neurons. Such cholinergic regulation of inflammatory response within the CNS may explain both the increase of pro-inflammatory cytokines under cholinergic imbalance (e.g. exposure to organophosphate compounds) [Svensson (2001) *id ibid.*; Dyer (2001) *id ibid.*] and the decrease of those same cytokines under retrieval of cholinergic balance (e.g. under antisense treatment, see Figure 6). This provides a new understanding of the improvement of survival and clinical status in EAMG rats receiving daily oral doses of EN101 as compared to the conservative AChE inhibitor (pyridostigmine) [Brenner (2003) *id ibid.*].

It is known in the literature that IL-1β induces arthritis in chondrocytes by suppressing Col2 gene expression [Hollander *et al.* (1994) *J. Clin. Invest.* **93**: 1722; Hollander *et al.* (1995) *J. Clin. Invest.* **96:** 2859; Bi *et al.* (1999) *Nat. Genet.* **22:** 85; Lefebvre *et al.* (1997) *Mol. Cell Biol.* **17**: 2336; Murakami *et al.* (2000) *J. Biol. Chem.* **275**: 3687; Tanaka *et al.* (2000) *Mol. Cell Biol.* **20:** 4428]. Therefore, the inhibition of IL-1β release by the antisense oligonucleotide herein described might result in cartilage regeneration. Thus, the invention also provides the use of an inhibitor of AChE expression, as defined herein, as an inducer of cartilage regeneration.

The antisense oligodeoxynucleotides used as anti-inflammatory agents in the present invention are preferably nuclease resistant. There are a number of modifications that impart nuclease resistance to a given oligonucleotide. Reference is made to WO 98/26062, which publication discloses that oligonucleotides may be made nuclease resistant e.g., by replacing phosphodiester internucleotide bonds with phosphorothioate bonds, replacing the 2'-hydroxy group of one or more nucleotides by 2'-O-methyl groups, or adding a nucleotide sequence capable of forming a loop structure under physiological conditions to the 3' end of the antisense oligonucleotide sequence. An example for a loop forming structure is the sequence 5' CGCGAAGCG, which may be added to the 3' end of a given antisense oligonucleotide to impart nuclease resistance thereon.

Phosphorothioate-modified oligonucleotides are generally regarded as safe and free of side effects. The antisense oligonucleotides of the present invention have been found to be effective as partially phosphorothioates and yet more effective as partially 2'-O-methyl protected oligonucleotides. WO 98/26062 teaches that AChE antisense oligonucleotides containing three phosphorothioate bonds out of about twenty internucleotide bonds are generally safe to use in concentrations of between about 1 and 10 μM. However, for long-term

applications, oligonucleotides that do not release toxic groups when degraded may be preferred. These include 2'-O-methyl protected oligonucleotides, but not phosphorothioate oligonucleotides. A further advantage of 2'-O-methyl protection over phosphorothioate protection is the reduced amount of oligonucleotide that is required for AChE suppression. This difference is thought to be related to the improved stability of the duplexes obtained when the 2'-O-methyl protected oligonucleotides are used [Lesnik, E.A. & Freier, S.M., *Biochemistry* 37, 6991-7, (1998)]. An alternative explanation for the greater potency of the 2'-O-methyl oligonucleotides is that this modification may facilitate penetration of the oligonucleotide chain through the cell membrane. A further advantage of 2'-O-methyl protection is the better protection against nuclease-mediated degradation that it confers, thus extending the useful life time of antisense oligonucleotides protected in this way.

Further, the inhibitor of AChE as defined above may also be used as an anti-pyretic. Preferably, said inhibitor of AChE is the antisense oligonucleotide denoted by any one of SEQ. ID. NO:1, SEQ. ID. NO:2 and SEQ. ID. NO:7.

In response to anesthesia, neural regulation induces rapid decrease in body temperature. As shown in Example 12, transgenic mice with host AChE-R elevation show inherently higher body temperature as compared to strain, gender and age-matched controls. Furthermore, their body temperature remains higher also under anesthesia, demonstrating impaired regulation and tentative association of AChE-R with pyrogenic responses. Thus, inhibitors of AChE-R expression would also have an effect in lowering the elevated body temperature that is characteristic of inflammatory reactions.

In accordance with the invention, the dosage of the antisense oligodeoxynucleotide is about 0.001 to 50 μg oligonucleotide per gram of body weight of the treated mammalian subject. Preferably, the dosage is about 0.01

to about 5.0 μg/g. More preferably, the dosage is between about 0.05 to about 0.7 μg/g. Thus, the optimal dose range is between 50-500 μg/kg of body weight of the treated subject, for rats, monkeys and also humans.

In a further aspect, the present invention intends to provide a pharmaceutical composition for the treatment of conditions triggering an inflammatory response in a mammalian subject in need, comprising as active agent the above-defined inhibitor of AChE expression. Optionally, the composition further comprises pharmaceutically acceptable additives, carriers and/or diluents. Preferably, said inhibitor of AChE expression is an antisense oligonucleotide directed against AChE.

More preferably, wherein said mammalian subject is a human, said antisense nucleotide has the sequence as denoted by any one of SEQ. ID. NO:1 and SEQ. ID. NO:7.

Alternatively, wherein said mammalian subject is a non-human mammalian, said antisense nucleotide has the sequence as denoted by any one of SEQ. ID. NO:2 and SEQ. ID. NO:3.

In a yet further aspect, the present invention provides a pharmaceutical composition for the treatment and/or prevention of inflammation in the joints, central nervous system, gastrointestinal tract, endocardium, pericardium, lung, eyes, skin and urogenital system in a mammalian subject in need, comprising as active agent the inhibitor of AChE expression as defined above, optionally further comprising pharmaceutically acceptable additives, carriers and/or diluents. Preferably, said inhibitor of AChE expression is an antisense oligonucleotide.

More preferably, wherein said mammalian subject is a human, said antisense nucleotide has the sequence as denoted by any one of SEQ. ID. NO:1 and SEQ. ID. NO:7.

Alternatively, wherein said mammalian subject is a non-human mammalian, said antisense nucleotide has the sequence as denoted by any one of SEQ. ID. NO:2 and SEQ. ID. NO:3.

The inhibitor of AChE expression, as defined above, is to be used in the preparation of the pharmaceutical composition of the invention.

Thus, the antisense oligonucleotide of the invention is generally provided in the form of pharmaceutical compositions. Said compositions are for use by injection, topical administration, or oral uptake.

Alternatively, the pharmaceutical composition of the invention may comprise as active agent a combination of at least two antisense oligonucleotides as defined in the invention, or functional analogs, derivatives or fragments thereof.

By "analogs and derivatives" is meant the "fragments", "variants", "analogs" or "derivatives" of said nucleic acid molecule. A "fragment" of a molecule, such as any of the oligonucleotide sequences of the present invention, is meant to refer to any nucleotide subset of the molecule. A "variant" of such molecule is meant to refer a naturally occurring molecule substantially similar to either the entire molecule or a fragment thereof. An "analog" of a molecule can be without limitation a paralogous or orthologous molecule, e.g. a homologous molecule from the same species or from different species, respectively.

Preferred uses of the pharmaceutical compositions of the invention by injection are subcutaneous injection, intraperitoneal injection, intravenous and intramuscular injection.

The pharmaceutical composition of the invention generally comprises a buffering agent, an agent which adjusts the osmolarity thereof, and optionally, one or more carriers, excipients and/or additives as known in the art, e.g., for the purposes of adding flavors, colors, lubrication, or the like to the pharmaceutical composition.

A preferred buffering agent is Tris, consisting of 10 mM Tris, pH 7.5-8.0, which solution is also adjusted for osmolarity.

For *in vivo* use, the antisenses are suspended is sterile distilled water or in sterile saline.

Carriers may include starch and derivatives thereof, cellulose and derivatives thereof, e.g., microcrystalline cellulose, xantham gum, and the like. Lubricants may include hydrogenated castor oil and the like.

Pharmaceutical compositions for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular

administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

The pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical compositions of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product. Such compositions may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams,

jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product.

In one embodiment, the pharmaceutical composition of the invention is for daily use by a subject in need of such treatment, at a dosage of active ingredient between about 0.001μg/g and about 50μg/g. Preferably, the treatment and/or prevention comprises administering a dosage of active ingredient of about 0.01 to about 5.0 μg/g. Most preferably, said dosage of active ingredient is of between about 0.05 to about 0.70 μg/g, and even most preferably , the dosage is from 0.15 to 0.50 μg/g of body weight of the subject in need.

Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the antisense oligonucleotide in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

The preparation of pharmaceutical compositions is well known in the art and has been described in many articles and textbooks, see e.g., Gennaro A. R. ed. (1990) *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, and especially pages 1521-1712 therein.

The results presented herein are the first demonstration of an organismal antisense response that affects primate CNS neurotransmission (Example 1). Positron Emission Tomography (PET) imaging studies in Rhesus monkeys demonstrated for 2'-O-methylated oligonucleotides limited, yet relatively long-term persistence in the brain as compared with phosphothioate agents [Tavitian (1998) *id ibid.*]. In addition, the blood-brain barrier of primates may

be more easily penetrated than that of rodents, which is compatible with the inventors' recent findings [Tomkins, O. *et al.* (2001) *Cell Mol Neurobiol* **21**: 675-691].

The antisense agent targeted toward the human ACHE sequence (see Examples) appeared effective in Cynomolgus monkeys at the same nanomolar dose as that of the corresponding agents in mice [Cohen *et al.* (2002) *id ibid.*] and rats Brenner *et al.* (2003) *id ibid.*]. Long-term AChE-R overproduction, as is the case in head-injured mice, is associated with impaired locomotion control that is susceptible to improvement under antisense suppression of AChE-R production [Shohami (2000) *id ibid.*]. In spite of the limited number of experimented animals used in the current study, delivery was appeared to be effective in both the intravenous and the oral administration mode, with dose dependence reflected by the more pronounced effects under 500 as compared to 150 μg/Kg/day of orally administrated hEN101.

Finally, the invention teaches a method of treatment of conditions triggering an inflammatory response, wherein said method comprises administering a therapeutically effective amount of an inhibitor of AChE expression to a mammalian subject in need, as defined herein, or a composition comprising as active agent an inhibitor of AChE expression, prepared as described above.

Preferably, said inhibitor of AChE expression to be used in the method of the invention is an antisense oligonucleotide, which, more preferably wherein said mammalian subject is a human, said antisense nucleotide has the sequence as denoted by any one of SEQ. ID. NO:1 and SEQ. ID. NO:7.

Alternatively, wherein said mammalian subject is a non-human mammalian, said antisense nucleotide has the sequence as denoted by any one of SEQ. ID. NO:2 and SEQ. ID. NO:3.

Said therapeutic effective amount, or dosing, is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}, found to be effective in *in vitro* as well as in *in vivo*.

The variant specificity, low dose and long duration efficacy of the antisense agents may be clear advantages over conservative drugs, both for interfering with acute stress-induced symptoms and inflammatory response, and hence for prevention of neurodeterioration. These considerations may be relevant to various disease conditions, including amyotrophic lateral sclerosis [Shaw, P.J. & Eggett, C.J. (2000) J. Neurol. 247 Suppl 1: I17-27], myasthenic syndromes [Becker et al. (1992) id ibid.], muscular dystrophy [Cifuentes-Diaz, C. et al. (2001) J. Cell Biol. 152: 1107-1114], spinal muscular atrophy [Sendtner, M. (2001) Curr. Opin. Neurol. 14: 629-634] and sepsis-mediated critical illness polyneuropathy [Hund, E. (2001) J. Neurol. 248: 929-934]. Antisense facilitation of the cholinergic attenuation of inflammatory responses in primate CNS neurons may thus offer interesting therapeutic advantages.

As shown in Examples 6-11, administration of a low dose of endotoxin to healthy volunteers induces secretion of proinflammatory cytokines and cortisol, compromises cholinergic homeostasis and alters memory. Both psychological [Maes M. et al. (1998) Cytokine 10:313-8], and physical [Goodman J. C. et al. (1990) J. Neuroimmunol. 30:213-7] stressors are likewise associated with the production of proinflammatory cytokines (including TNF-α and IL-6) in humans. Exposure to stressful stimuli exerts profound effects on cholinergic homeostasis in general and on the production and cellular distribution of

AChE-R in particular. Therefore, experimental endotoxemia emerges as a valid model for studying the interactions between cytokines and the changes in cholinergic homeostasis (as those are reflected by AChE-R modulations) as well as the impact of these interactions on memory functions. No subjective feelings of illness were involved, so that the endotoxin-induced memory alterations could not be attributed to a perceived physical-illness-associated distress. The selectivity of the observed memory changes was compatible with reports by others that cortisol does not affect attention, verbal executive function or vigilance [Lupien et al. (1999) Rev. Neurosci. 10: 117-39].

Figure 11 presents a scheme summarizing the kinetic follow-up for the different parameters that were measured and the postulated associations between them, predicting potentially causal relationships between the induction of cytokines, hormone secretion, AChE modulations and the resultant memory changes. Interestingly, during the first testing period the endotoxin-induced impairment in declarative memory was highest and correlated positively with cytokine secretion, whereas the improvement in working memory became prominent at 3hr post-treatment and showed no correlation with cytokine secretion. In contrast, both types of memory changes were significantly correlated with AChE-R cleavage, although cholinergic control over working memory seemed to begin earlier than for declarative memory (3 hr vs. 9 hr post-injection, Fig. 11B and Fig. 11C, respectively).

Previous reports have documented decrements in declarative memory following endotoxin administration to healthy volunteers [Reichenberg (2001) id ibid.], as well as following cytokine (especially interferon and interleukin-2) therapy [Meyers C. A. (1999) Adv. Exp. Med. Biol. 461:75-81; Capuron L. et al. (2001) Psychosom. Med. 63:376-86], viral (e.g., influenza) infection [Capuron (1999) id ibid.] or cortisol administration [de Quervain, D. J. et al. (2000) Nat. Neurosci. 3:313-4]. In this study, the endotoxin-induced decrease in declarative memory performance was associated with cytokines secretion only in the first testing

period. In contrast, it was associated with AChE activity and AChE-R cleavage levels during the last period, when cytokine concentrations have returned to baseline yet the differences between AChE activity and AChE-R cleavage were maximal between the endotoxin and the placebo conditions. These findings may suggest that immune-mediated processes are prominent in the early endotoxin-induced memory impairments, whereas the later effects are probably mediated by the cholinergic system.

This study demonstrates that changes in memory functioning following endotoxin exposure are co-associated with the induction of proinflammatory cytokines and AChE-R cleavage. The tentative pathway through which these changes may occur involves alterations in cholinergic neurotransmission and elevation in cytokine secretion (Figure 11). These are associated with many medical conditions that involve inflammatory processes, particularly within the brain (e.g., stroke, brain trauma and neurodegenerative disease, such as vascular dementia) [McGeer P. L. and McGeer E. G. (1995) Brain Res. Rev. 21:195-218; Saito H. et al. (1995) Clin. Exp. Pharmacol. Physiol. Suppl. 22:S257-9; Levin and Simon (1998) id ibid. For example, closed head injury results in the production of TNF-α and other proinflammatory cytokines [Goodman et al. (1990) id ibid.; Trembovler V. et al. (1999) J. Interferon Cytokine Res. 19:791-5] as well as in excessive accumulation of AChE-R within the brain [Shohami et al. (2000) id ibid.]. The findings presented herein suggest that cytokine-cholinergic interactions play an important role in the memory alterations that accompany these conditions, and may provide insights into the development of novel preventive and therapeutic procedures that will counteract the corresponding memory impairments without harming the improved capacities.

Disclosed and described, it is to be understood that this invention is not limited to the particular examples, process steps, and materials disclosed herein as such process steps and materials may vary somewhat. It is also to be

understood that the terminology used herein is used for the purpose of describing particular embodiments only and not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The following Examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

Examples

The basic working hypothesis guiding this study was that stimulus-induced modulations in the levels and composition of neuronal AChE variants, ChAT and VAChT together contribute toward the maintenance of cholinergic homeostasis in primate motoneurons. This predicted neuronal AChE-R overproduction as well as ChAT and VAChT suppression also under mild stress (e.g. handling or injection). To test this hypothesis, the inventors measured plasma AChE activities and labeled AChE-R mRNA, ChAT and

VAChT in lumbar spinal cord sections of cynomolgus monkeys with and without treatment with hEN101. There was no indication of change in the motor functioning of hEN101-treated monkeys following daily administration of nanomolar doses of hEN101 for one week, as assessed by general follow-up of motor behavior, clinical signs or electrocardiography. No treatment-related toxicity or inflammatory effect was observed in white blood cell (WBC) counts or post-mortem, suggesting that the modulations induced by this oligonucleotide reflected solely the consequences of its antisense effect and indicating general maintenance of cholinergic balance under such effects. Because of its specificity towards AChE-R mRNA, the inventors predicted that hEN101 would alter the level and/or composition of peripheral AChE. The inventors further whether AChE, ChAT and VAChT levels in motoneurons are changed under handling stresses and, if so, whether antisense suppression of AChE-R would attenuate neuronal IL-1β accumulation.

Experimental Procedures

Experimental procedures employed in studying the anti-inflammatory effects of hEN101 in the primate spinal cord

Animals: 15 month-old purpose-bred cynomolgus monkeys were supplied by Charles River (UK) Ltd. Antisense administration was performed at Huntingdon Life Sciences Ltd. (Huntingdon, UK), in compliance with all of the relevant regulations for animal experimentation in the UK.

Test substance: Human (h) HPLC-purified, GLP grade EN101 (purity 95% as verified by capillary electrophoresis) was purchased from Avecia Biotechnology (Milford, MA). The primary hEN101 sequence, 5'CTGCCACGTTCTCCTGCA*C*C*3' (SEQ. ID. NO:1) is complementary to the coding sequence of human AChE mRNA (GeneBank Accession No. NM 000665, nucleotide positions 733-752) within exon 2, common to all three AChE variants [Soreq, H. & Zakut, H. (1993) *Human cholinesterases and*

anticholinesterases, Academic Press, INC. San Diego; Ben Aziz-Aloya, R. *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2471-2475]. The three 3'-terminal residues (*) were protected against nuclease attack with oxymethyl groups at the 2' position. The sequence representing hEN101 with the three 3'-terminal bases modified is denoted by SEQ. ID. NO:7. Lyophilized oligonucleotides were resuspended in sterile double distilled water (24 mg/ml), and stored at –20 °C.

Several modes of chemical protection for antisense agents are currently being clinically tried in human studies [for recent review see Opalinska, J.B. & Gewirtz, A.M. (2002) *Nat. Rev. Drug Discov.* **1**: 503-514]. The chemical protection protocol used in the current study (namely, three 3'-terminal 2'-Oxymethyl groups) combines maintenance of the oligonucleotide's capacity to recruit RNase H to its unprotected part while tightening the hybridization bonds through the 2'-O-methyl groups [Soreq and Seidman (2001) *id ibid.*], and offering improved intestinal permeability [Geary, R. S. *et al.* (2001) *J. Pharmacol. Exp. Ther.* **296:** 890-7]. An additional benefit of this protection scheme is that removal of the protected 3' end will leave behind a naked and hence vulnerable oligonucleotide that will be rapidly degraded. Unlike other AS-ONs [Bennett, C.F. (2002) *Antisense Nucleic Acid Drug Dev.* **12**: 215-224; Braasch, D.A. & Corey, D.R. (2002) *Biochemistry* **41**: 4503-4510; Sazani, P. *et al.* (2002) *Nat. Biotechnol.* **20**: 1228-1233] gradual nucleolytic breakdown would not lead in this case, to non-specific interactions, of shortened ON agents.

hEN101 stability: Stability of freeze-dried hEN101 was tested by HPLC during storage at -20 ±5°C, 4 ±°C and 25 ±2°C (60 ±5% relative humidity) in the dark. Three samples from each storage condition were collected after 3, 6 and 9 months and their stability analyzed by HPLC. hEN101 was found to be stable for at least 6 months at -20°C under these storage conditions.

hEN101 administration: Three pairs of 1.5 to 2.5 Kg cynomolgus monkeys, 1 male and 1 female, were administered hEN101 for 7 days: 150 µg/Kg daily *per*

os (*p.o.*) by oral gavage (15 μg/ml in 0.9% saline) or 500 μg/Kg daily (p.o., 50 μg/ml in saline) or by intravenous (*i.v.*) injection (100 μg/ml in saline). Plasma samples were removed at the noted hours following the second day of treatment and kept at -20°C until use. Following 1 week of daily treatment, animals were euthanized and lumbar spinal cord preparations were paraffin-embedded by standard procedures. One male naïve monkey served as control.

Toxicology: Potential toxicity of hEN101 was tested at Huntingdon before, during and following treatment. Among the parameters noted were body weight, food consumption, general locomotor behavior, electrocardiography and blood pressure, blood count, prothrombin time and standard blood chemistry (Hitachi 917 Clinical Chemistry Analyzer). Post mortem observation included organ weights and scanning of hematoxylin and eosin-stained sections of brain, heart, kidneys, liver, lungs, spinal cord and stomach.

***In situ* hybridization:** Tissues were fixed in 4% paraformaldehyde and cut into 7 μm paraffin-embedded sections. Lumbar spinal cord sections were deparaffinized, rehydrated using serial ethanol dilutions and permeabilized with proteinase K (10 μg/ml, 10 min at 37°C). Slides were exposed to 5' biotinylated, fully 2'-oxymethylated AChE-R or AChE-S- specific 50-mer cRNA probes complementary to human *ACHE* pseudointron 4 or exon 6, respectively (Microsynth, Belgach, Switzerland). The following probes were employed:

- human AChE-R probe (nucleotide positions 88-38 in GenBank Accession No. S 71129; SEQ. ID. NO:5):

5'-
CUAGGGGGAGAAGAGAGGGGUUACACUGGCGGGCUCCCACUCCCCCUCC
UC-3';

- human AChE-S probe (nucleotide positions 2071-2022 in GenBank Accession No. NM 000665; SEQ. ID. NO:6):

5'-

CCGGGGGACGUCGGGGUGGGGUGGGGAUGGGCAGAGUCUGGGGCUCG UCU-3'.

Hybridization was performed overnight at 52°C in hybridization mixture containing 10 µg/ml probe, 50 µg/ml yeast tRNA, 50 µg/ml heparin and 50% formamide in 375 mM Na chloride, 37.5 mM Na citrate, pH 4.5. Slides were washed to remove unhybridized probe, blocked with 1% skim milk containing 0.01% Tween-20 and 2 mM levamisol, an alkaline phosphatase inhibitor used to suppress non-specific staining and incubated with streptavidin-alkaline phosphatase (Amersham Pharmacia, Little Chalfont Bucks, UK). Fast Red™ substrate (Roche Diagnostics, Mannheim, Germany) was used for detection.

Immunohistochemistry: Re-hydrated spinal cord sections were subjected to heat-induced antigen retrieval by microwave treatment in 0.01 M citrate buffer, pH 6.0. Non-specific binding was blocked by 4% naive goat or donkey serum in PBS with 0.3% Triton X-100 and 0.05% Tween 20. Slides were incubated with primary antibodies diluted in the same buffer (1 h, room temp., overnight, 4°C). Sections were rinsed and incubated with biotin-conjugated secondary antibody, diluted (1:200) in the same blocking buffer (3 h, room temp.). The primary antibodies included rabbit polyclonal anti-VAChT (1:100, Sigma, St. Louis, Mo), goat polyclonal anti-ChAT (1:50, Chemicon International, Temecula, CA) and goat anti-IL-1β (1:20, R&D systems, Minneapolis, MN). Biotinylated secondary antibodies were donkey anti-rabbit (Chemicon) and donkey anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA), both used at 1:200 dilutions. Detection was with Fast Red substrate for anti-VAChT and ChAT antibodies and with Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) for the anti-IL-1β antibody.

Confocal microscopy: was carried out using a Bio-Rad MRC 1024 confocal scanhead (Hemel Hempsted, Hertfordshire, U.K.) coupled to an inverted Zeiss Axiovert 135 microscope (Oberkochen, Germany) equipped with a Plan Apochromat 40X1.3 immersion objective. Fast Red was excited at 488 nm and emission was measured through a 580df32 interference filter (580 ± 16 nm). Immunolabeled sections were scanned every 0.5 μm and projections analyzed using the Image Pro Plus 4.0 (Media Cybernetics, Silver Spring, MD) software.

Cholinesterase activity measurements: Plasma samples were subjected to cholinesterase catalytic activity measurements [Ellman, G.L. *et al.* (1961) *Biochem. Pharmacol.* 7, 88-99] adapted to a multi-well plate reader. Acetylthiocholine (ATCh) hydrolysis rates were measured following prior incubation for 30 min with $5x10^{-5}M$ of the specific butyrylcholinesterase (BuChE) inhibitor tetraisopropylpyrophosphoramide, iso-OMPA. Total plasma cholinesterase activities were measured in the absence of inhibitors.

Experimental procedures employed in studying the relationships between AChE-R, cytokines and memory

Subjects of the memory study: Ten male subjects participated in the study, which was approved by an independent ethics committee. Subjects recruitment as well as physical and psychiatric screening, were described in detail elsewhere [Reichenberg A. *et al.* (2001) *id ibid.*]. The current study involved a subset of the subjects included in the previous project, with serum AChE and working memory tests added. Interviews by experienced psychiatrists excluded the presence and the history of any axis I psychiatric disorder according to the DSM-IV [American Psychiatric Association (1994) *Diagnostic and statistical manual for mental disorders*, 4th ed. Washington DC]. Only subjects who successfully passed the screening procedure, and signed an informed consent form, were considered eligible to participate. Comprehensive assessment was performed, and involved each subject going through a number of physical and

neuropsychological tests in a clinical research unit using a balanced, randomized, double-blind, cross-over design.

Procedure for the memory tests: All technical equipment, including the blood sampling device, was housed in a room adjacent to the sound-shielded experimental room. Every subject passed two 10 days apart testing sessions and spent the night before each experimental session in the research unit. A battery of neuropsychological tests, assessing memory, learning, and attention was given for adaptation upon their first arrival in the evening, minimizing subsequent practice effects [McCaffrey, R.J. and Lynch, J.K. (1992) *Neuropsychol. Rev.* **3**:235-48]. Alternate versions of these tests were used in the experimental testing sessions. In the next morning, an intravenous cannula was inserted into an antecubital forearm vein for intermittent blood sampling and intravenous (i.v.) injection of endotoxin (0.8 ng *Salmonella abortus equi* endotoxin per Kg body weight) in one session or the same volume of 0.9% NaCl (saline) solution on the other occasion (placebo). The order of injections was balanced, so that half of the subjects received the saline injection and half received the endotoxin injection first. No significant differences were found between the groups defined by the treatment order in either age, years of education, or body weight. The experimenter and the subject were blind with respect to the group assignment. During each session, subjects were tested three times, at 1-2, 3-4 and 9-10 hr post-injection. Blood was collected at baseline before i.v. injection, and at the beginning of each testing period. Rectal temperature was measured continuously using a thermistor probe. Self-reported physical sickness symptoms (headaches, muscle pain, shivering, nausea, breathing difficulties, and fatigue) were assessed at the end of each testing period, by a questionnaire using a 5-point Leikart scale (0-no symptoms, 4-very severe symptoms).

***Salmonella abortus equi* endotoxin**: Prepared for use in humans, this endotoxin was available as a sterile solution free of proteins and nucleic acids.

The endotoxin preparation employed has proven to be safe in various studies of other groups [Burrell R. (1994) *id ibid.*] and in studies at the Max Planck Institute of Psychiatry, including more than 100 subjects since 1991 [Pollmacher T. *et al.* (1996) *J. Infect. Dis.* **174**:1040-5].

Plasma levels of AChE and its degradation product, cytokines and cortisol: Blood was collected in tubes containing Na-EDTA and aprotinin and was immediately centrifuged. Plasma was aliquoted and frozen to -80°C. AChE catalytic activity was measured as the capacity for acetylthiocholine (ATCh) hydrolysis in the presence of 1 x 10^{-5} M tetraisopropylpyrophosphoramidate (iso-OMPA), a selective inhibitor of serum butyrylcholinesterase, BChE [Soreq H. and Glick D. (2000): Novel roles for cholinesterases in stress and inhibitor responses. In: Giacobini E. (ed.) *Cholinesterases and Cholinesterase Inhibitors: Basic, Preclinical and Clinical Aspects*. London, Martin Dunitz, pp 47-61]. Endotoxin-induced differences were calculated by subtracting activities in the absence of endotoxin, with each individual serving as its own control and daily hour carefully matched. To evaluate AChE-R concentrations and integrity, plasma proteins (40μg) were subjected to 4-20% polyacrylamide gel electrophoresis under fully denaturing conditions (BioRad Laboratories, Hercules, CA), blotted to nitrocellulose filters, incubated with rabbit anti-AChE-R antibodies [Sternfeld M. *et al.* (2000) *Proc. Natl. Acad. Sci. USA* **97**:8647-8652] and peroxidase-conjugated anti-rabbit immunoglobulins, and subjected to ECL™ detection (Amersham Pharmacia Biotech, UK), densitometric analysis and quantification as described [Shohami (2000) *id ibid.*]. The plasma levels of cortisol were determined by a radioimmunoassay, and the plasma levels of cytokines and soluble cytokine receptors were assessed by commercial enzyme-linked immunoabsorbent assays [Mullington J. *et al.* (2000) *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**:R947-55]. Labeling AChE-R mRNA and its protein product in vascular endothelial cells: Fluorescent in situ hybridization and immunohistochemistry of AChE-R mRNA and AChE-R protein were performed and quantified as reported [Cohen

(2002) *id ibid.*; Perry, C. *et al.* (2002) *Oncogene* **21**:8428-8441] using paraffin-embedded tissue sections from surgically-removed biopsies of patients with or without clinical inflammation due to non-specific kidney vasculitis or following kidney rejection.

MALDI-TOF-MS analysis of immunolabeled proteins: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was employed in an attempt to identify the protein and peptide bands labeled by anti-AChE-R antibodies in blotted membranes. Proteolytic degradation of the gel - eluted peptide was performed using the endoprotease LysC from Achromobacterlyticus (Wako Chemicals, Inc., USA) at a substrate to enzyme ratio of 200:1. Digestion was carried out overnight in 0.05M Tris HCl, pH 9.0, containing 4M urea at 30°C.

Neuropsychological assessment: Declarative memory was assessed using the *Story Recall* test [Green P. and Allen L. M. (1995): *Manual for the CogniSyst Story Recall test* CogniSyst Inc., Durham, NC]. Subjects were requested to repeat a 25-item story from memory immediately, and 30 min after presentation. The total number of correct verbatim recall was counted. Memory span and working memory were assessed using the *Digit Span forward & backward* [Wechsler D. (1987): *Wechsler Memory Scale, Revised Manual* The Psychological Corp, San Antonio, TX]. Subjects were requested to repeat lists of digits with increased number of digits every two lists either in the correct order of presentation (*forward condition*-assessment of span), or in a reversed order (*backward condition*- assessment of working memory). The number of lists correctly repeated was counted. Attention was assessed using the Ruff 2&7 cancellation test [Ruff R. M. and Allen C. C. (1996): *Ruff 2&7 Selective Attention Test: Professional Manual.* Psychological Assessment Resources Inc., Lutz, FL]: Subjects were instructed to mark either the digit 2 or the digit 7, which are randomly placed either between letters or between digits. The numbers of correct responses in a 5 minute trial were counted.

Statistical analyses: The main hypotheses concerning treatment effects on AChE activity, AChE-R levels, and neuropsychological performance were tested using repeated measure analysis of variance models (ANOVAs). Repeated measure ANOVAs were also used to examine the treatment effect on physical sickness symptoms, on plasma levels of cytokines and cortisol and on body temperature. The level of significance was set at the critical value of p=0.05 (two tailed). Whenever significant treatment-by-time interactions were found, the simple effects were analyzed as suggested [Winer B. *et al.* (1991): *Statistical Principles in Experimental Design*, 3rd ed. McGraw-Hill, New York], and Tukey's adjustments were applied.

To assess the associations between changes from the placebo to the endotoxin condition in AChE activity, AChE-R levels, and physiological (cytokines and cortisol secretion), and neuropsychological parameters, Pearson's correlation coefficients were calculated.

No deviation from normal distributions was evident for any of the dependent variables. No univariate outliers were found using Z-scores and no multivariate outliers were found using the Mahalanobis distance [Tabachnick B. G. and Fidell L. S. (2001) *Using Multivariate Statistics*, 4th ed. Allyn and Bacon, Boston, MA]. To adjust for any non-homogeneity of covariance for the within-subject effects, we used p values that were adjusted using the Huynh-Feldt method [Norusis M. J. (1994) *SPSS advanced statistics 6.1.* SPSS Inc., Chicago, IL]. Analyses were carried out using SPSS 10.

Linear rank Wilcoxon test for two related samples was used for the analysis of AChE-R- and IL-1β-positive fractions of analyzed neurons, measured on at least 4 sections from each group. Differences were considered significant when a p value of ~0.05 or less was obtained using the SAS 8.0 software. Student's t

test was used for analyzing the numbers and volume of VAChT-containing terminals in spinal cord sections.

Example 1

Treatment-reduced VAChT and ChAT labeling in spinal cord motoneurons

VAChT was predictably concentrated in cholinergic (C) terminals surrounding motoneurons [Weihe (1996) *id ibid.*], where it loads neural vesicles with ACh. Confocal microscopy projections of spinal cord motoneurons (cell diameter = 40μm) from hEN101-treated monkeys as compared with the naïve state showed small but significant dose-independent decreases ($p<0.01$, Student's t test) in the average number of VAChT-positive C-terminals per cell (Fig. 1A, 1B), suggesting a handling stress effect on loading C-terminals with ACh. VACh-T-labeled C-terminals were significantly smaller (<60 μm^3) under *p.o.* administration of 150 μg/Kg/day as compared to control sections (Fig. 1B and 1C, $p<0.01$, Student's t test), perhaps reflecting changes in VAChT translocation into vesicles and/or VAChT stability.

VAChT production is largely co-regulated with that of ChAT [Usdin, T.B.*et al.* (1995) *Trends Neurosci.* **18**, 218-224], since both are produced from one gene complex (the so called "cholinergic locus") [Erickson, J.D. *et al.* (1996) *Prog. Brain Res.* **109**, 69-82]. ChAT staining of C-terminals on motoneurons indeed presented similar changes to those observed for VAChT staining (data not shown). In addition, anti-ChAT antibodies labeled in control sections several partition cells (Fig. 1D), from which cholinergic terminals emerge to motoneurons [Barber, R.P. *et al.* (1984) *J. Comp. Neurol.* **229**, 329-346]. Lumbar spinal cord sections from hEN101-treated monkeys, regardless of the dose or mode of administration, revealed conspicuously decreased staining intensity of ChAT-positive partition cells (Fig. 1E), again indicating handling stress-related suppression of ACh production and slowdown of vesicle recycling.

Example 2

EN101 prevention of stress–induced increases in plasma AChE activity

Cholinesterase activities were measured in plasma samples taken during the second day of hEN101 administration. ATCh hydrolysis in plasma is largely due to serum BuChE, the primary serum cholinesterase encoded by a non-homologous mRNA which remained generally unchanged. However, plasma also includes a minor, but significant AChE activity [Zakut, H. *et al.* (1998) *Cancer* **61**, 727-737], measurable following pre-incubation in the presence of $5x10^{-5}$M of the BuChE-specific inhibitor, iso-OMPA. AChE activity increased, as compared with the values before treatment (pre-dose), within the 5 hr following the stressful oral gavage administration of 150 μg/Kg EN101 (Table 1), potentially reflecting increased production under handling. This further indicates a short half life for primate AChE-R mRNA *in vivo*, compatible with previous findings [Chan (1991) *id ibid*; Brenner *et al.* (2003) *id ibid.*]. Increases were effectively suppressed by the higher oral dose of 500 μg/Kg EN101, and yet more so following *i.v.* of administration of 500 μg/Kg EN101 (Table 1), possibly reflecting dose-dependent hEN101 prevention of AChE-R synthesis.

Table 1: hEN101-induced prevention of treatment-associated increases in plasma AChE activity [1]

hEN101 dose (μg/Kg)		150	500	500
Mode of administration		*p.o.*	*p.o.*	*i.v.*
	hr post-treatment			
Total ChE activity (% of pre-treatment[2])	0	100 ± 1	100 ± 2	100 ± 1
	3	92 ± 9	105 ± 1	89 ± 2
	6	102 ± 3	96 ± 2	94 ± 1
	12	98 ± 2	96 ± 1	93 ± 1
AChE activity (% of pre-treatment[3])	0	100 ± 4	100 ± 6	100 ± 4
	3	117 ± 2	114 ± 6	105 ± 4
	6	135 ± 1	100 ± 5	89 ± 5
	12	123 ± 3	112 ± 4	94 ± 3

[1] Percent changes in the ATCh hydrolysis rates in plasma samples from monkeys treated twice on 2 consecutive days with the noted amounts and administration routes of hEN101.
[2] In the absence of inhibitors, hydrolysis rates reflect activity of the abundant cholinesterase in plasma, BChE.
[3] AChE specific activity, measured in the presence of $5x10^{-5}$M of the specific BChE inhibitor, iso-OMPA. Values represent average ± SEM from six measurements in plasma samples derived from 2 monkeys. Mean AChE and BChE absolute activity.

Example 3

EN101 effects on AChE-R and AChE-S mRNAs in monkey spinal cord neurons

Paraffin-embedded sections of lumbar spinal cord from *Cynomolgus* monkeys treated for 7 days once daily with hEN101 were subjected to high resolution fluorescent *in situ* hybridization (FISH). Variant-specific FISH probes (Fig. 2A) revealed AChE-S more than AChE-R mRNA labeling in numerous punctuate areas and longitudinal threads, possibly cross-sections and longitudinal sections through neuronal processes (Fig. 2B-2C). This difference, albeit statistically non-significant was compatible with previous observations demonstrating AChE-S, but not AChE-R mRNA in murine neuronal processes

under normal conditions [Meshorer (2002) *id ibid.*]. The higher oral and *i.v.* dose yielded reduced AChE-R mRNA labeling (Fig. 2G and 2I as compared with the lower dose, Fig. 2E). AChE-S mRNA-labeled neurons displayed limited EN101-induced suppression (Fig. 2H, 2J as compared to 2D), with reduced process labeling (Fig. 2F, 2H and 2J). Positron Emission Tomography (PET) imaging studies in *Rhesus* monkeys demonstrated for 2'-O-methylated oligonucleotides limited, yet relatively efficient penetrance to the brain as compared with phosphorothioate agents [Tavitian *et al.* (1998) *id ibid.*]. In addition, the blood-brain-barrier of primates may be more easily penetrated than that of rodents [Tomkins *et al.* (2001) *Cell Mol. Neurobiol.* **21**: 675-91]. Nevertheless, this is the first demonstration of an organismal antisense response that affects primate CNS neurons.

At the same nanomolar dose as that of the corresponding agents in mice [Cohen (2002) *id ibid.*], and rats [Brenner (2003) *id ibid.*], delivery of human EN101 appeared in *Cynomolgus* monkeys to be effective in both the intravenous and the oral administration mode, as it did in rats [Brenner (2003) *id ibid.*]. Albeit in a limited number of animals, dose dependence was reflected by the more pronounced effects under 500 as compared to 150 μg/Kg/day of orally administrated hEN101.

Example 4

Antisense destruction of AChE-R mRNA is inversely related to perikaryon size

Similarly sized neurons in hematoxylin-eosin stained spinal cord sections (Fig. 3A) were sorted into three size groups according to their cell body diameter (Fig. 3B): motoneurons (=40 μm, 20-35% of total counted neurons, localized to motor nuclei in the ventral horn and intermediate zone), medium-sized neurons (20-40 μm, about 60%, dispersed throughout the spinal cord, mainly in the ventral horn and intermediate zone), and small neurons (10-20 μm, 5-20%, located primarily in the dorsal horn). AChE-S and AChE-R mRNA labeled cell

fractions from each group were evaluated in adjacent sections of small and medium sized AChE-R positive cells (<40 μm diameter) by over 4-fold as compared to the naïve state (p=0.057 for small cells, Wilcoxon test).

AChE-R-positive smaller neuron fractions dropped significantly under the higher hEN101 oral dose (p=0.033, Wilcoxon test), compared to the 150 μg/Kg/day treatment, and even further under its *i.v.* administration (p=0.015). Medium sized fractions dropped significantly following *i.v.* 150 μg/Kg/day as compared to *p.o.* administration of 150 μg/Kg/day (p=0.030). Reduced staining intensity suggested a certain antisense effect in motoneurons, as well, albeit with relatively limited efficacy. However, there was no discernable reduction in the total fractions of labeled large cell bodies by any treatment (p>0.100). This possibly reflects distinct membrane and/or metabolic properties, different cell volumes or a combined contribution of these properties. For AChE-S mRNA, the number of large positive cell bodies remained unchanged, whereas positive small and medium sized neurons, were reduced by 50% and 20%, respectively under either low or high dose of hEN101 as compared to naïve. The apparent dose-independence of changes in AChE-S mRNA is compatible with the hypothesis that these changes were not antisense driven, but could possibly reflect the effect of handling stress of shifting splicing from AChE-S to AChE-R [Kaufer (1998) *id ibid.*].

Example 5

hEN101 suppression of neuronal pro-inflammatory cytokines

Lumbar sections from hEN101-treated monkeys contained a higher fraction of both large and medium-sized IL-1β positive cell bodies than naïve sections, suggesting stress-induced inflammatory response (Fig. 4A, p=0.051 and 0.034 respectively, Wilcoxon test). Lower fractions of IL-1β labeled cell bodies were shown in sections from 500 μg/Kg/day hEN101-*i.v.* as compared to 150 μg/Kg/day *p.o.* treated monkeys (Fig. 4A, p=0.067 for both size groups, Wilcoxon test). Association analysis demonstrated a putative correlation

between neuronal AChE-R and IL-1β levels in medium-sized, but nor large cells (Fig. 4B and data not shown). IL-6 labeling as well was suppressed significantly following *i.v.* administration of 500 μg/Kg hEN101 (Fig. 4C, p=0.03 and 0.015 for medium and large neurons, respectively) as compared to 500 μg/Kg –*p.o.*-treated monkeys.

Example 6

Endotoxin induces impairments in AChE-R activity and integrity

Endotoxin administration produced a time-dependent decrease in plasma AChE activity, measured by quantifying the rate of ATCh hydrolysis in the presence of the butyrylcholinesterase (BChE) inhibitor iso-OMPA. This reduction displayed a significant treatment-by-time interaction (Fig. 5A) [$F(2,16)=3.94$, $p=0.04$]. Saline administration (placebo) caused no change in AChE activity, excluding the possibilities that it was induced by the injection stress or by circadian influences. The decline in hydrolytic activity could potentially reflect losses in the AChE protein. To test this possibility, electrophoretically separated plasma proteins were immune-reacted with antibodies selective for the C- terminal peptide unique to AChE-R [Sternfeld *et al.* (2000) *id ibid.*]. These antibodies labeled a 66kd protein, likely to be full-length AChE-R, as well as a shorter peptide with an apparent size of 6.5 kD. A parallel labeling pattern in the serum of stressed mice [Grisaru *et al.* (2001) *id ibid.*] raised the suggestion that this was an immunopositive C- terminus cleavage product of AChE-R. Endotoxin administration induced a slight, yet persistent, increase in the AChE-R cleavage product (Fig. 5B, 5C). This increase did not reach statistical significance [$F(1,8)=2.32$, $p=0.16$, for treatment effect] (Fig. 5C). However, at 9 hr post-treatment, the endotoxin-induced decrease in AChE activity was significantly correlated with endotoxin-induced increase in AChE-R cleavage ($r=-0.65$) (Fig. 5D).

Example 7

MALDI-TOF-MS analysis of AChE-R cleavage product

To further characterize the AChE-R cleavage product, larger plasma samples (180 μg/lane) were resolved by electrophoresis. Protein bands that co-migrated with the bands labeled with anti AChE-R antibodies were cut out of the gel and subjected to MALDI-TOF-MS analyses. The elution product of the larger band was identified as being mainly composed of serum albumin (molecular weight, 69367), compatible with the assumption that AChE-R is only a minor component in this size fraction of human serum proteins. The shorter peptide eluted from the excised band, however, revealed a single peak with a molecular mass of 3613-3615. Figure 6 demonstrates the MALDI-TOF-MS profile of this eluted peptide. Peptide property calculations positioned the presumed proteolytic cleavage site 36 residues from the C- terminus of AChE-R, with a calculated mass of 3614. Under these assumptions, cleavage could occur between asparagine and arginine residues upstream to the AChE-R diversion site (Fig. 6).

Parallel size peptides were observed in gel-eluted products from several individuals, demonstrating consistent cleavage processes. LysC proteolysis failed to further shorten this peptide. Edman degradation was unsuccessful, perhaps due to N- terminal blockade, and further experiments were prevented because of lack of material. The mass spectrometry approach thus pointed, although inconclusively, at an AChE-R cleavage site in human plasma under endotoxic stress near the C-terminal splice site that marks the deviation between human AChE splice isoforms.

Example 8

Vascular endothelial cells produce AChE-R

In search for the potential cell type origin of plasma AChE-R, the inventors performed fluorescent in situ hybridization (FISH) and immunohistochemistry

on human tissues from patients with or without inflammatory diseases (e.g. kidney vasculitis). Vascular endothelial cells displayed labeling with both AChE-R cRNA and anti AChE-R antibodies (Fig. 7A, 7B). Quantification of signal intensities revealed considerable similarities between AChE-R mRNA and AChE-R protein levels in patients with or without inflammatory vasculitis, so that tissues with less pronounced mRNA labeling also displayed fainter protein labeling (Fig. 7C). This pointed at vascular endothelial cells, which also harbor non - neuronal nicotinic acetylcholine receptors [Heeschen *et al.* (2002) *J. Clin. Invest.* **110**:527-36] as a probable site of continuous plasma AChE-R production.

Example 9

AChE-R cleavage is associated with cytokines secretion

Endotoxin induced a transient, significant increase in the plasma levels of cortisol, TNF-α and IL-6 (Fig. 8A-8C), although at the employed dose it does not produce any significant effects on the subjective rating of physical or behavioral sickness symptoms [Reichenberg (2001) *id ibid.*]. The selective increase in peripheral cytokine levels in the absence of subjective CNS effects on cognitive or intellectual function, suggested that changes in memory functions under these conditions would reflect objective endotoxin-induced alterations. Cortisol levels increased during the first and second testing periods, TNF-α and IL-6 peaked during the first testing period and decreased thereafter and rectal temperature (not shown) peaked during the second period. These time-dependent effects were reflected by significant treatment-by-time interactions [$F(2,16)=41.2$, 10.6, 10.5, 3.2, respectively, all $p<0.05$, by H-F].

At each testing period, correlation analysis enabled the comparison between the biochemical and functional responses of tested individuals. Thus, endotoxin-induced AChE-R cleavage (computed as the change in a certain individual from the endotoxin to the placebo condition) was significantly

($p<0.05$) and positively correlated with the secretion of cortisol, during the last testing period ($r=0.70$) (Fig. 8A). AChE-R cleavage was significantly ($p<0.01$) and negatively correlated with the secretion of TNF-α and IL-6 during the first ($r=-0.72$ and -0.66, respectively) (Fig. 8B, 8C), but not later testing periods.

Example 10

AChE-R cleavage is associated with endotoxin-induced impairments in declarative memory

Endotoxin administration decreased the performance in tests of declarative memory during all testing periods. This was reflected by decreased immediate recall of story items [$F(1,8)=6.5$, $p=0.03$] (Fig. 9A) and reduced delayed story recall [$F(1,8)=3.5$, $p=0.09$] (data not shown). Endotoxin-induced decrease in immediate and delayed recall of story items was significantly ($p<0.05$) and negatively associated with TNF-α and IL-6 secretion ($r=-0.59$ to -0.67) during the first, but not during other testing periods (data not shown), suggesting the potential involvement of additional mechanism(s) in endotoxin-induced impairments in declarative memory. At the last testing period, the endotoxin-induced decrease in immediate recall of story items was significantly ($p<0.05$) and negatively ($r=-0.63$) associated with AChE-R cleavage (Fig. 9B), indicating that the consequent increase in ACh levels, perhaps in conjunction with continuously suppressed cytokine production, interferes with declarative memory. This notion was supported by the positive ($r=0.68$) association of declarative memory impairments with the decrease in AChE activity during the last testing period (Fig. 9C), when cytokine levels already receded, but not during earlier testing periods.

Example 11

AChE-R cleavage association with improved working memory

Endotoxin administration induced a significant improvement in working memory performance, reflected by an increased score in the digit span backward test during all testing periods [$F(1,8)=12.3$, $p=0.008$] (Fig. 10A). No

significant changes in the digit span forward test (assessing memory span) or on the attention test (Ruff 2&7 cancellation test) were evident (data not shown), emphasizing the selectivity of the observed differences.

The endotoxin-induced improvement in working memory performance showed no significant association with the secretion of TNF-α, IL-6 or cortisol, yet was negatively associated with AChE-R cleavage. Association was significant ($p<0.05$) during the second and third testing periods ($r=-0.84$ and -0.64, respectively) (Fig. 10B and data not shown). Thus, subjects with a greater endotoxin-induced elevation in AChE-R cleavage (and, presumably, larger increases in ACh levels) showed both lower endotoxin-induced improvement in working memory functioning, and greater endotoxin-induced impairment in declarative memory.

Example 12

AChE-S Transgenic mice display elevated body temperature

Fever is one of the consequences of higher levels of circulating pro-inflammatory cytokines. In order to verify whether the constitutive expression of human synaptic AChE (hAChE-S) [Beeri *et al.* (1995) *id ibid.*] and the consequent over-expression of murine AChE-R [Cohen *et al.* (2002) *id ibid.*] influenced the release of pro-inflammatory cytokines in the animal, the inventors measured body temperature. Five transgenic FVB/N hAChE-S and mAChE-R overexpressing females, 3-5 months old, had their temperature measured between 5 and 55 minutes after anesthesia, which was administered in order to induce a change in body temperature. As shown in the graph (Fig. 12A-B), body temperature decreased with post-treatment time. Interestingly, the average body temperature of the transgenic mice was always 2°C higher than in the control mice. This suggests that their inherited cholinergic imbalance impaired their control over body temperature. These finding are compatible with the inventors' previous report of impaired hypothermic

response of these transgenic mice to the administration of paraoxon [Beeri *et al.* (1995) *id ibid.*].

Example 13

Effects of Tacrine on LPS-induced IL-1 secretion in the hippocampus and IL-1 and TNF-α secretion in the serum.

Male C57 mice were injected (i.p.) with either saline or tacrine (1.5 mg/Kg), immediately followed by an injection of either saline or LPS (1.0 mg/Kg) (n=5 animals per group). Two hours later, mice were deeply anesthetized with 24 μg Nembutal per mouse, blood was taken by heart puncture and the hippocampus was excised and placed in tubes containing 500 μl of RPMI + 100 KIU aprotinin. The levels of IL-1β in the hippocampus (Fig. 13A) and IL-1β (Fig. 13B) and TNF-α (Fig. 13C) in the serum were assessed with commercial ELISA kits (R&D Systems). LPS induced a significant increase in the hippocampal and serum IL-1β, which was significantly attenuated in tacrine-treated mice. In contrast, tacrine produced a small and non-significant attenuation of LPS-induced TNF-α secretion in the serum.

Example 14

Effects of Rivastigmine on LPS-induced IL-1 secretion in the hippocampus and IL-1 and TNF-α secretion in the serum.

Male C57 mice were injected (i.p.) with either saline or one of three doses of rivastigmine (0.5, 1.5 and 3.0 mg/Kg), immediately followed by an injection of either saline or LPS (1.0 mg/Kg) (n=5 animals per group). Two hours later, mice were deeply anesthetized with 24 μg Nembutal per mouse, blood was taken by heart puncture and the hippocampus was excised and placed in tubes containing 500 μl of RPMI + 100 KIU aprotinin. The levels of IL-1β and TNF-α were assessed with commercial ELISA kits (R&D Systems). LPS induced a significant increase in the hippocampal IL-1β, which was significantly attenuated only by the high dose of rivastigmine (Fig. 14A). LPS-induced IL-

1β secretion within the blood was dose-dependently suppressed by the 1.5 and 3.0 mg/Kg doses of rivastigmine (Fig. 14B). LPS-induced TNF-α secretion in the blood was not affected by rivastigmine treatment, even at a high dose (Fig. 14C).

Example 15

Cytokines as mediators of emotional and cognitive effects of stress caused by surgery

Several lines of evidence indicate that stress influences a variety of cognitive functions, including memory. In particular, exposure to stress was found to impair declarative memory, while leaving procedural memory intact. It is also well known that stress influences many immune functions, including the production and secretion of cytokines. Following exposure to various stressors, there is an increase in peripheral IL-6, as well as IL-1β and TNFα, accompanied by decrease in IL-2, in both humans and experimental animals.

The study was designed to examine the role of cytokines in mediating the affective and cognitive effects of stress. Two types of stressful situations were investigated in the same subjects: Psychological stress – while waiting for a surgery (i.e., in the morning of the surgery day), and surgical stress – in the day after surgery.

Twenty generally healthy volunteers were administered with a comprehensive neuropsychological test battery, assessing emotional and cognitive parameters, before and after a minor surgery (Laparoscopic Cholecystectomy or Hernia). Each subject was tested in three occasions: (a) Several days before surgery (baseline) = t0, (b) In the morning of the surgery day = t1, (c) A day after surgery = t2. Blood samples were collected in each session, and serum levels of cytokines (IL-1β, IL-6) were measured. Fifteen control subjects went through the same procedure.

In the morning of the surgery day, there was a significant increase in the levels of both anxiety (STAI) (Fig. 15A) and depression (DACL) (Fig. 15B) ($F(2,82)=3.871$, $p<0.025$ and $F(2,82)=11.189$, $p<0.0001$, respectively) . No change was found in the levels of fatigue and pain (Figs. 15C and 15D, respectively). In the morning following surgery there was further increase in depression, but not in anxiety, alongside a significant increase in pain and fatigue ($F(2,80)=24.588$, $p<0.0001$ and $F(2,80)=10.148$, $p<0.0001$, respectively).

With regards to the cognitive parameters (Fig. 15E-15H), in the morning of the surgery day tests showed a significant decline in performance of the word list recall task (HVLT) ($F(2,70)=4.120$, $p<0.021$). In the morning following surgery, an additional decline was found in the word list recall as well as in the performance of a visual memory task involving a complex figure reconstruction (MCG) ($F(2,70)=3.973$, $p<0.023$).

For each parameter (psychological performance, cytokine level, etc) differences were computed between each stressful situation (t1, t2) and baseline (t0). Pearson correlations were computed between cytokines levels and psychological variables (Fig. 16A-C).

In the morning of the surgery day (t1), there was a significant correlation between increased levels of IL-1β and the elevation in depressed mood ($r=0.525$) (Fig. 16b).

In the morning following the surgery (t2), there were significant correlations between increased IL-1 levels and impaired immediate and delayed Logical memory (story recall test) ($r=-0.627$ and -0.532, respectively). Significant correlations were obtained between increased IL-6 levels and improved delayed recall in the Word List Recall (HVLT) test ($r=0.386$), as well as improved immediate and delayed Complex Figure recall test (MCG) ($r=0.502$ and 0.590,

respectively). There was a significant increase in IL-6 ($F(2,38)=29.114$, $p<0.0001$) (Fig. 16C).

Example 16

Selective elimination of AChE-R mRNA in the brain of EN301-treated mice

Experimental procedure:

3 month old FVB/N female mice were injected intra-peritoneally daily with 500μg/Kg of EN301 (n=7) or with vehicle (PBS, n=6). EN301 corresponds to mEN101, defined herein as SEQ. ID. NO:2. This antisense oligonucleotide is targeted to a sequence within exon 2 of mouse AChE exon 2 sequence. EN301 was produced by Microsynth, Switzerland, at relatively large quantities for animal tests. The treatment persisted for 3 consecutive days, and the mice were sacrificed on day 4. Brain was collected, flash frozen in liquid nitrogen and stored at -70°C.

Total RNA was extracted from the brain and RT-PCR reaction was conducted using primers targeting the common sequence in Exon 2 of murine AChE cDNA or the unique sequence in Exon 6, specific to the AChE-S variant. 5 μl samples were removed from the 50μl PCR reaction mixture at cycles 25, 31 and 35. Samples were run on a 1.5% Agarose gel. The results of the PCR specific for the exon 2 sequence, after 31 cycles, are shown in Fig. 17A. Photographs were saved and fluorescence quantified using the PhotoShop software, and the results expressed in histograms (Figs. 17B-17C).

Results:

The goal of the present experiment was to test for reduction in AChE gene expression under EN301 treatment, while ensuring that AChE-S mRNA levels are maintained reflecting sustained cholinergic neurotransmission.

Normalized to RNA quantities, EN301-treated brains showed a significant 25% reduction ($p=0.01$, Student's T-Test) in the common transcript levels (Fig. 17B), whereas the S variant showed a non-significant 17% increase (Fig. 17C), reflecting a relatively larger fraction of AChE-S mRNA out of the total content of mRNA as compared with the untreated brain.

The ratio between AChE-S:common (S/Com) transcripts showed that in the EN301-treated brain, the S/Com ratio is significantly increased (from 0.65 to 0.98). RT-PCR data cannot be used as such for comparing the absolute quantities of the analyzed transcripts, because different primer pairs may function with different efficacies. However, that these two tests point at the same direction (namely, that AChE-R but not AChE-S mRNA was reduced in the EN301-treated brains and that the relative concentration of AChE-S mRNA increased, albeit insignificantly, under treatment) supports the notion that this agent affects brain gene expression as well.

The present results lead to the conclusion that EN301 treatment causes selective destruction of AChE-R mRNA in the EN301 treated brains while maintaining essentially unmodified AChE-S levels. Note that to exert such an effect, EN301 does not necessarily have to cross the blood-brain barrier. Rather, by reducing the levels of peripheral AChE it would increase acetylcholine levels, suppressing the production by macrophages of pro-inflammatory cytokines e.g. IL-1 [Wang, H. *et al.* (2003) *Nature* **421**, 384-8]. Because IL-1 promotes AChE gene expression [Li *et al.* (2000) *J. Neurosci.* **20**, 149-155], and since the peripheral pro-inflammatory cytokines are known to affect the brain [Pick *et al.* (2004) *Annals NY Acad Sci.* **in press**], such an effect will eventually reduce AChE-R levels in the brain as well.

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2___

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JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

Claims:

1. Use of an inhibitor of AChE expression, as an anti-inflammatory agent.

2. Use of an inhibitor of AChE expression, as a suppressor of pro-inflammatory cytokines release.

3. The use as defined in any one of claims 1 and 2, wherein said inhibitor of AChE expression is any one of an AChE-specific ribozyme, an RNA sequence used for RNA interference of the AChE gene, and an antisense oligonucleotide directed against AChE.

4. The use as defined in any one of claims 1 and 2, wherein said inhibitor of AChE expression is a nuclease resistant antisense nucleotide directed against AChE.

5. The use as defined in any one of claims 3 and 4, wherein said inhibitor of AChE expression is an antisense oligonucleotide directed against AChE, having the sequence as denoted by any one of SEQ. ID. NO:1, SEQ. ID. NO:2 and SEQ. ID. NO:7.

6. Use of an inhibitor of AChE expression, as an anti-pyretic.

7. The use as defined in claim 6, wherein said inhibitor of AChE expression is any one of an AChE-specific ribozyme, an RNA sequence used for RNA interference of the AChE gene, and an antisense oligonucleotide directed against AChE.

8. The use as defined in claim 7, wherein said inhibitor of AChE is an antisense oligonucleotide directed against AChE, having the sequence as denoted by any one of SEQ. ID. NO:1, SEQ. ID. NO:2 and SEQ. ID. NO:7.

9. The use as defined in claims 2 to 5, wherein said pro-inflammatory cytokine is any one of IL-1β, TNFα, IL-6, IL-8, IL-12 and IL-18.

10. The use as defined in claim 9, wherein said pro-inflammatory cytokine is IL-1β.

11. The use as defined in claims 2 to 5, wherein said pro-inflammatory cytokine release is triggered by any one of stress, bacterial infection, drugs, irradiation, exposure to AChE inhibitors, stroke, auto-immune diseases, multiple chemical sensitivity, and any cumulative age-dependent damages.

12. A pharmaceutical composition for the treatment of conditions triggering an inflammatory response in a mammalian subject in need, comprising as active agent an inhibitor of AChE expression, optionally further comprising pharmaceutically acceptable additives, carriers and/or diluents.

13. A pharmaceutical composition for the treatment and/or prevention of inflammation in the joints, central nervous system, gastrointestinal tract, endocardium, pericardium, lung, eyes, skin and urogenital system in a mammalian subject in need, comprising as active agent an inhibitor of AChE expression, optionally further comprising pharmaceutically acceptable additives, carriers and/or diluents.

14. The pharmaceutical composition as defined in any one of claims 12 and 13, wherein said inhibitor of AChE expression is any one of an AChE-specific ribozyme, an RNA sequence used for RNA interference of the AChE gene, or an antisense oligonucleotide directed against AChE.

15. The composition as defined in claim 14, wherein said mammalian subject in a human, and said inhibitor of AChE expression is an antisense oligonucleotide directed against AChE, as denoted in any one of SEQ. ID. NO:1 and SEQ. ID. NO:7.

16. The composition as defined in claim 14, wherein said mammalian subject in a non-human mammalian, and said inhibitor of AChE expression is an antisense oligonucleotide directed against AChE, as denoted by SEQ. ID. NO:2.

17. The pharmaceutical composition of any one of claims 12-16, which is for daily use by a subject in need of a dosage of active ingredient between about 0.001μg/g and about 50μg/g.

18. The pharmaceutical composition of anyone of claims 12-16, wherein the treatment and/or prevention comprises administering a dosage of active ingredient of about 0.01 to about 5.0 μg/g.

19. The pharmaceutical composition of any one of claims 12-18, wherein the treatment and/or prevention comprises administering a dosage of active ingredient of about 0.15 to about 0.50 μg/g.

20. Use of an inhibitor of AChE expression, for the preparation of a pharmaceutical composition as defined in any one of claims 12 to 19, wherein said inhibitor of AChE expression is any one of an AChE-specific ribozyme, an RNA sequence used for RNA interference of the AChE gene, and an antisense oligonucleotide directed against AChE.

21. A method of treatment of conditions triggering an inflammatory response comprising administering a therapeutic effective amount of an inhibitor of AChE expression to a mammalian subject in need, or a composition as

defined in any one of claims 12 to 19, wherein said inhibitor of AChE expression is any one of an AChE-specific ribozyme, an RNA sequence used for RNA interference of the AChE gene and an antisense oligonucleotide directed against AChE.

22. The method as defined in claim 21, wherein said mammalian subject is a human, and said inhibitor of AChE expression is an antisense oligonucleotide as denoted by any one of SEQ. ID. NO:1 and SEQ. ID. NO:7.

23. The method as defined in claim 21, wherein said mammalian subject is a non-human mammalian, and said inhibitor of AChE expression is an antisense oligonucleotide as denoted by SEQ. ID. NO:2.

24. The method as defined in any one of claims 21 to 23, wherein said conditions are selected from any one of stress, bacterial infection, drugs, irradiation, exposure to AChE inhibitors, stroke, auto-immune diseases, multiple chemical sensitivity and any cumulative age-dependent damages.

Fig. 1A

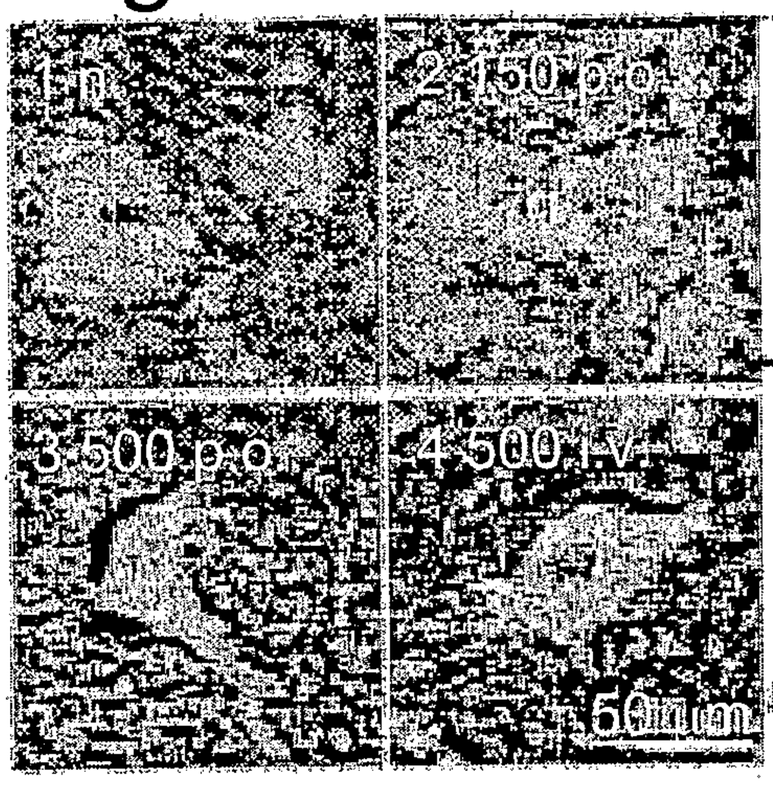


Fig. 1B

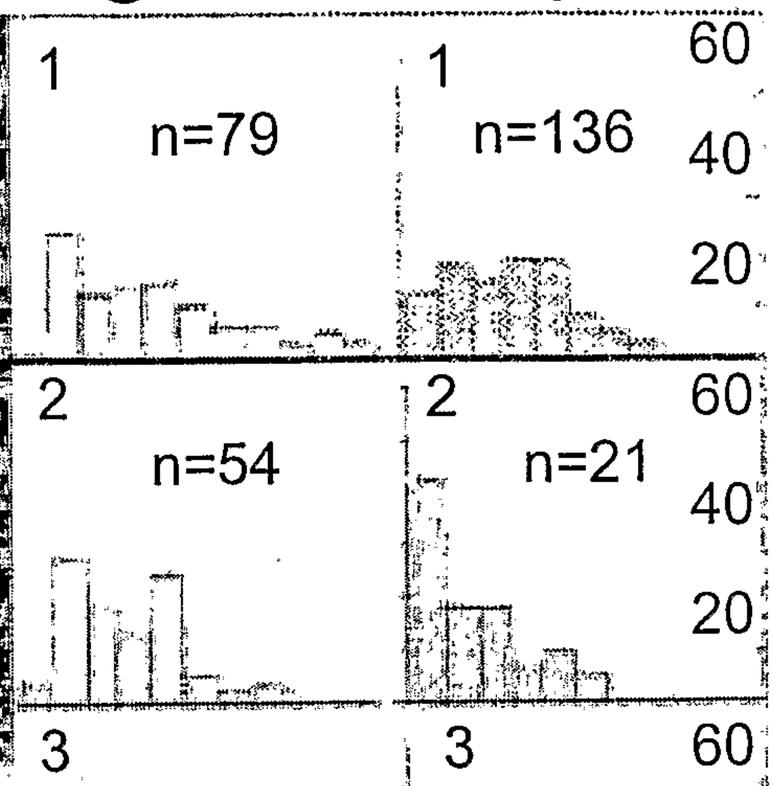


Fig. 1C

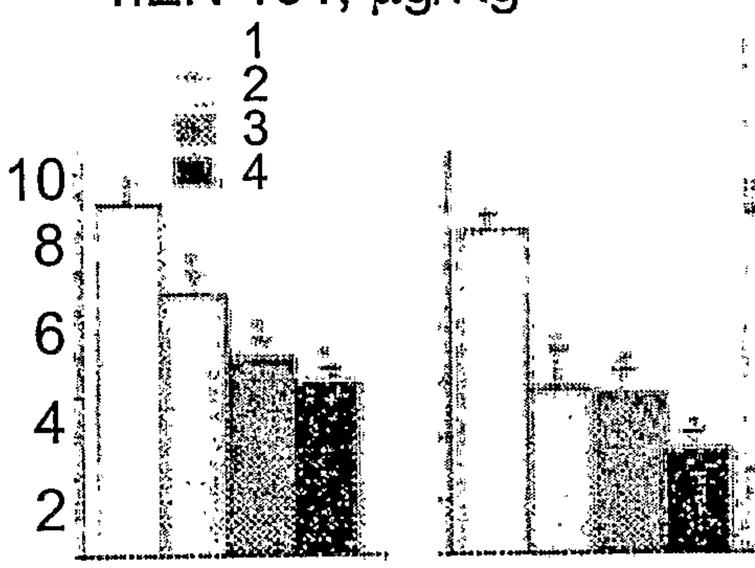


Fig. 1D

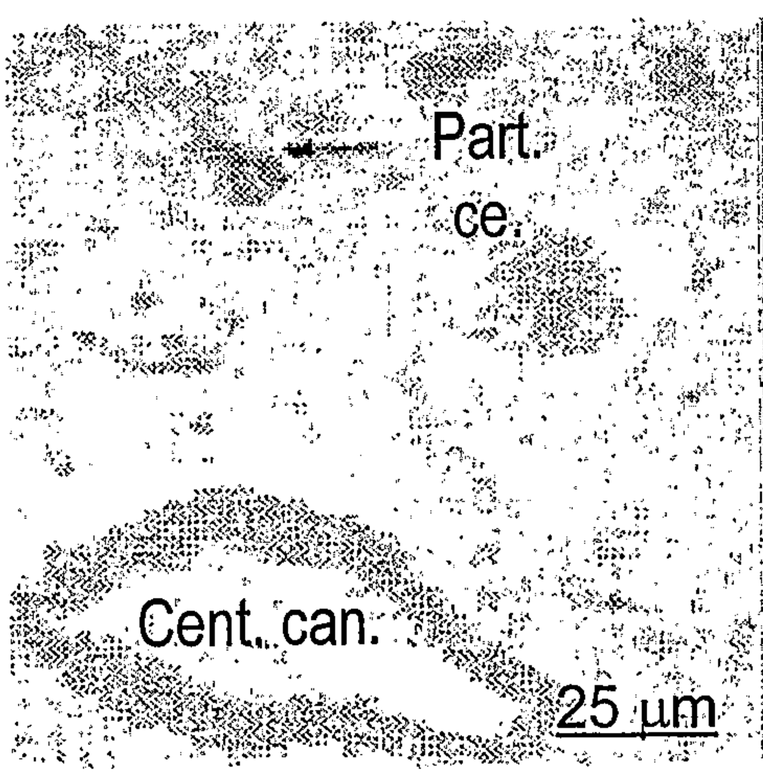


Fig. 1E

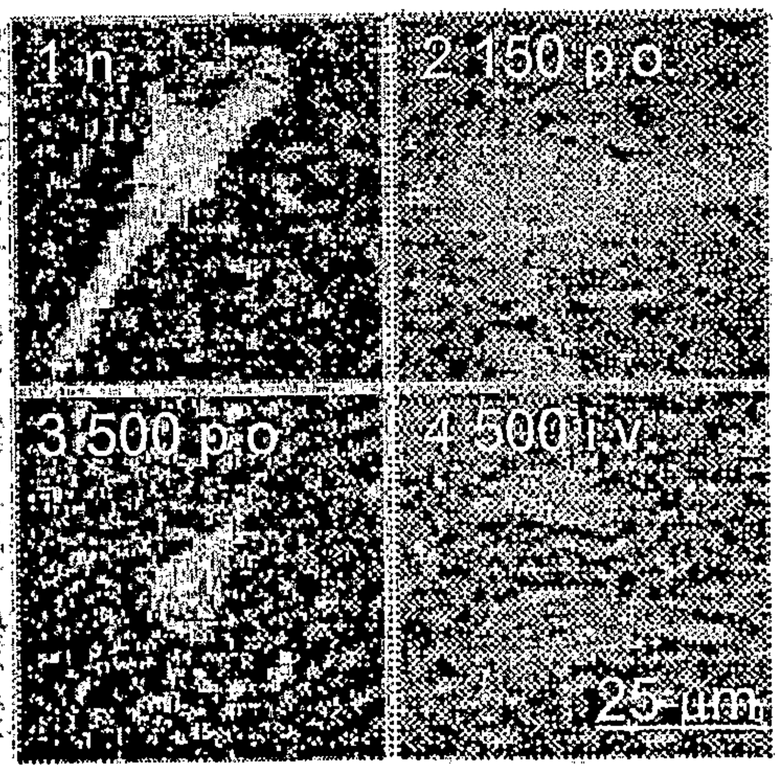


Fig. 1F

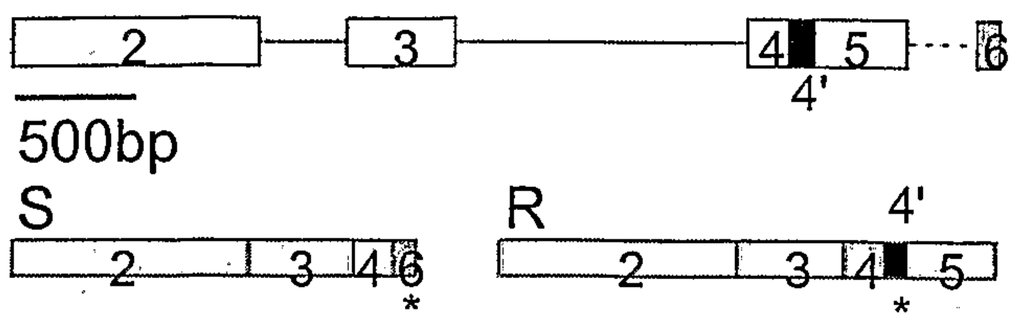


Fig. 2A

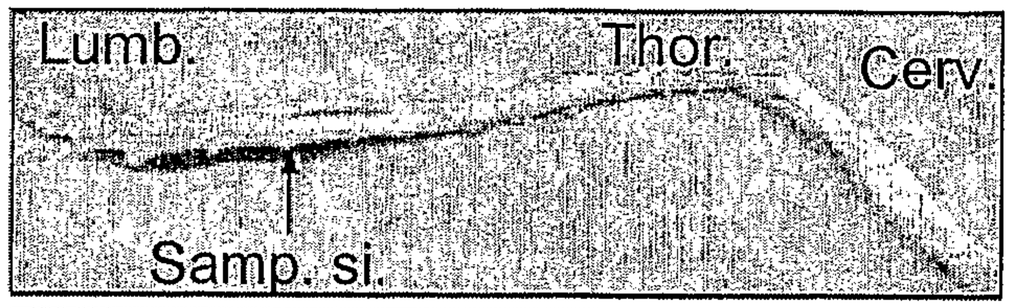


Fig. 2B

Fig. 2C

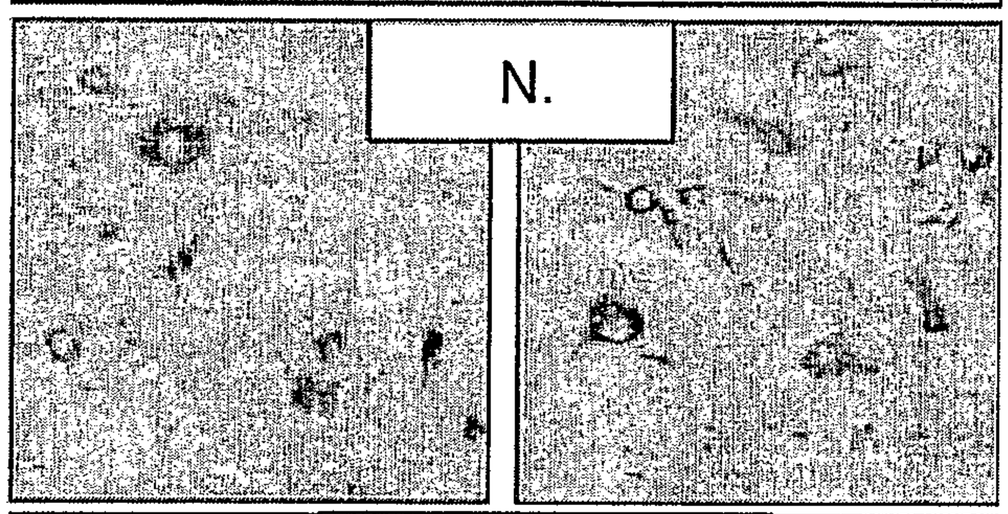


Fig. 2D

Fig. 2E

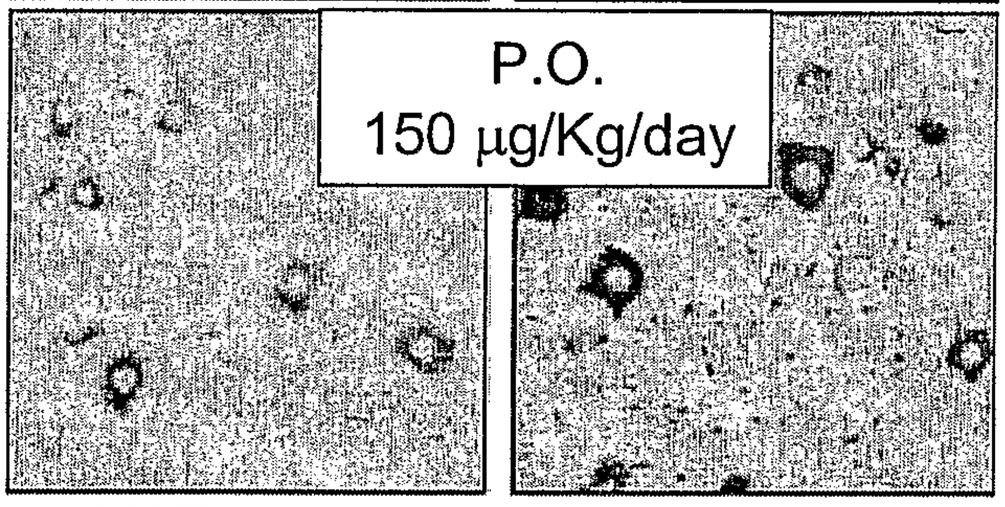


Fig. 2F

Fig. 2G

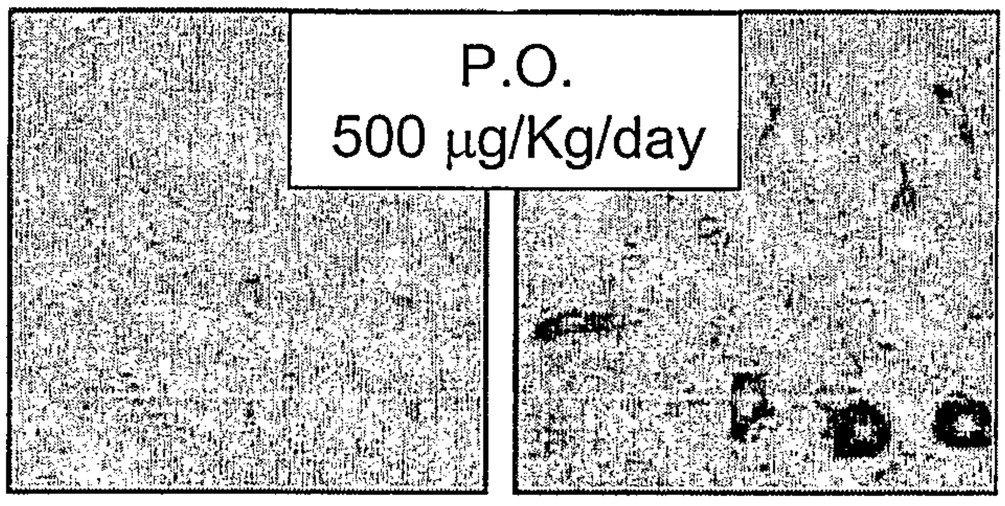


Fig. 2H

Fig. 2I

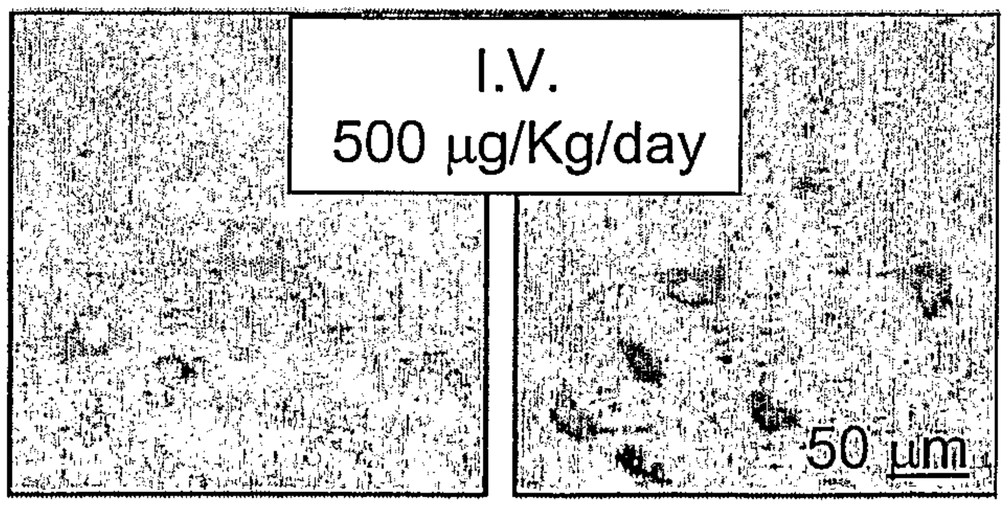


Fig. 2J

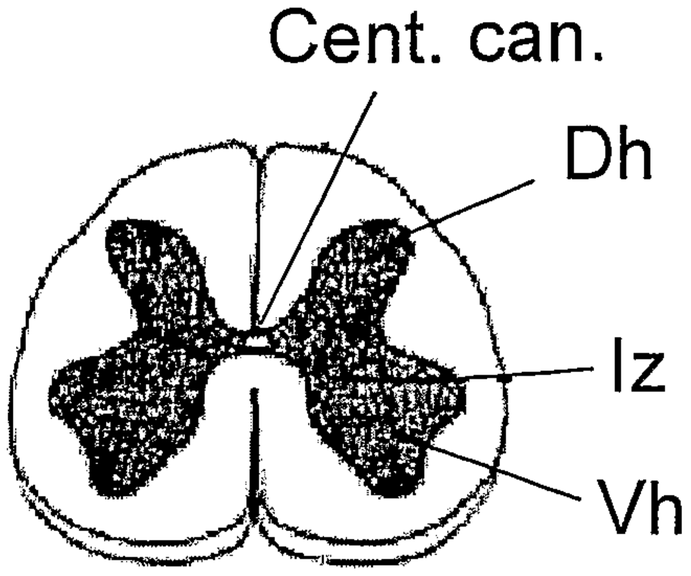


Fig. 3A

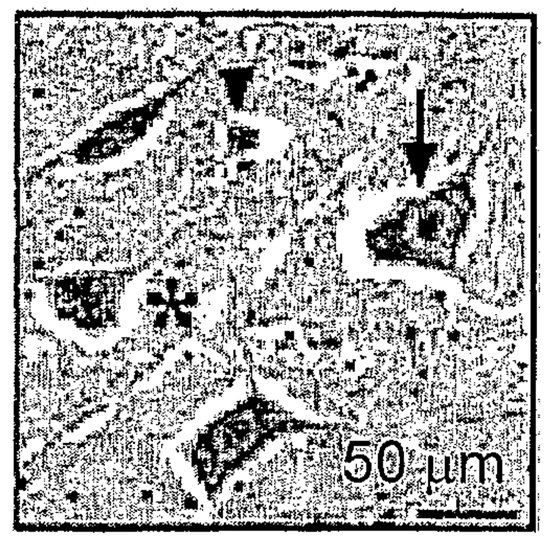


Fig. 3B

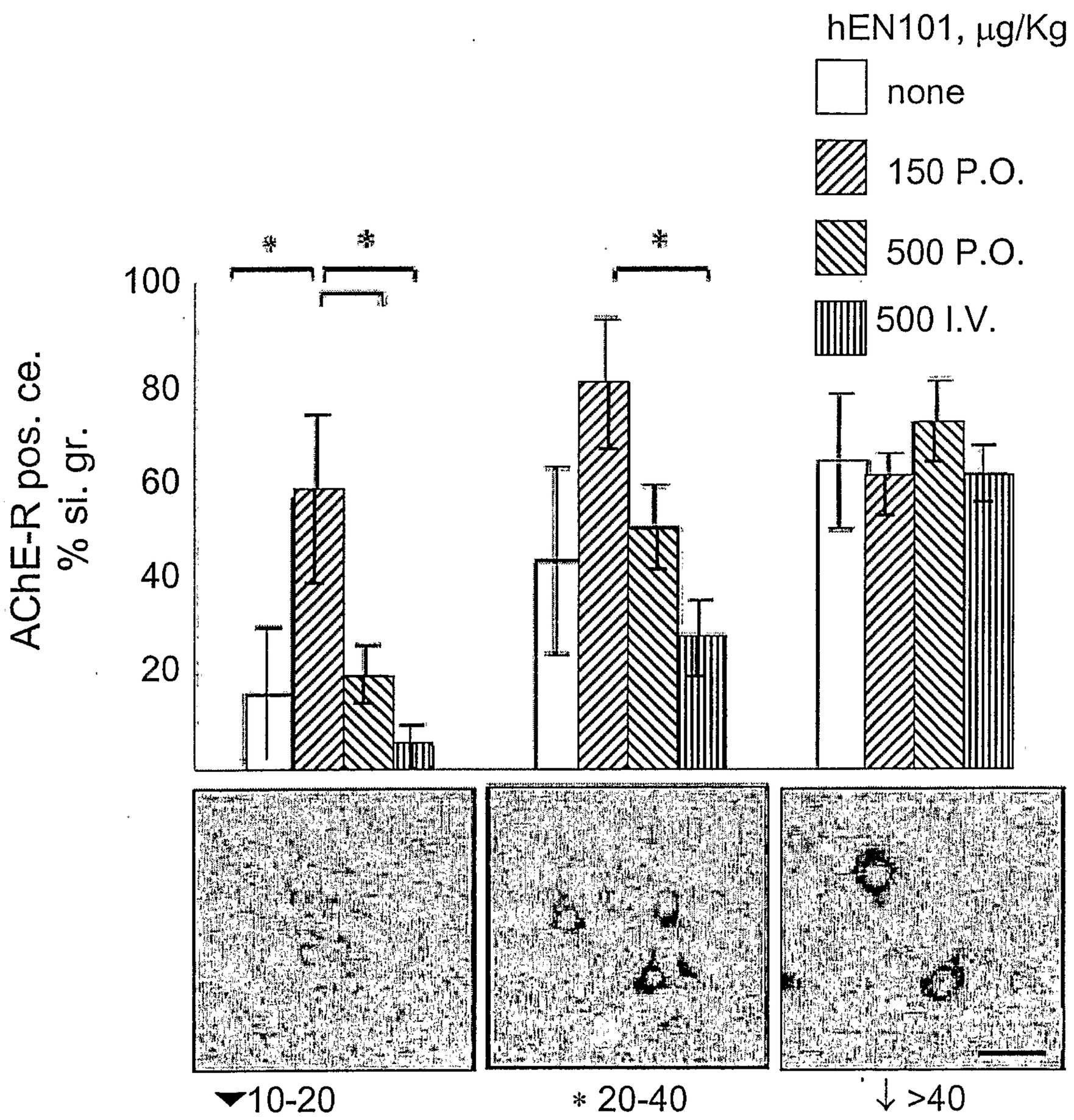


Fig. 3C

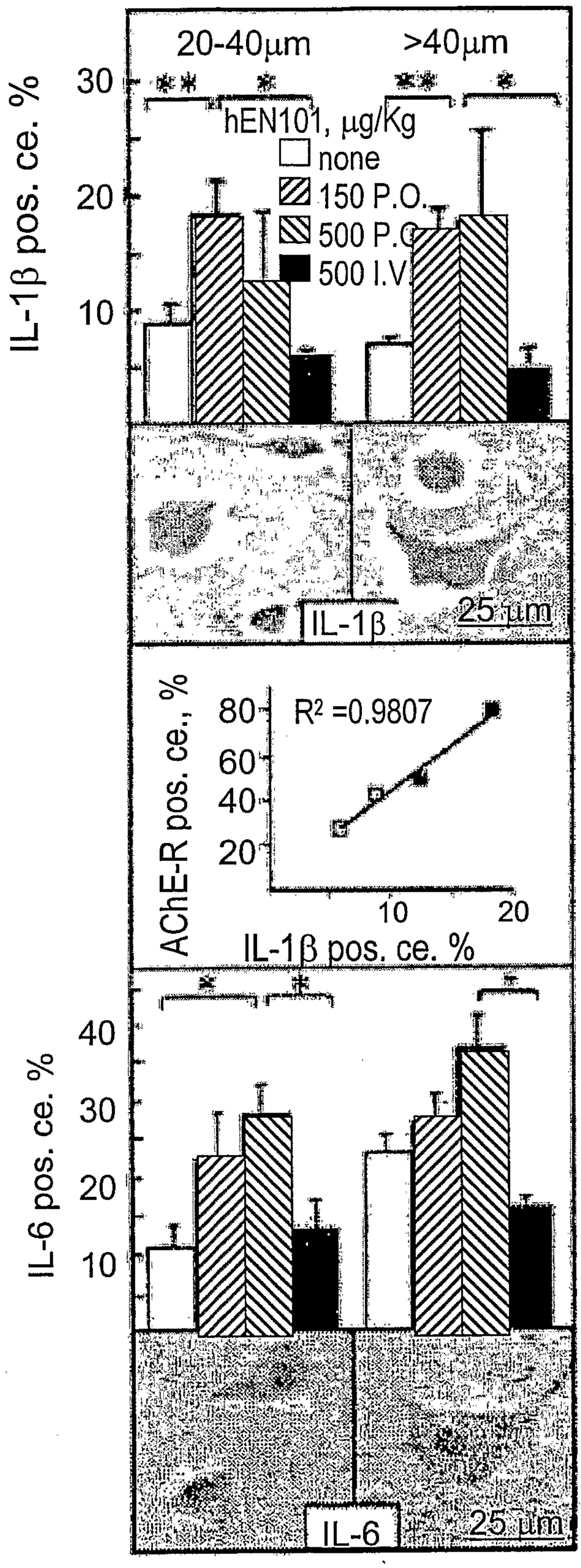


Fig. 4A

Fig. 4B

Fig. 4C

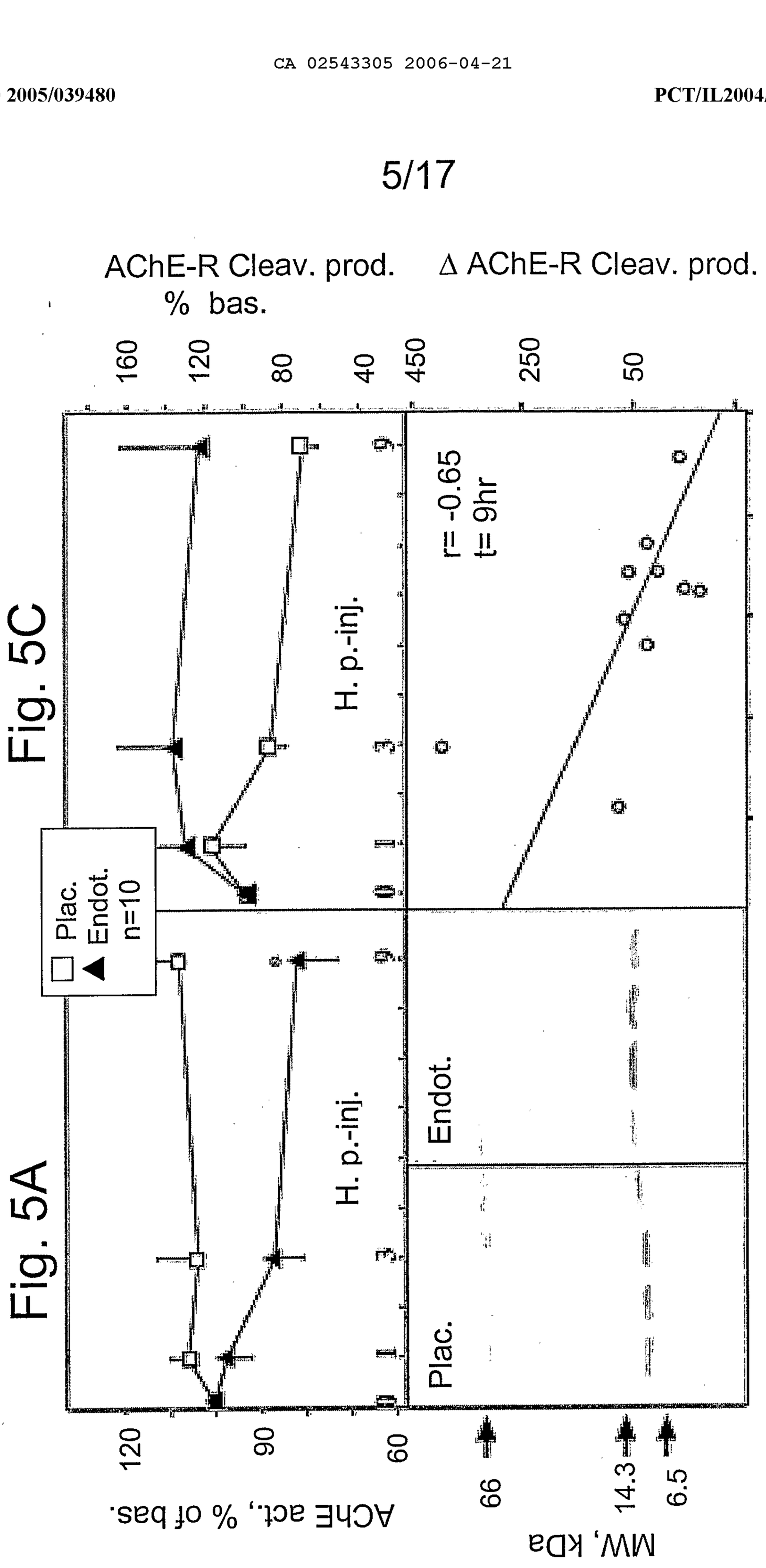
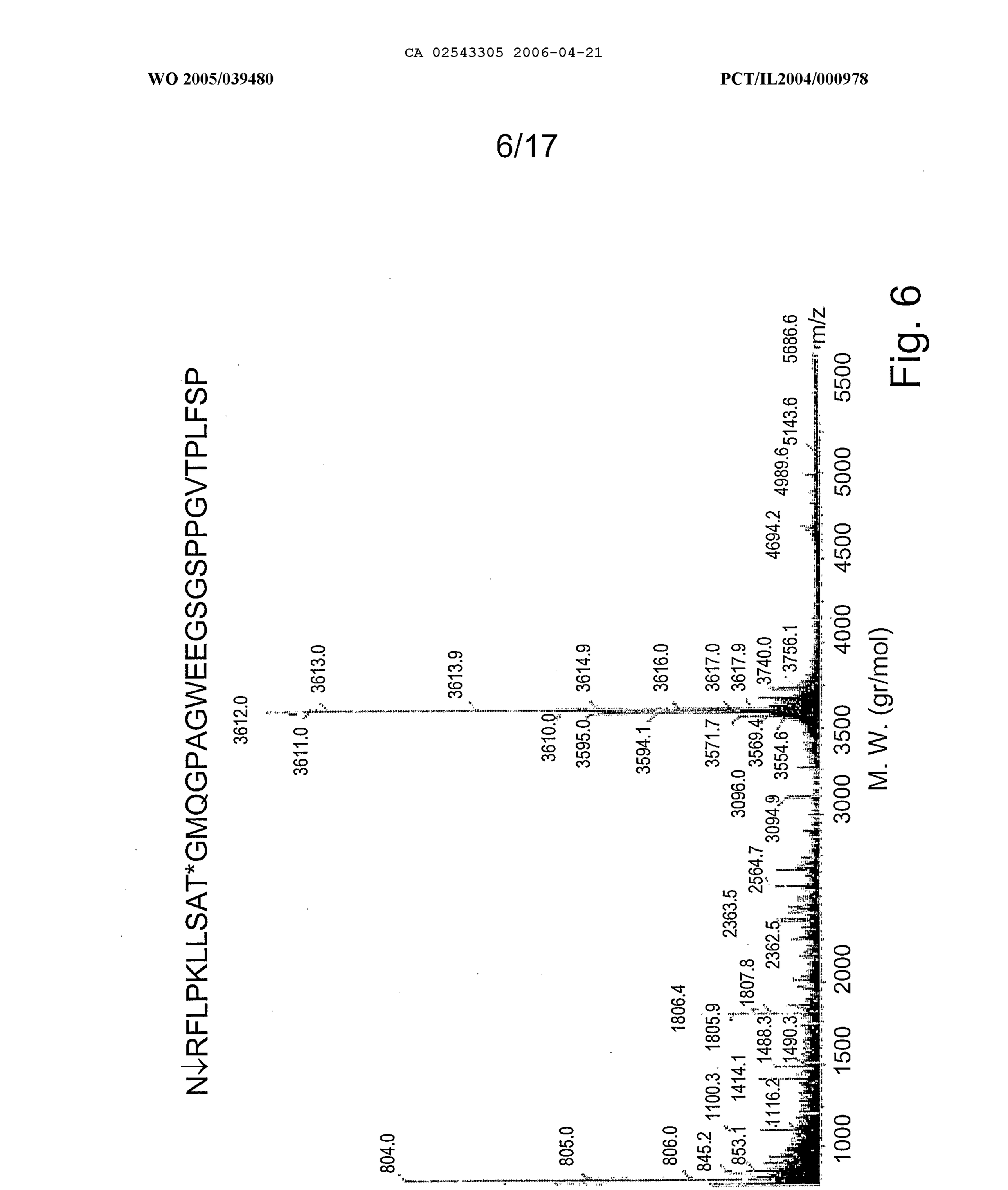


Fig. 5A

Fig. 5B

Fig. 5C

Fig. 5D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Rel. abund.
m/z
M. W. (gr/mol)
3612.0
3611.0
3613.0
3613.9
3610.0
3595.0
3614.9
3594.1
3616.0
3571.7
3617.0
3569.4
3617.9
3554.6
3740.0
3756.1
4694.2
4989.6
5143.6
5686.6
1806.4
1100.3
1805.9
1414.1
1807.8
1116.2
1488.3
2363.5
1490.3
2362.5
2564.7
3096.0
3094.9
804.0
805.0
806.0
845.2
853.1
1000
1500
2000
3000
3500
4000
4500
5000
5500

Fig.

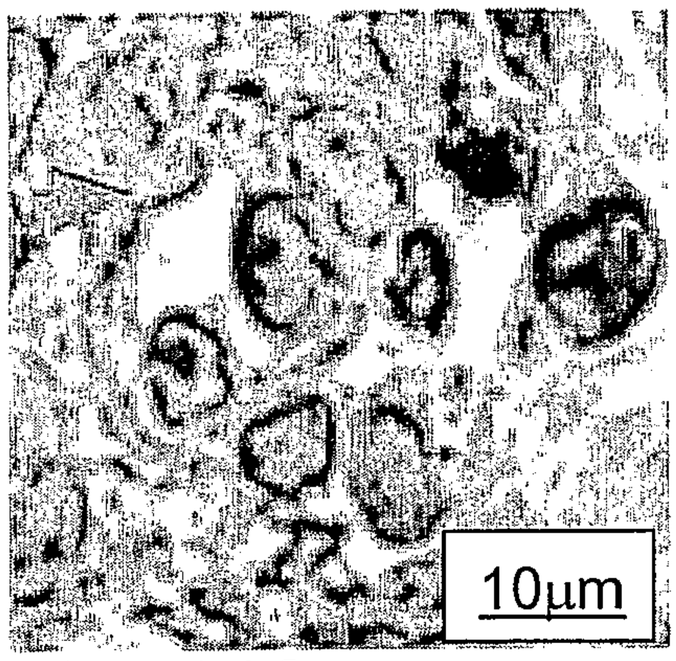


Fig. 7A

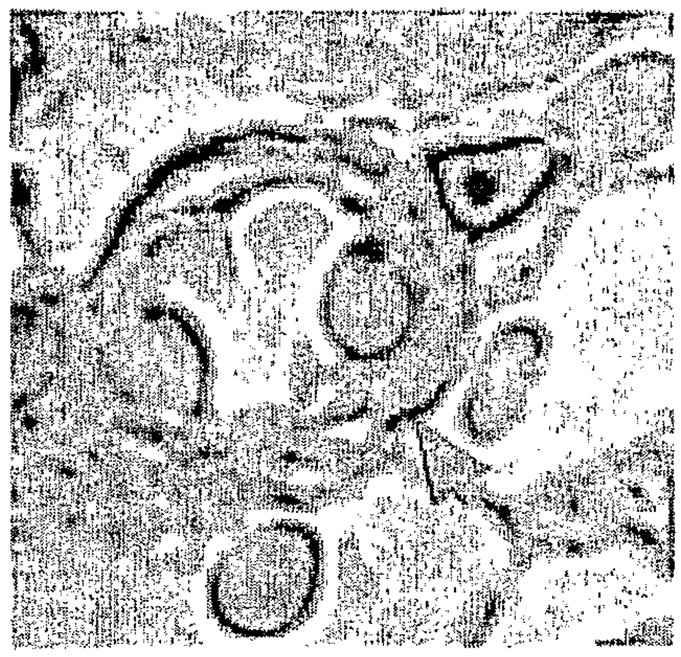


Fig. 7B

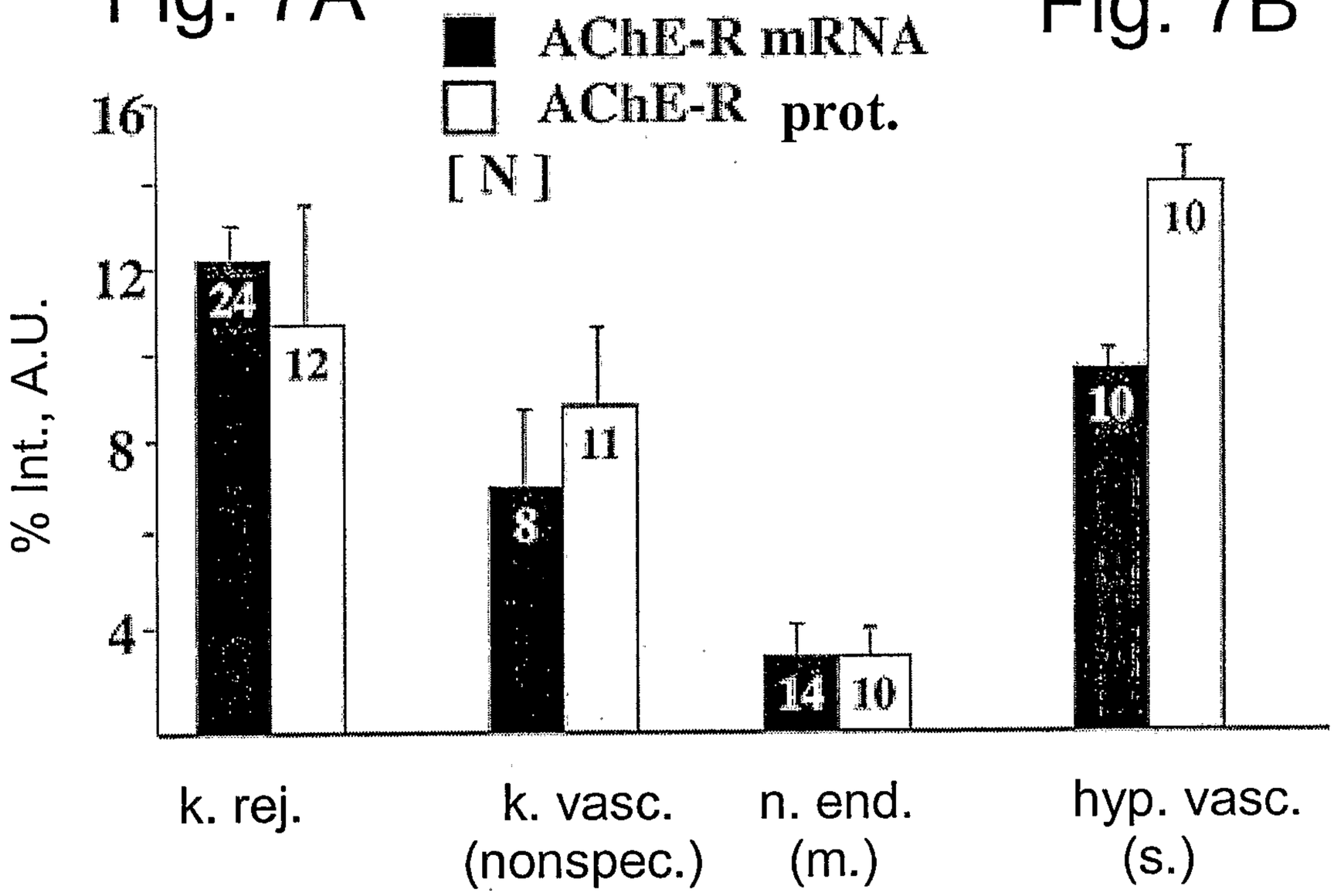


Fig. 7C

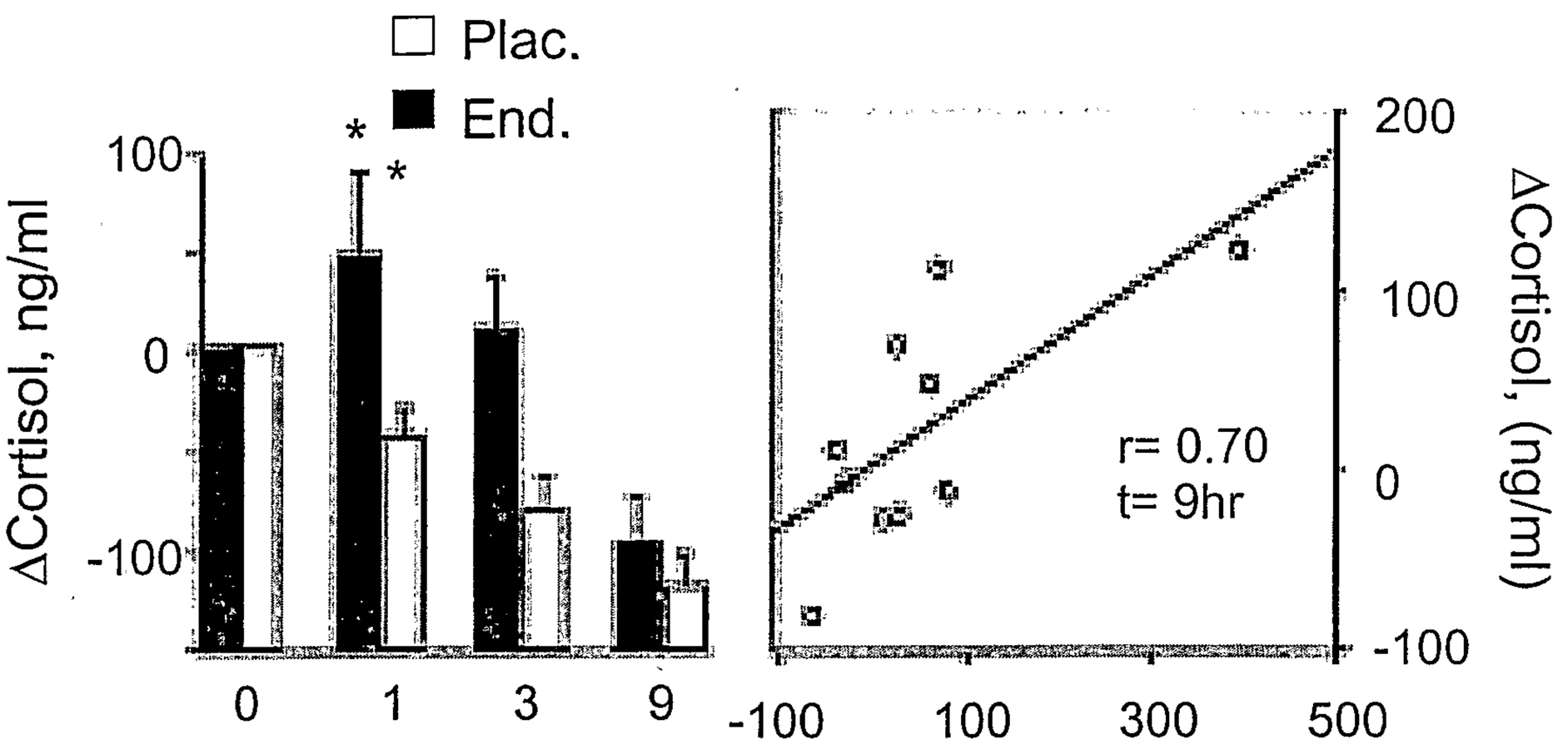


Fig. 8A

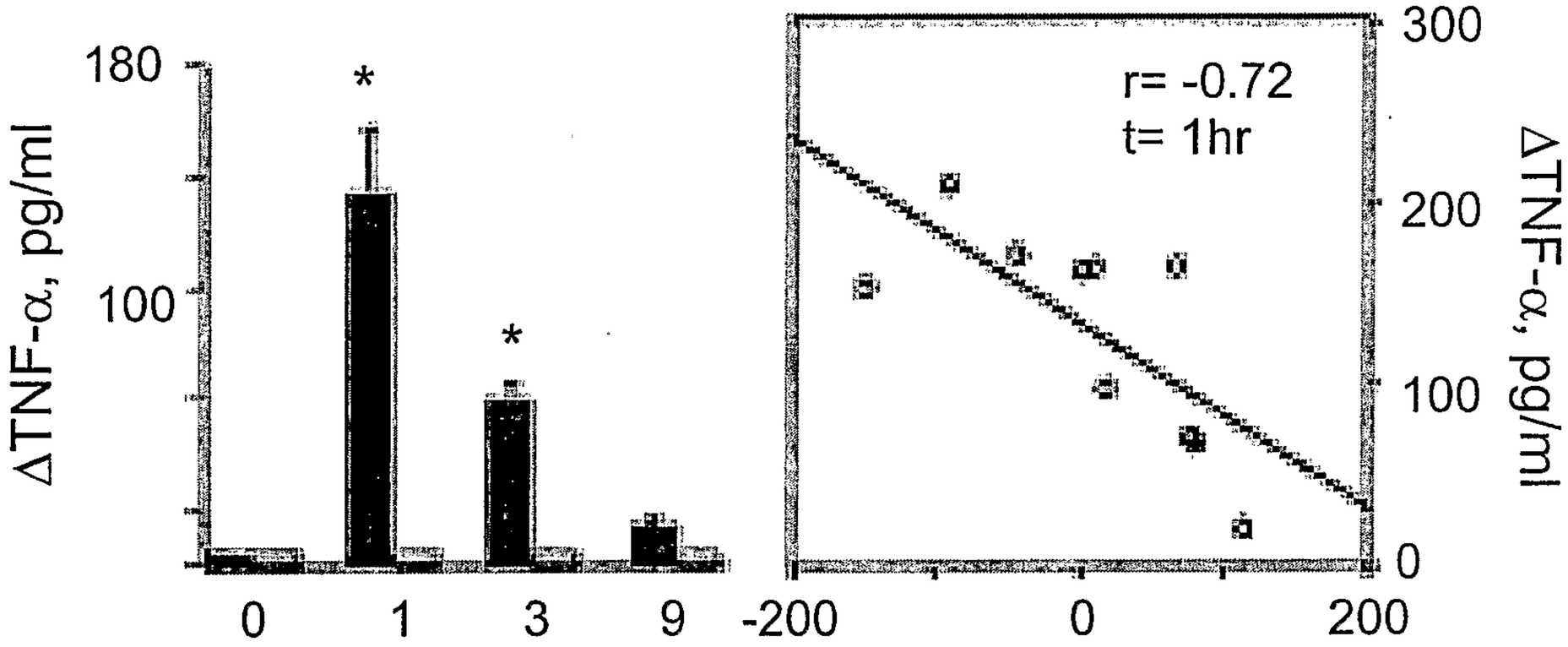


Fig. 8B

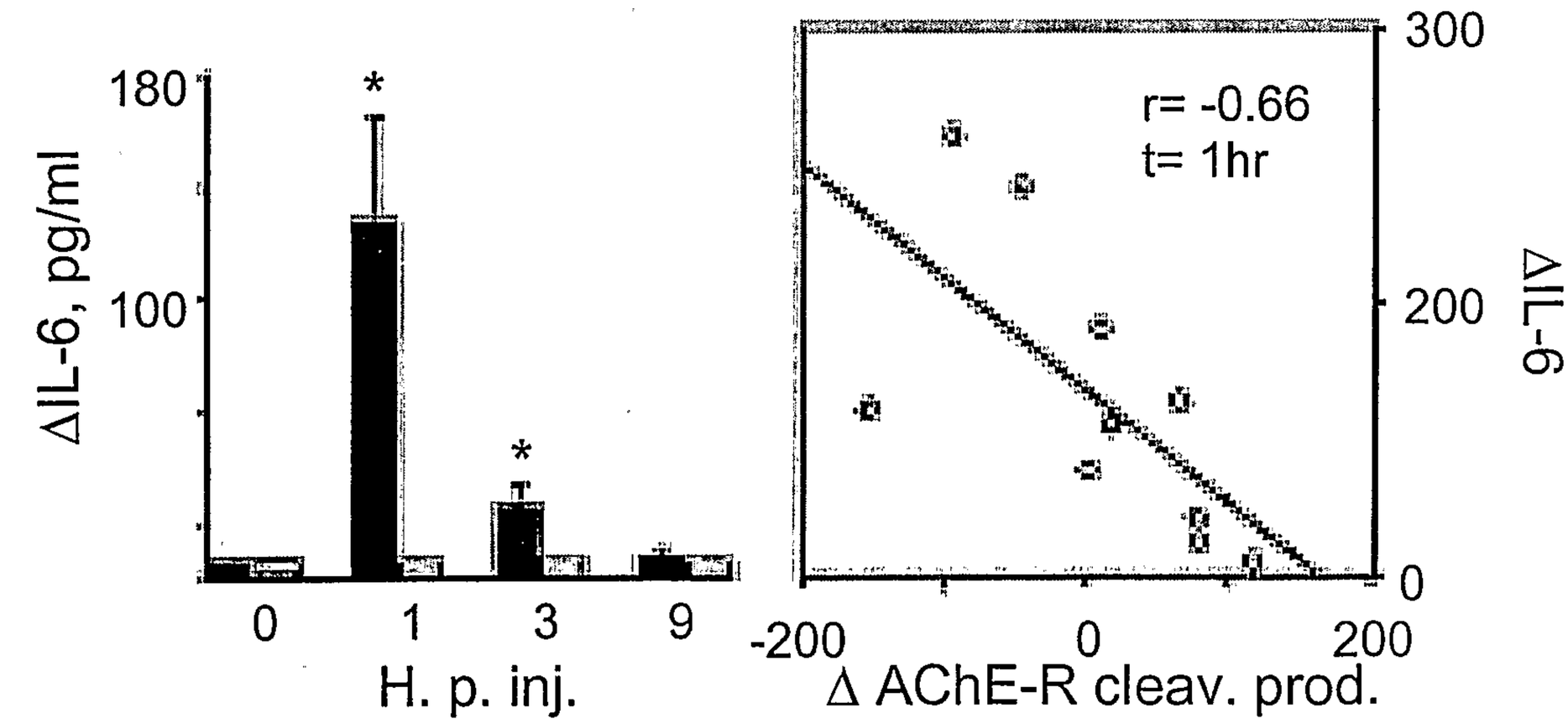


Fig. 8C

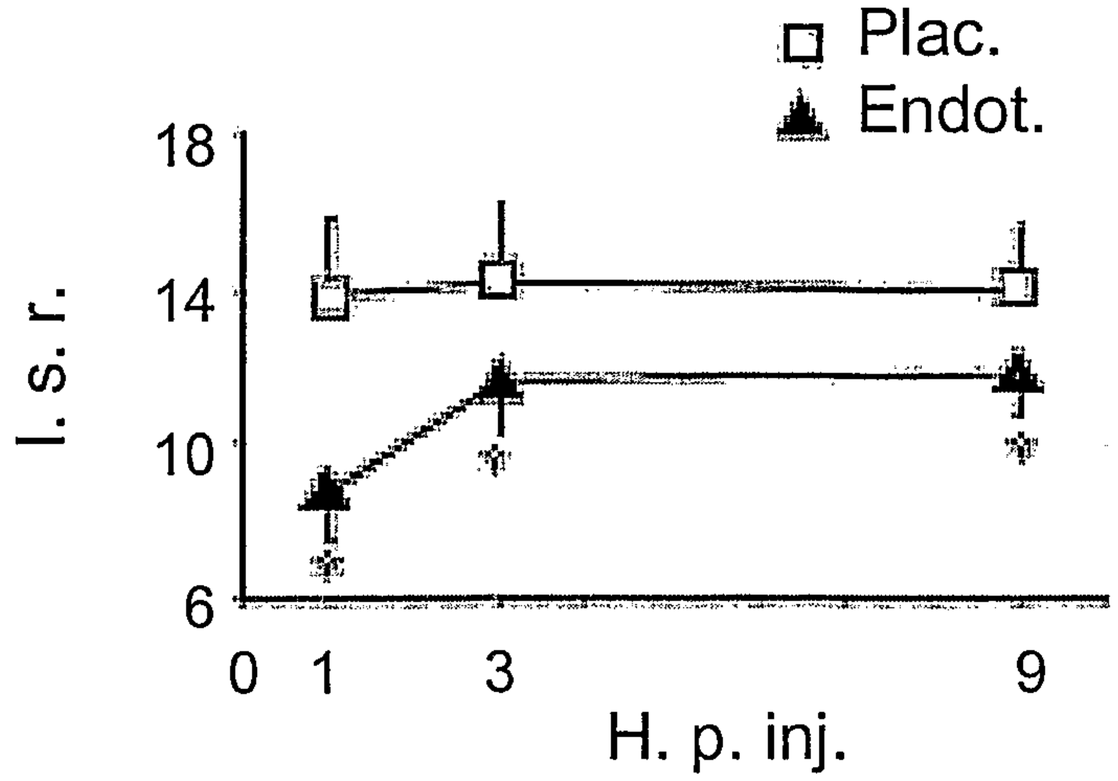


Fig. 9A

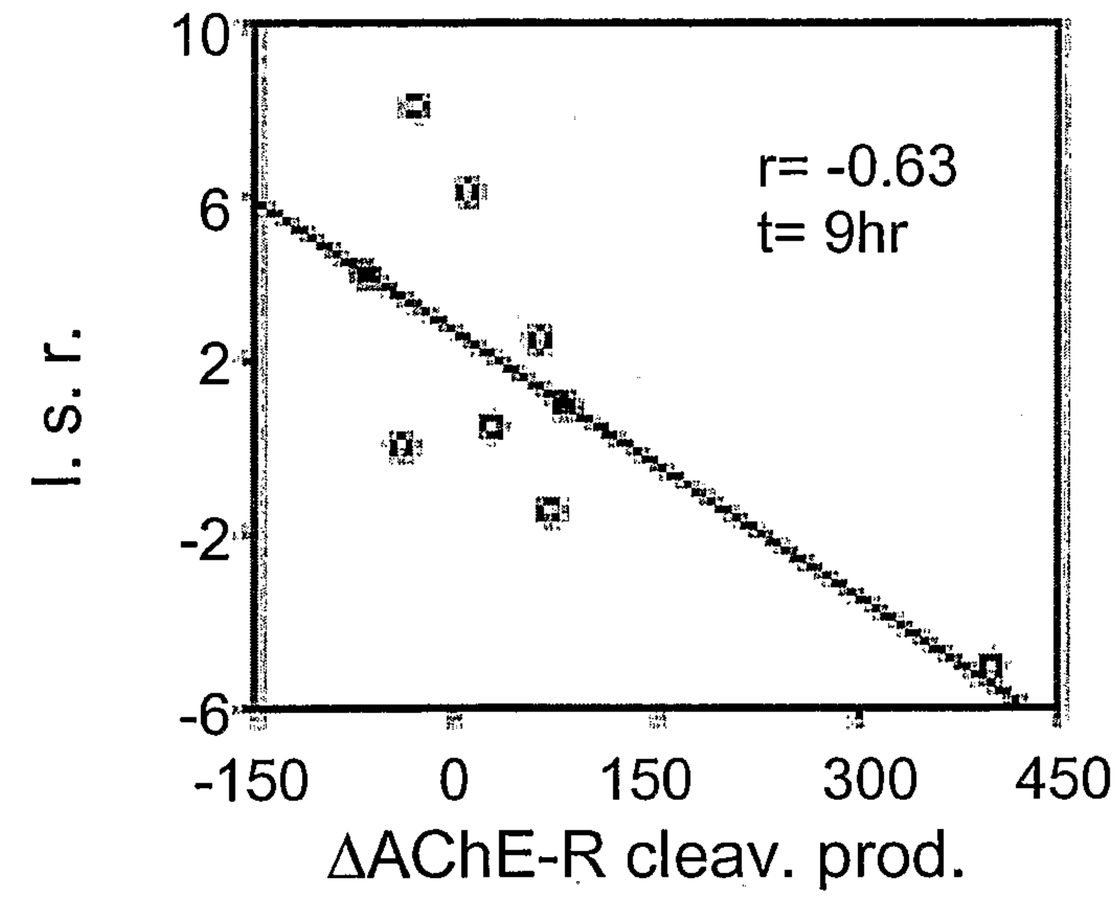


Fig. 9B

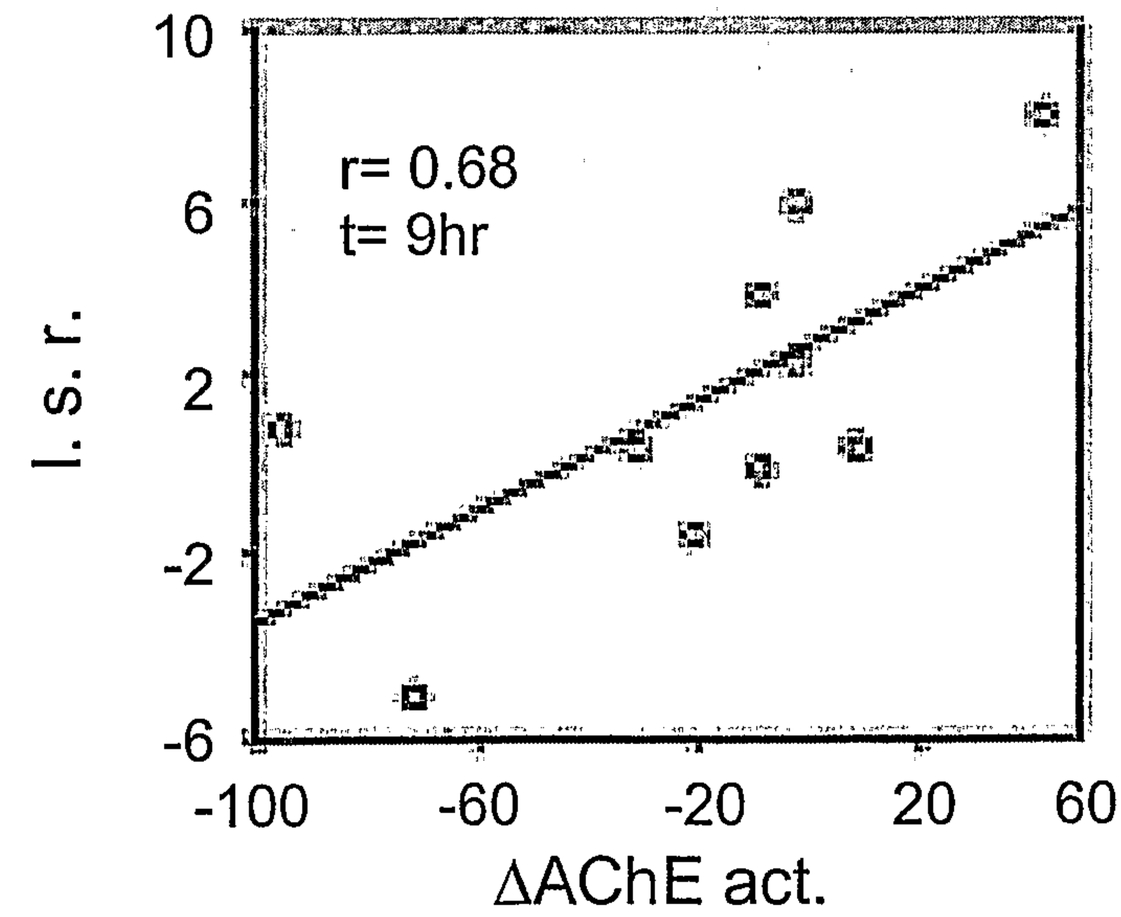


Fig. 9C

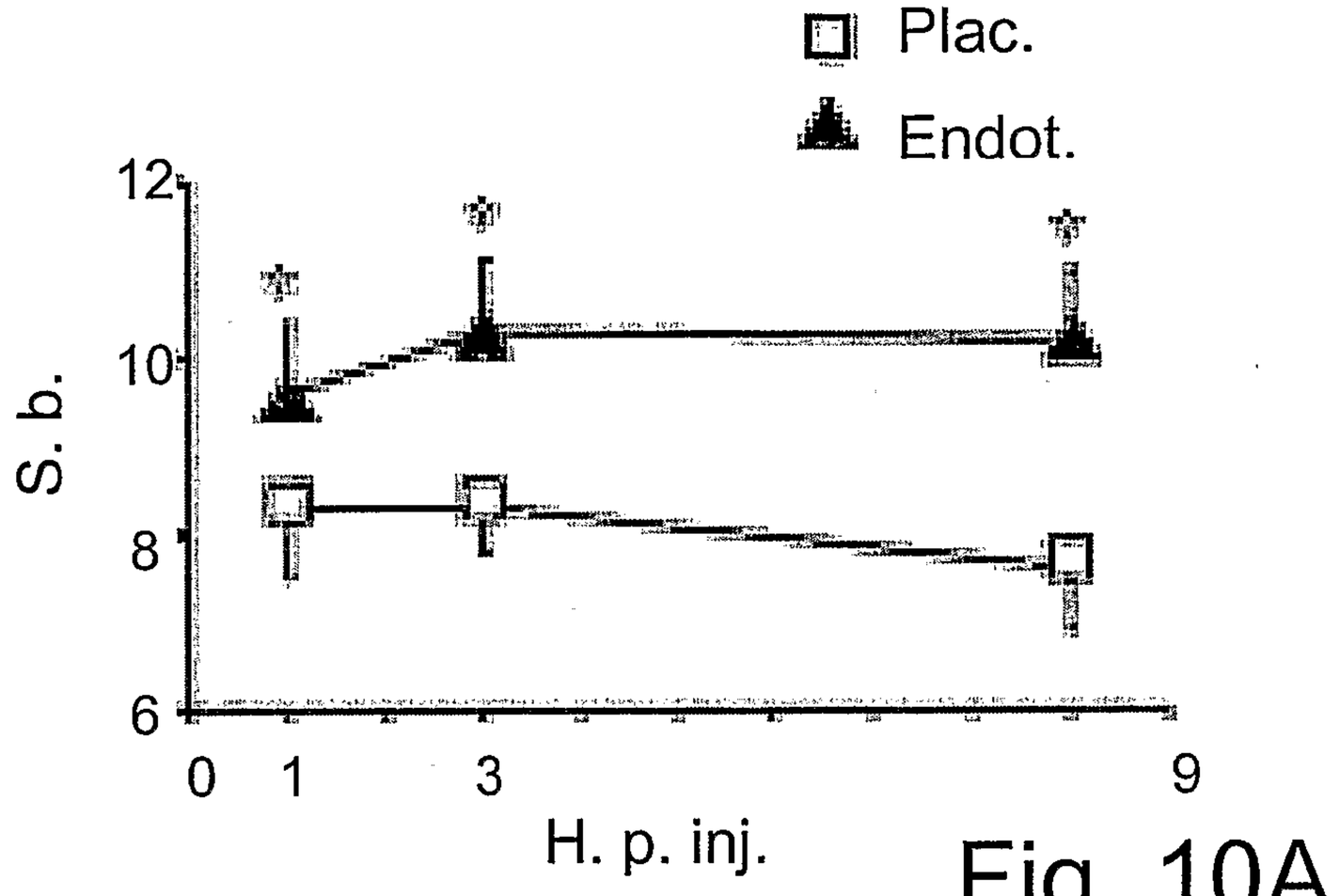


Fig. 10A

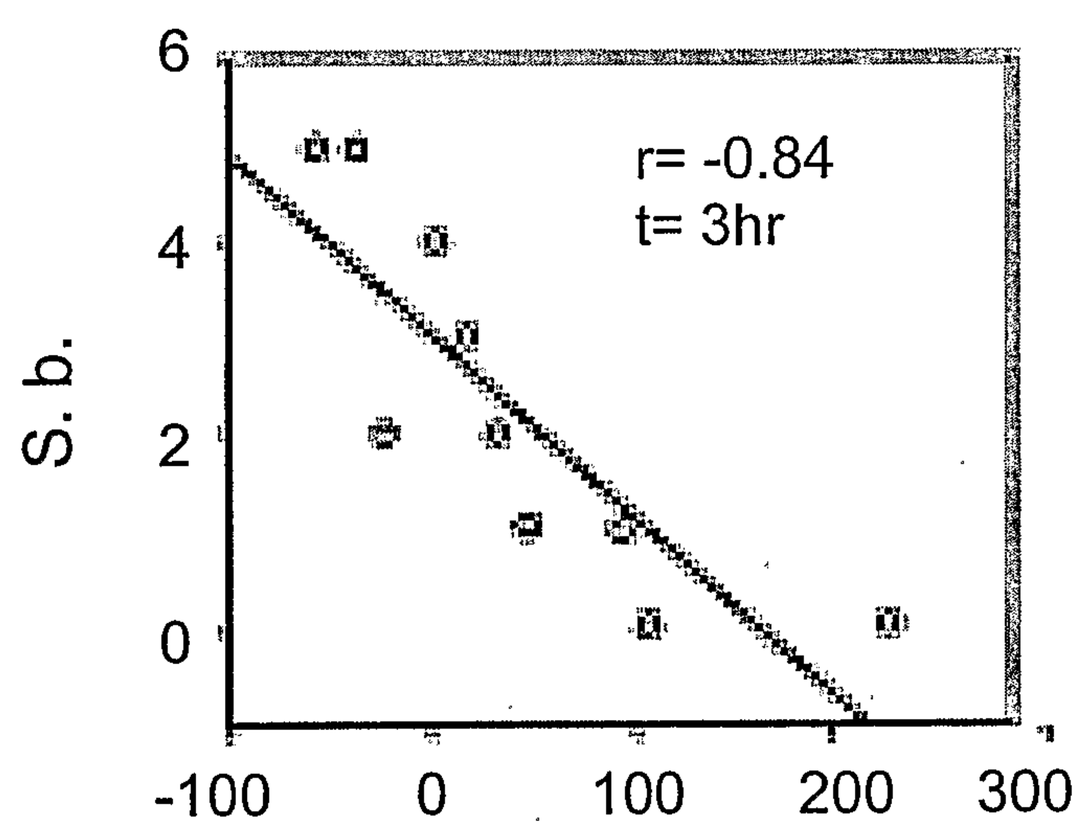


Fig. 10B

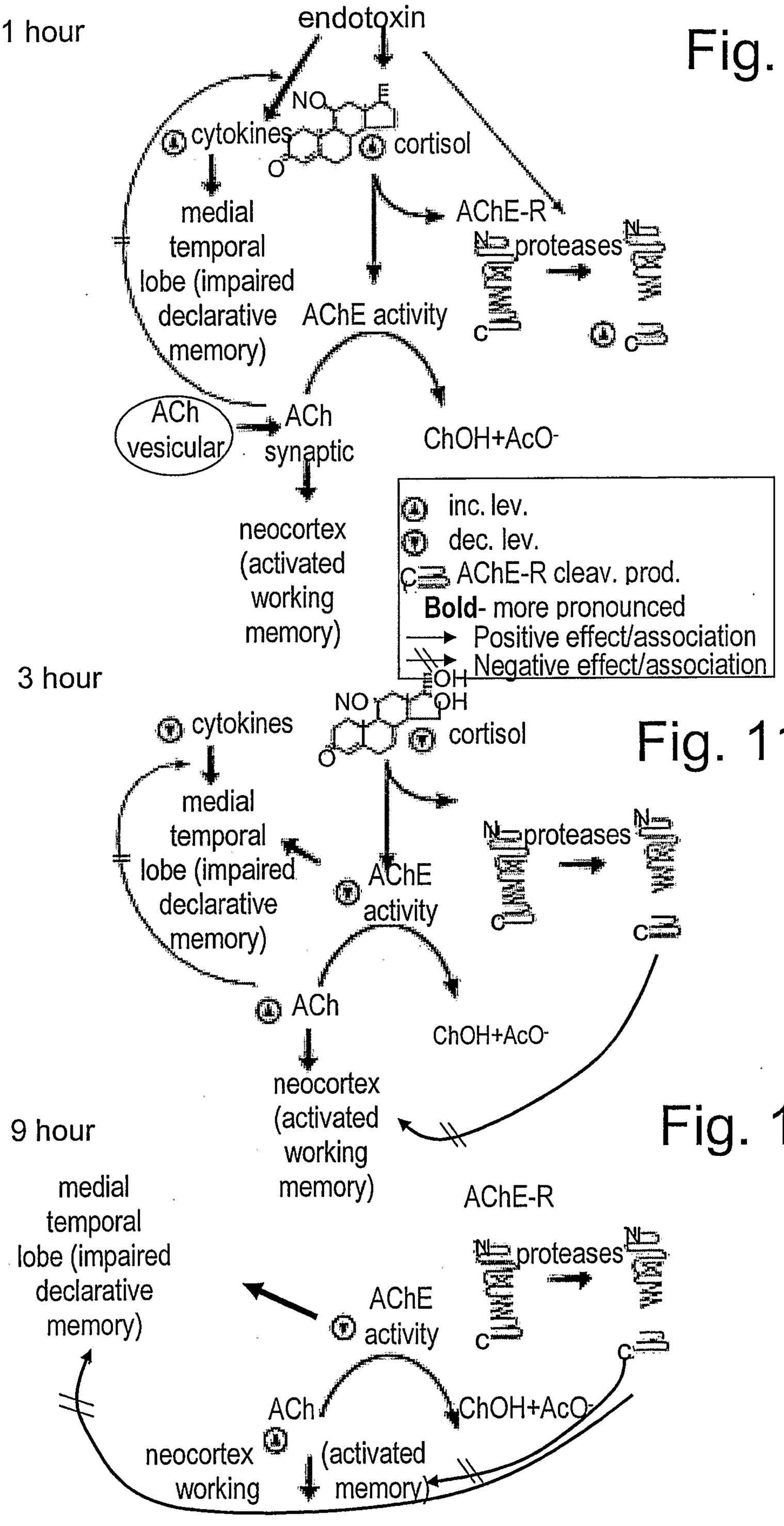


Fig. 11A

Fig. 11B

Fig. 11C

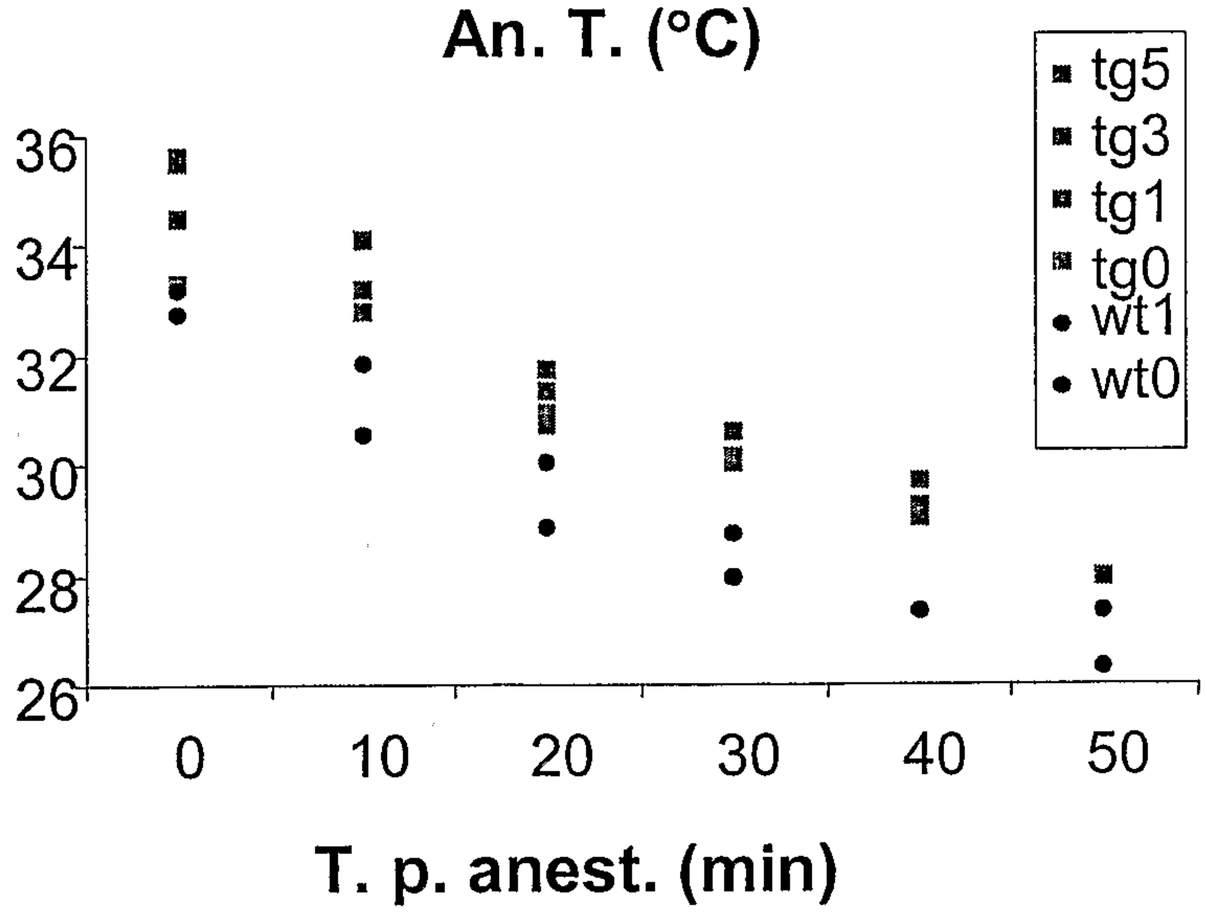


Fig. 12A

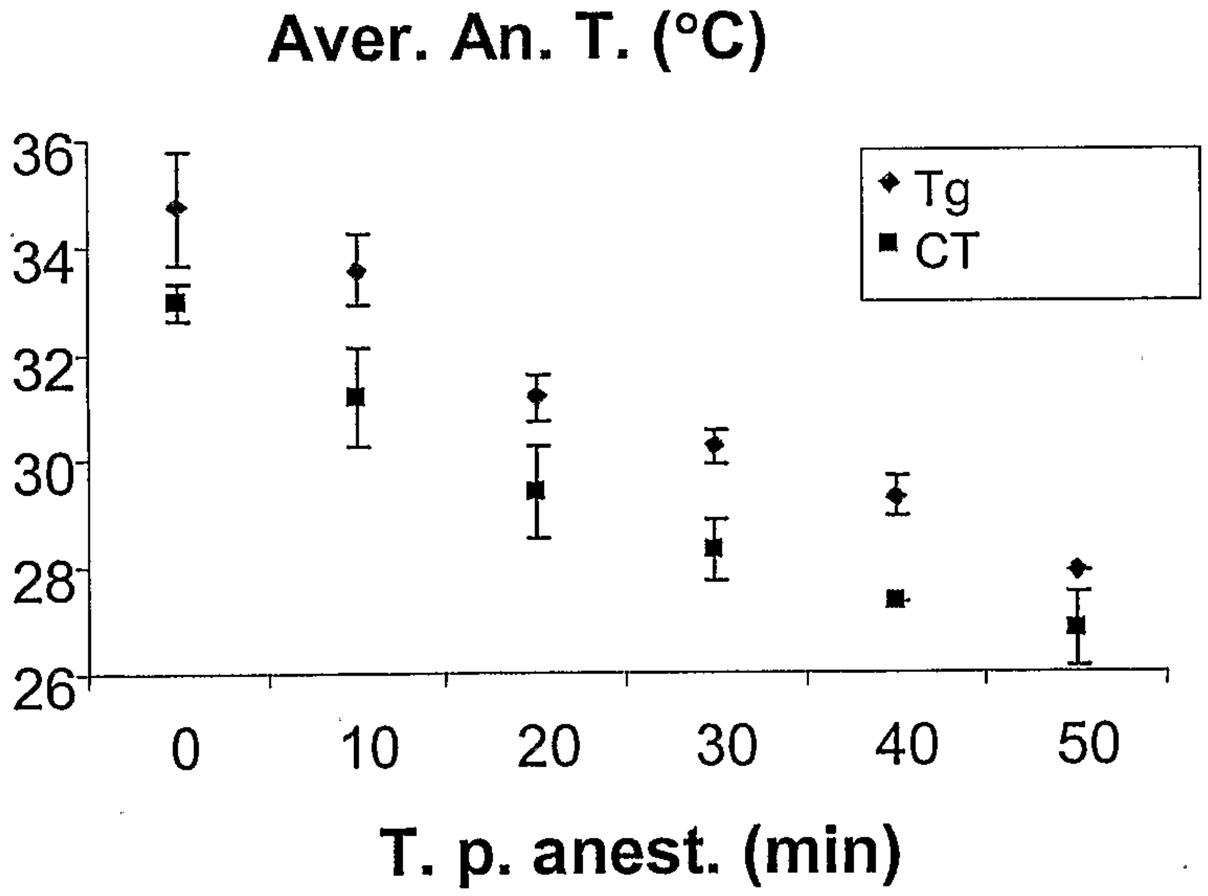


Fig. 12B

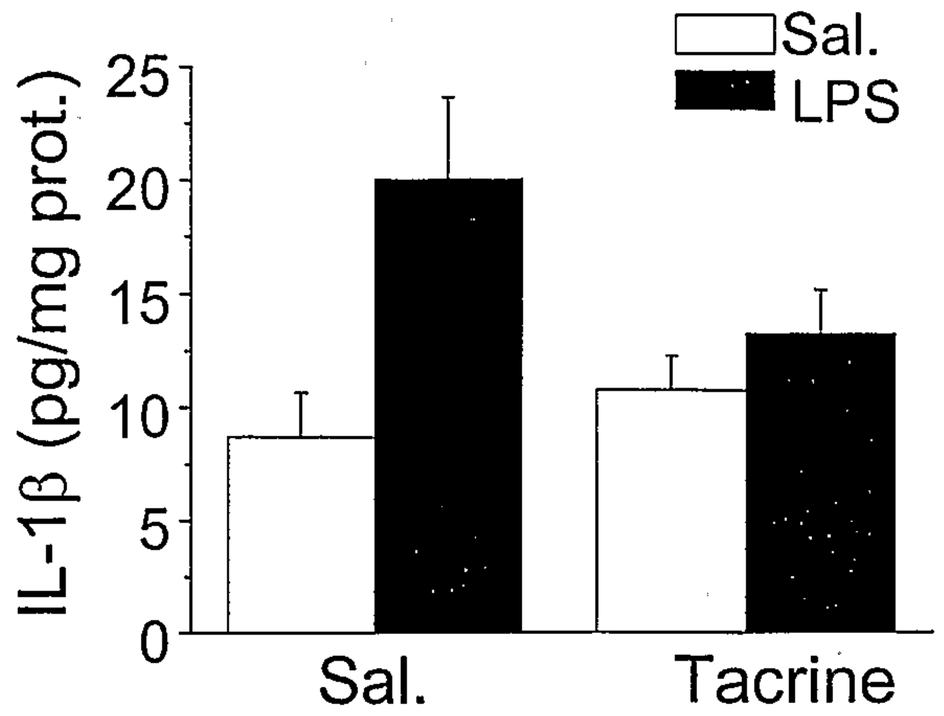


Fig. 13A

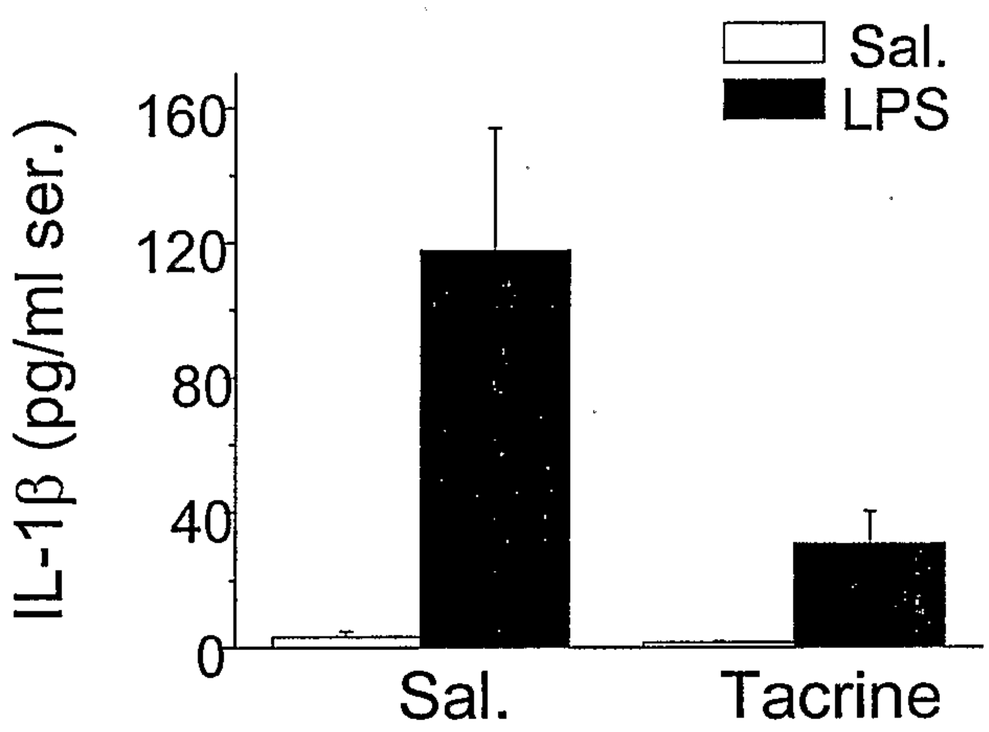


Fig. 13B

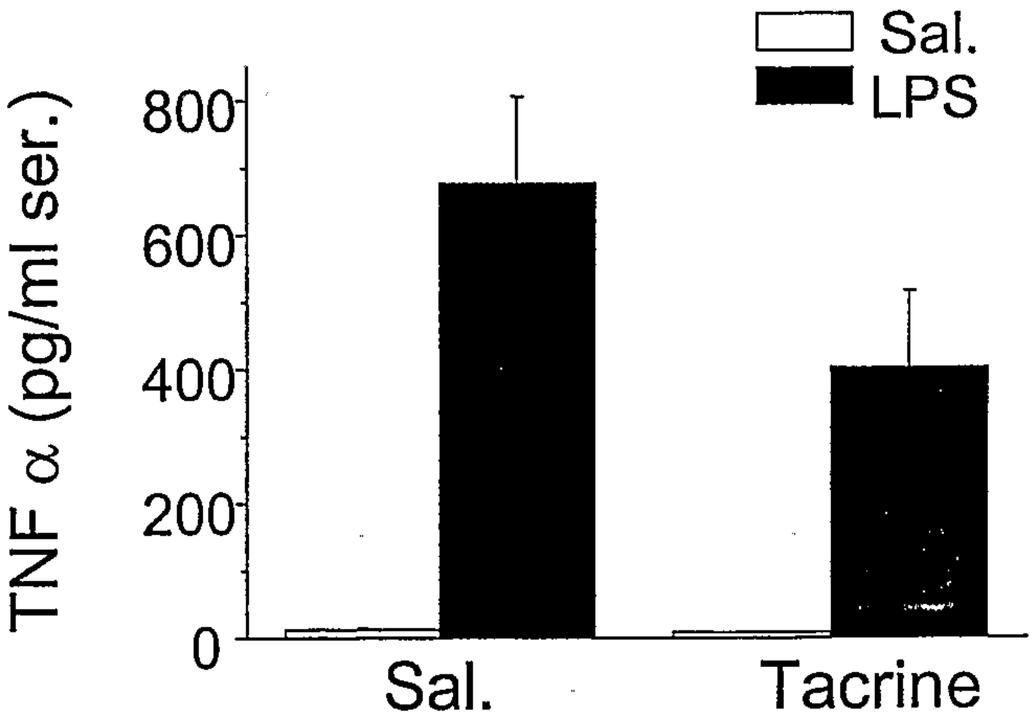


Fig. 13C

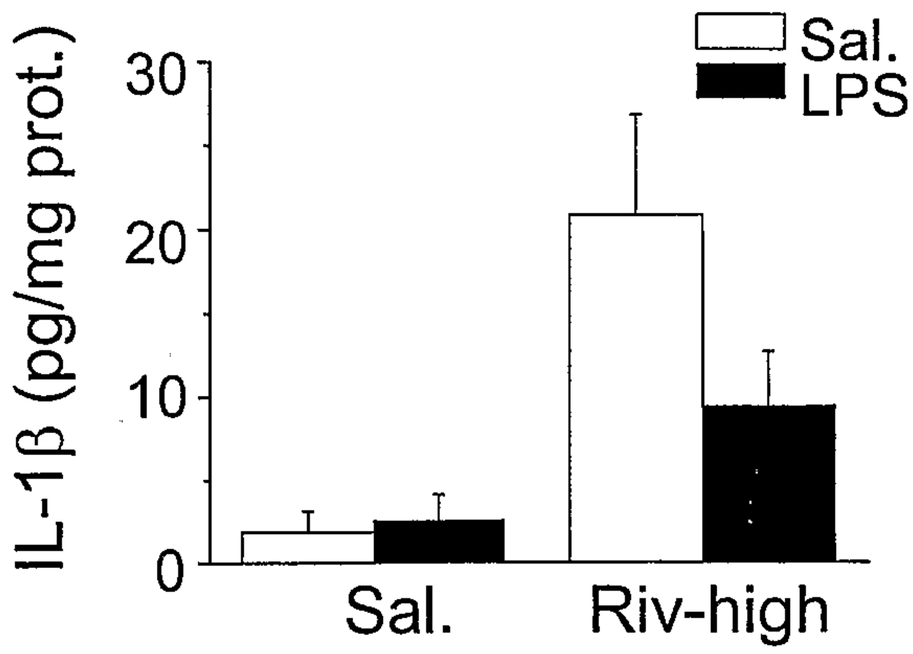


Fig. 14A

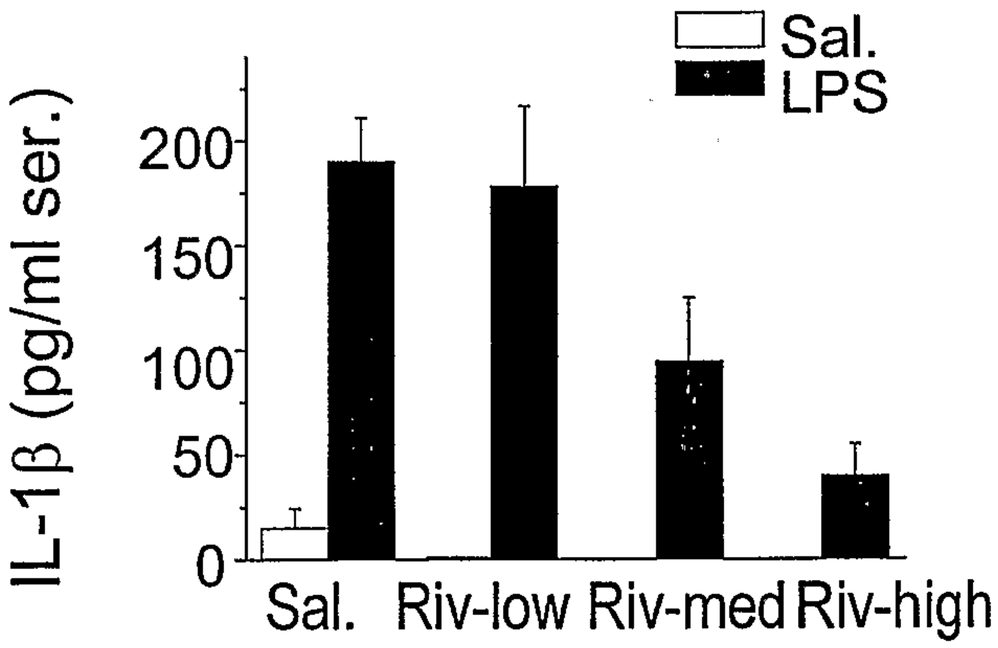


Fig. 14B

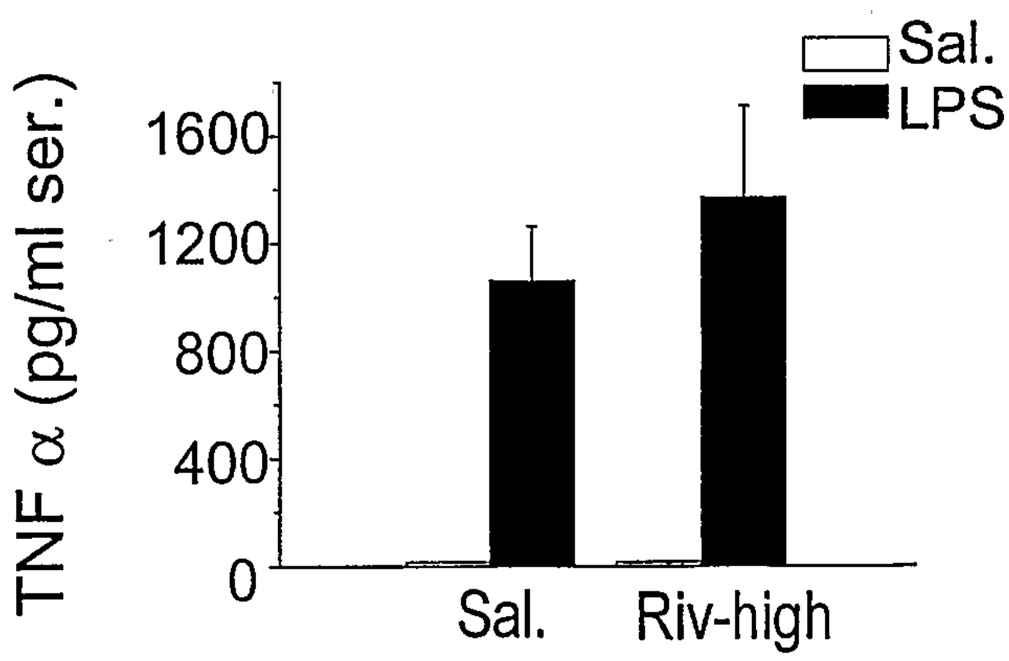


Fig. 14C

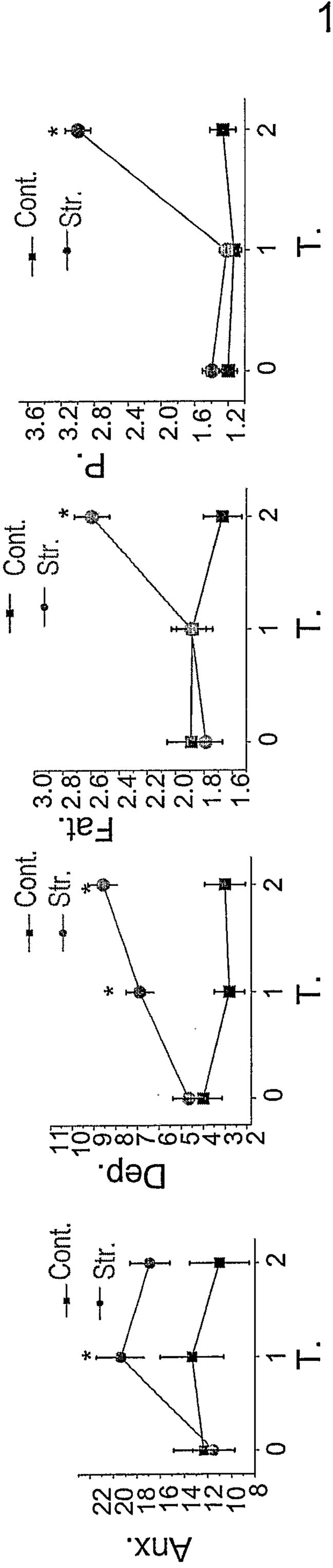


Fig. 15A Fig. 15B Fig. 15C Fig. 15D Fig. 15E Fig. 15F Fig. 15G Fig. 15H

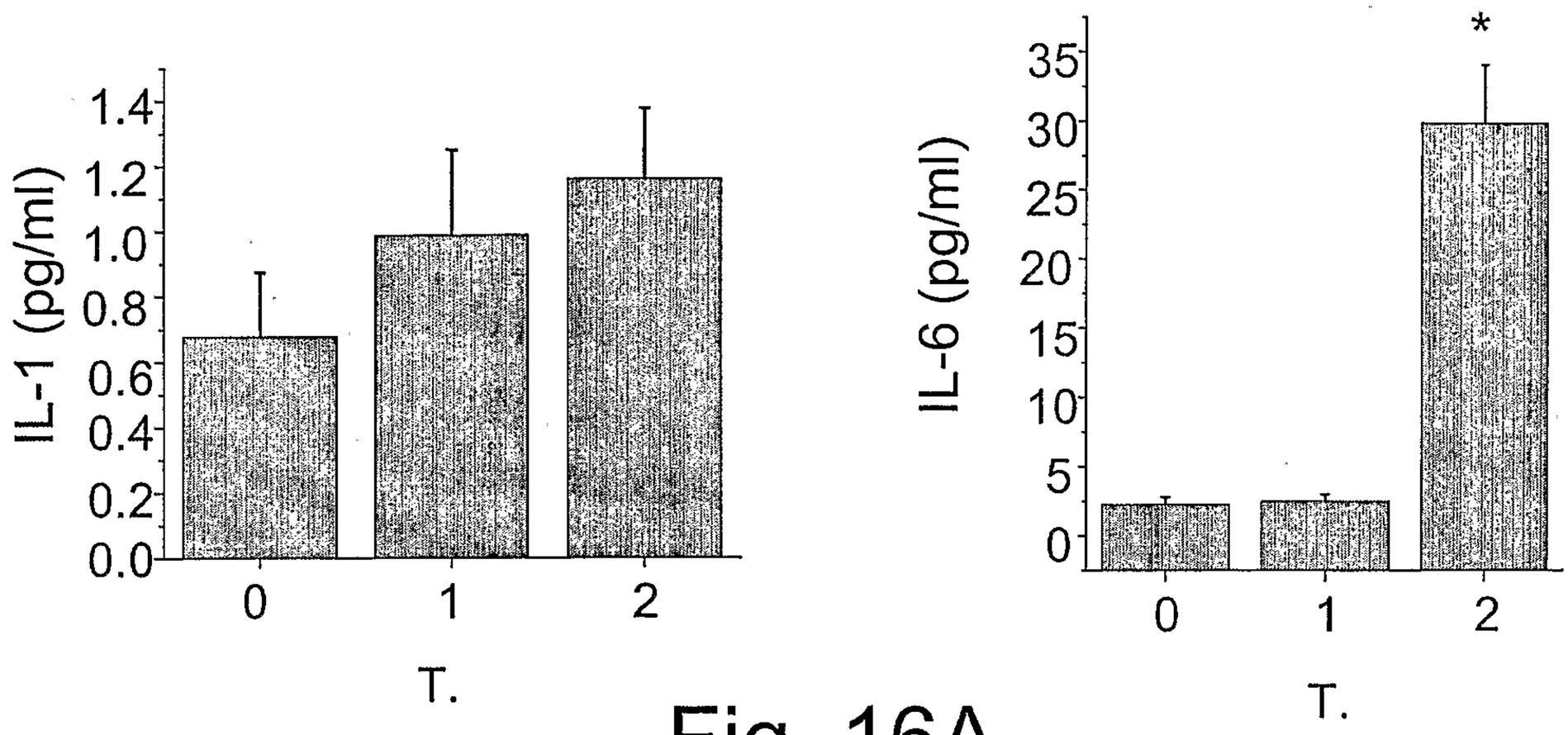


Fig. 16A

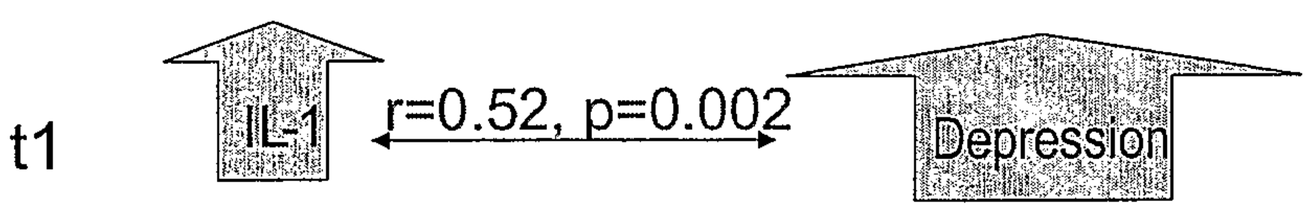
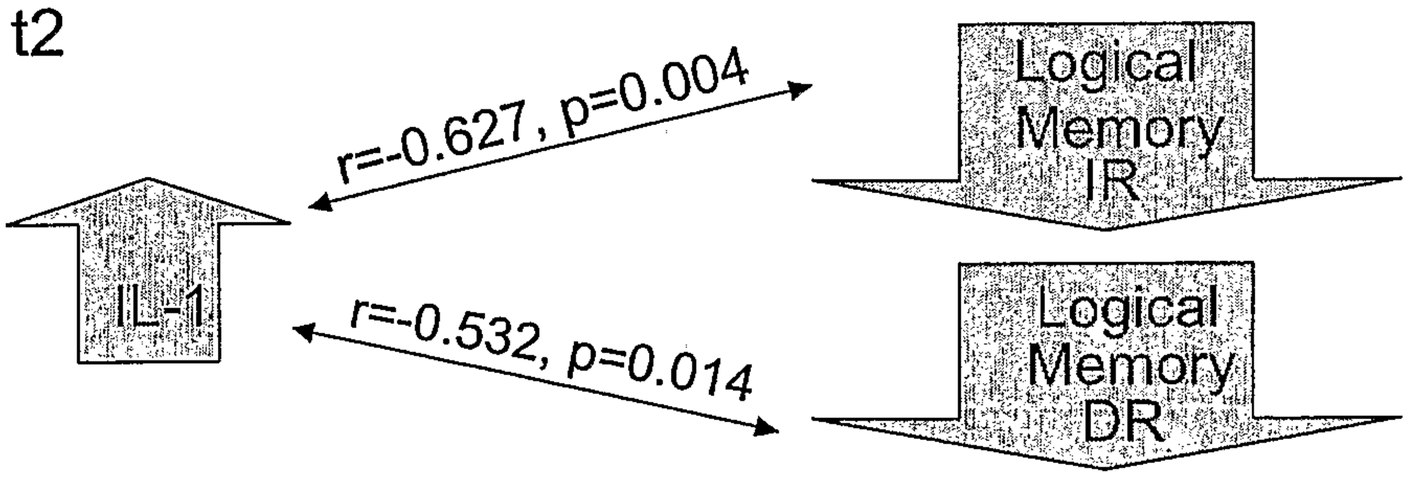


Fig. 16B



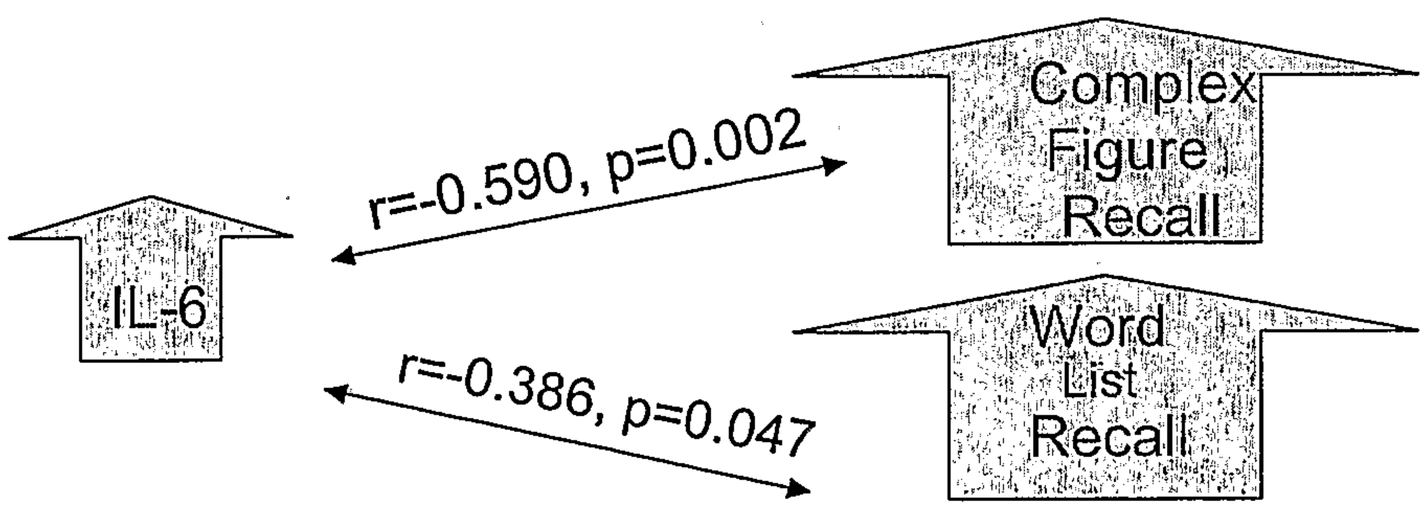


Fig. 16C

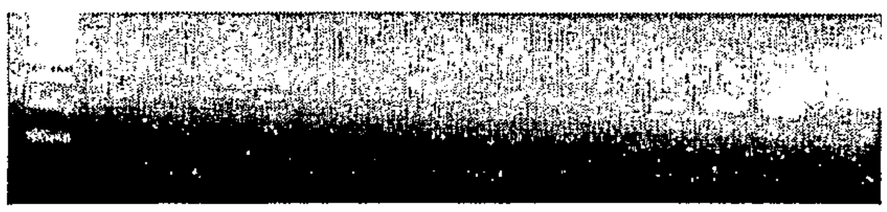


Fig. 17A

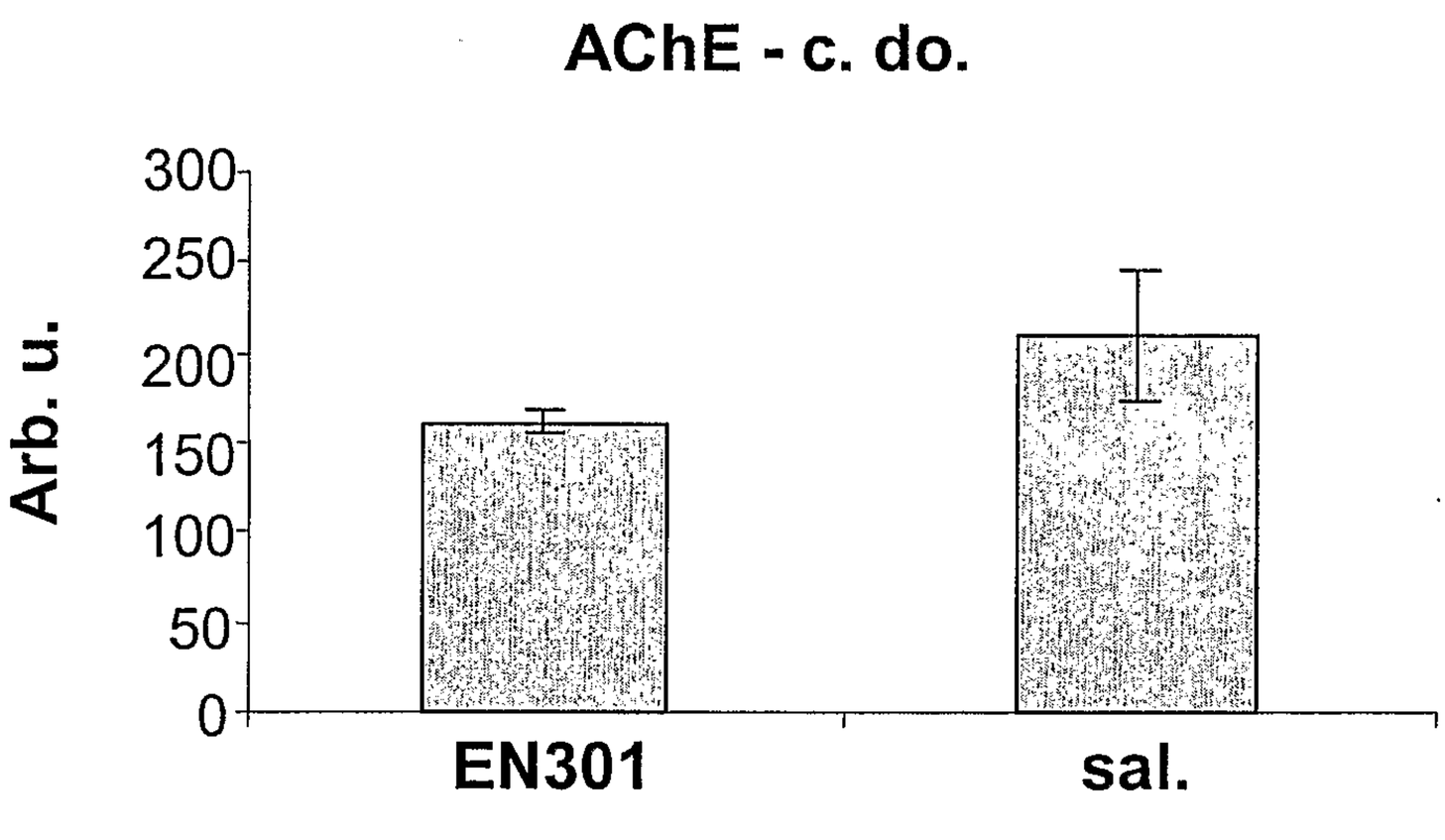


Fig. 17B

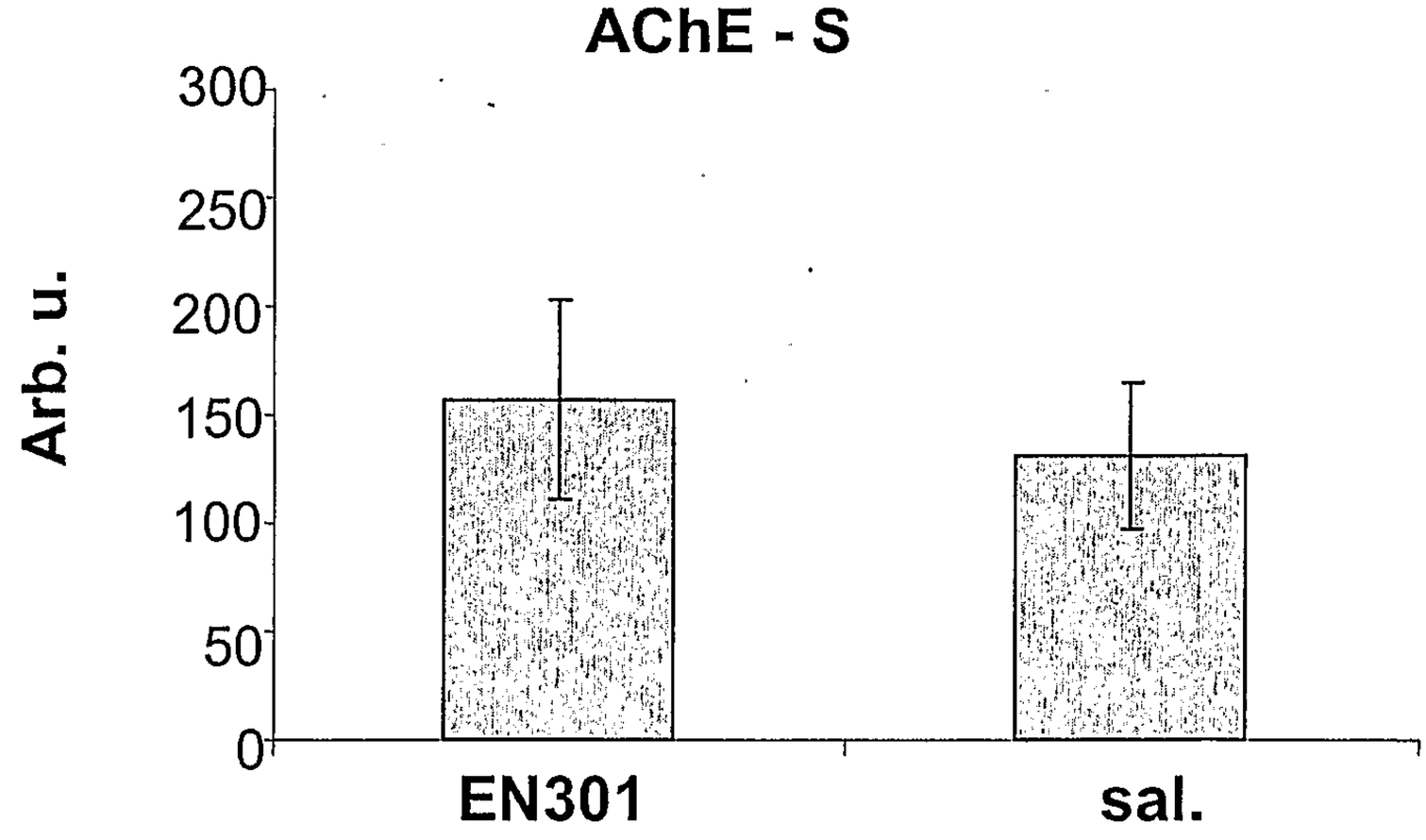


Fig. 17B