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(54) Title: PHARMACEUTICAL COMPOSITIONS COMPRISING ACETYLCHOLINESTERASE ANTISENSE DEOXYNUCLEOTIDES FOR THE TREATMENT OF MUSCULAR AND NEUROMUSCULAR DISORDERS

(57) Abstract: The present invention relates to a pharmaceutical composition for the treatment and/or prevention of a progressive neuromuscular disorder, and for use in decreasing muscle fatigue, comprising low concentrations of a synthetic antisense oligonucleotide targeted to the common coding domain of acetylcholine esterase (AChE) mRNA. The present invention further relates to a novel method for the diagnosis of progressive neuromuscular disorders.

PHARMACEUTICAL COMPOSITIONS COMPRISING ACETYLCHOLINESTERASE ANTISENSE DEOXYNUCLEOTIDES FOR THE TREATMENT OF MUSCULAR AND NEUROMUSCULAR DISORDERS

Field of the Invention

The present invention relates to a pharmaceutical or medical composition for the treatment and/or prevention of a progressive neuromuscular disorder, comprising as active ingredient a synthetic antisense oligonucleotide targeted to the common coding domain of acetylcholine esterase (AChE) mRNA. The present invention further relates to a novel method for the diagnosis of progressive neuromuscular disorder.

Background of the Invention

Neuromuscular junctions (NMJ) are highly specialized, morphologically distinct, and well-characterized cholinergic synapses (Hall and Sanes, *Cell* 72 Suppl., 99-121, 1993). Chronic impairments in NMJ activity induce neuromuscular disorders characterized by progressive deterioration of muscle structure and function. The molecular and cellular mechanisms leading from compromised NMJ activity to muscle wasting have not been elucidated.

Inhibitors of AChE, the acetylcholine-hydrolyzing enzyme, induce neuromuscular pathologies similar to those observed in diseases of neuromuscular transmission, including degeneration of synaptic folds, terminal nerve branching, enlargement of motor endplates, and disorganization of muscle fibers (Laskowski *et al.*, Exp. Neurol. 47, 290-306, 1975; Kawabuchi *et al.*, Experientia 32, 632-635, 1976). Cholinesterase inhibitors promoting delayed myopathy include organophosphate poisons such as DFP and paraoxon (an insecticide) and carbamate drugs like

pyridostigmine and neostigmine. Muscle weakness is also prominent among the complaints of Gulf War veterans having received 90 mg per day pyridostigmine as a prophylactic guard against anticipated exposure to chemical weapons (Haley, R.W. *et al.*, Jama 277, 215-222, 1997). Similarly, the only drugs currently approved for Alzheimer's disease are potent AChE inhibitors. However, it is significant that a promising candidate anticholinesterase Alzheimer's drug was recently withdrawn from clinical trials after muscle weakness was reported by some patients (SCRIP World Pharmaceutical News, No. 2374, p. 19, 1998).

Despite the serious side effects of AChE inhibitors, these drugs are today the treatment of choice for a number of disorders involving neuromuscular impairment. One of these disorders is Myasthenia gravis (MG), which is caused by a defect in neuromuscular transmission due to autoantibody-mediated attack upon the muscle nicotinic acetylcholine receptors (AChR). It is characterized by fluctuating weakness that may be transiently improved by inhibitors of acetylcholinesterase (AChE). The clinical severity of myasthenia gravis is usually graded functionally and regionally, according to an adaptation of a scale devised by Osserman: Grade I involves focal disease (e.g., restricted to ocular muscles); Grade II, generalized disease that is either mild or moderate; Grade III, severe generalized disease; and Grade IV, a crisis, with life-threatening impairment of respiration (Drachman D.B. *et al.*, N. Engl. J. Med. 330, 1797-810, 1994; Osserman. K., *et al*, Myasthenia Gravis, G. Stratton, ed. New York,. 80, 1958). The basic abnormality in MG is a decrease in the density of nAChRs at neuromuscular junctions. In general, the degree of reduction in nAChRs correlates with the severity of MG.

The characteristic electrodiagnostic abnormality is a progressive, rapid decrement in the amplitude of muscle action potentials evoked by repetitive

nerve stimulation at 3 or 5 Hz. This myasthenic fatigue is caused by decrease in the number of AChR molecules available at the post-synaptic site. Inhibiting anti-AChR antibodies are present in 85% to 90% of patients.

Patients with MG, but not with congenital myasthenias due to other causes (Triggs *et al.*, Muscle Nerve 15, 267-72, 1992), display a transient clinical response to AChE inhibitors such as edrophonium. The available anti-AChE drugs are the first line of treatment, but most patients require further help. This includes drastic measures, such as plasma exchange, thymectomy and immunosuppression. Unfortunately, all of the currently employed MG drug regimens are associated with deleterious long-term consequences. These include disturbance of neuromuscular transmission, exacerbation and induction of MG symptoms. In addition, the otherwise safe use of common drugs such as anti-infectives, cardiovascular drugs, anticholinergics, anticonvulsants, antirheumatics and others has been reported to worsen the symptoms of MG patients (Wittbrodt, Arch. Intern. Med., 157, 399-408, 1997).

While the neuromuscular malfunctioning associated with MG can be transiently alleviated by systemic chronic administration of carbamate acetylcholinesterase (AChE) inhibitors (e.g. pyridostigmine), the inventors have found that pyridostigmine induces a feedback response leading to excess AChE accumulation (Friedman *et al.*, Nature Medicine 2, 1382-1385, 1996; Kaufer *et al.*, Nature 393, 373-377, 1998).

Acetylcholinesterase (AChE) is commonly known for its role in terminating cholinergic neurotransmission by hydrolyzing the neurotransmitter acetylcholine. In all mammals, AChE is encoded by one gene but alternative splicing at its 3'-end yields three different mRNA transcripts which encode proteins with distinct carboxyl termini (Ben Aziz-Aloya *et al.*, Prog. Brain

Res. 98, 147-53, 1993; Karpel. R., *et al.*, Exp. Cell Res. 210, 268-77, 1994) (Fig. 1). The three proteins are all catalytically active: they include the "synaptic" form, AChE-S (S), encoded by the transcript that ends with exon 6, the hematopoietic form bound to the erythrocyte membrane, AChE-E (E), encoded by the transcript ending with exon 5 and the readthrough form, AChE-R, encoded by the transcript containing pseudointron 4. This transcript accumulates under multiple stress insults through the feedback response described above in brain (Kaufer. D., *et al.*, Chem. Biol. Interact. 119-120, 349-60, 1999), muscle (Lev-Lehman. E., *et al.*, J. Mol. Neurosci. 14, 93-105, 2000) and intestine (Shapira. E., *et al.*, Hum. Mol. Genet. 9, 1273-1281, 2000). Beside its catalytic function, AChE has morphogenic, non-catalytic capacities (Grisaru *et al.*, 2000; Grisaru *et al.*, 1999).

Transgenic mice overexpressing human AChEs in spinal cord motoneurons, but not in muscle, displayed progressive neuromotor impairments that were associated with changes in NMJ ultrastructure (Andres, C. *et al.* Proc. Natl. Acad. Sci. USA 94, 8173-8178, 1997). However, it was not clear whether the moderate extent of overexpressed AChE in muscle was itself sufficient to mediate this severe myopathology. In rodent brain, the inventors found previously that both traumatic stress and cholinesterase inhibitors induce dramatic calcium-dependent overexpression of $AChE_R$ (Kaufer, *et al.*, Nature 393, 373-377, 1998).

Chronic AChE excess was found to cause in transgenic mice and amphibian embryos, progressive neuromotor deterioration (Ben Aziz-Aloya *et al*, Proc. Natl. Acad. Sci. USA, 90, 2471-2475, 1993; Seidman *et al.*, J. Neurochem. 62, 1670-1681, 1994; Seidman, *et al.*, Mol. Cell. Biol. 15, 2993-3002, 1995; Andres, C., *et al.*, Proc. Natl. Acad. Sci. USA 94, 8173-8178, 1997; Sternfeld *et* al., J. Neurosci. 18, 1240-1249, 1998). Also, myasthenic patients suffer acute crisis events (average annual incidence: 2.5%, see Berrouschot *et al.*,

Crit. Care Med. 25, 1228-35, 1997) associated with respiratory failure reminiscent of anti-AChE intoxications.

The Prior Art

In one approach, the prior art teAChEs that chemically protected RNA aptamers capable of blocking the autoantibodies to the nicotinic Acetylcholine Receptor (nAChR) may be developed and used to treat Myasthenia gravis. This approach has several drawbacks in that the RNA aptamers do not have the amplification power characteristic of the RNAse-inducing antisense agents and in that it fails to address the problem of the feedback responses in MG.

Antisense oligodeoxynucleotides (AS-ODNs) are powerful tools for sequence-dependent suppression of target genes (Agrawal. S., and Kandimalla, E.R., Mol. Med. Today 6, 72-81, 2000; Crooke, S.T., Methods Enzymol 313, 3-45, 2000). AS-ODNs are presumed to act by facilitating the action of ribonuclease on mRNA-ODN hybrids (Ma, M., *et al.*, Nat. Biotechnol. 18, 58-61, 2000; Wu. H., *et al.*, J. Biol. Chem. 274, 28270-8, 1999). To exert their effects, AS-ODNs must enter the cell, interact with their target mRNAs long enough for the nuclease to act, and then attach to another mRNA. Much effort has been devoted to understanding the cellular uptake and mechanism of action of AS-ODNs (Beltinger *et al.*, J. Clin. Invest. 95, 1814-23, 1995). In contrast, less is known about host cell responses to this process. For example, it is not known whether AS-ODN-treated cells compensate for lost mRNA. This issue is important, since feedback upregulation of a targeted gene may mask antisense effects and encourage the use of excessively high concentrations of ODN.

Another important issue to address relates to the effects of foreign DNA and AS-ODN degradation products such as free nucleotides on cellular

physiology. Nucleotides and their analogs modulate cell volume through several independent mechanisms (Galietta, L.J., *et al.*, FEBS Lett. 304, 61-5, 1992). Under physiological steady-state conditions, cell volume is held constant by a "pump-leak-mechanism". The osmotic pressure arising from impermeable cytoplasmic solutes is balanced by the Na^+/K^+ pump, accompanied by the constant expenditure of metabolic energy (Galyam, N., *et al.*, accepted to Antisense Nucleic Acid. Drug. Dev., 2001). Micromolar or lower adenosine concentrations such as those expected to accumulate by degradation of ODN were shown to activate the A1 adenosine receptor and a Cl^- channel in the apical membrane of RCCT-28A endothelial cells (Light, D.B., *et al.*, Am. J. Physiol. 258, F273-80. 1990; Schwiebert, E.M., *et al.*, J. Clin. Invest. 89, 834-41, 1992). Extracellular ATP, UTP and related compounds similarly stimulate Cl^- secretion and affect cell volume in the nasal epithelium of both normal and cystic fibrosis patients (Knowles *et al.*, N. Engl. J. Med. 325, 533-8, 1991). Once they enter the cell, nucleotides affect the nucleocytoplasmic shuttle of proteins and RNA (Gerace, L., Cell 82, 341-4, 1995), while non-hydrolyzable GTP analogues inhibit this shuttle (Melchior, F., *et al.*, J. Cell. Biol. 123, 1649-59, 1993). Changes in nuclear protein import may affect several levels of cellular metabolism (Gorlich, D., EMBO J. 17, 2721-7, 1998), but were not yet examined under AS-ODN treatment. These observations predict sequence non-specific cellular responses to AS-ODN treatments. Therefore, both the balance of ion homeostasis and nuclear-cytoplasmic interactions must be considered in AS-ODN studies.

The present inventors have previously found that antisense oligonucleotides against the common coding region of AChE are useful for suppressing AChE production (see WO 98/26062). This PCT publication also teAChEs that antisense oligonucleotides against the human AChE are useful in the treatment of memory deficiencies as observed in transgenic mice that

expressed human AChE in their brain.

The observed effects (see Table 4-5 in WO 98/26062) are similar to the effect of the prior art AChE inhibitor Tacrine (see Fig. 9B in WO 98/26062).

In view of the above, it is desirable to improve the prior art treatment approaches for MG and other diseases involving an impairment in neuromuscular transmission. The prior art treatment involving the use of Acetylcholinesterase inhibitors is afflicted with undesirable side effects because of the induction of AChE and neuromuscular impairments by such inhibitors.

It has now been surprisingly found that morphological and functional changes in the NMJ are correlated to overexpression of a specific isoform of AChE mRNA, viz., the "readthrough" isoform containing the pseudointron I4 in the mature mRNA. More surprisingly, it has now been found, and that is an object of the invention, that antisense oligonucleotides directed to the common coding region of AChE may be used to specifically destroy "readthrough" AChE mRNA. It has further been surprisingly found, and that is another object of the invention, that AChE antisense agents are by far superior to conventional AChE enzyme inhibitor drugs in the treatment of neuromuscular disorders. The superiority of the present antisense agents may be due to the fact that conventional enzyme inhibitors actively induce I4 AChE mRNA overexpression. According to the teaching of the present invention, this may lead to detrimental changes in the neuromuscular junction. This consequence of treatment may be entirely avoided by using the present antisense agents.

Summary of the invention

The invention relates to a pharmaceutical or medical composition for the treatment and/or prevention of a progressive neuromuscular disorder, comprising as active ingredient an antisense oligonucleotide targeted to AChE mRNA.

The antisense oligonucleotide preferably causes preferential destruction of I4 AChE mRNA.

The oligonucleotide is preferably an oligodeoxynucleotide. However, also ribonucleotides, nucleotide analogues, or mixtures thereof are contemplated by the invention.

The oligonucleotide of the invention may also be a ribozyme, preferably a hammerhead ribozyme, which comprises a sequence complementary to AChE mRNA sequence, and which is capable of destroying AChE mRNA, preferably I4 AChE mRNA. The complementary sequence is preferably selected from a sequence as described further below for antisense oligonucleotides against AChE.

The pharmaceutical composition is preferably for a once daily use by a patient and preferably comprises between about 0.1 and about 175 mg, more preferably between about 1 and about 70 mg, most preferably between about 15 and about 50 mg of active ingredient.

The pharmaceutical composition is preferably for treatment and/or prevention comprising a dosage of active ingredient of about 0.01 to about 2.5 mg/kg, more preferably about 0.1 to 1.5 mg/kg, most preferably about 0.25 to about 0.75 mg/kg.

The neuromuscular disorder is preferably associated with an excess of AChE mRNA or protein. More preferably, the neuromuscular disorder is associated with an excess of I4 AChE mRNA. The I4 AChE mRNA excess is preferably caused by enhanced transcription. The enhanced transcription preferably involves the activity of an early immediate gene, more preferably the *fos* gene. Alternatively, or in addition, the excess is due to impaired 3' splicing, e.g., because of the lack of specific splicing factors necessary therefor (see Lev-Lehman et al., Brain Res. 661, 75-82, 1994).

The invention relates, in one embodiment thereof, to the pharmaceutical or medical composition for use in treating a progressive neuromuscular disorder, wherein said disorder is associated with impairment of cholinergic transmission. In a preferred embodiment, the progressive neuromuscular disorder results from impairment of cholinergic transmission, or is caused by impairment of cholinergic transmission or causes impairment of cholinergic transmission.

The progressive neuromuscular disorder further preferably involves muscle distortion, muscle re-innervation or neuromuscular junction (NMJ) abnormalities. Also preferably, the progressive neuromuscular disorder involves changes in intracellular Ca^{++} levels. Examples for conditions that may be treated or prevented by the pharmaceutical composition of the invention are *Myasthenia gravis*, Muscular dystrophy, amyotrophic lateral sclerosis, post-traumatic stress disorder (PTSD), multiple sclerosis, Dystonia, post-stroke sclerosis, post-injury muscle damage, Eaton-Lambert disease, excessive re-innervation, post-exposure to AChE inhibitors, and the like conditions.

The synthetic antisense oligonucleotide may be targeted to one or more of exons 1, 2, 3, 4, 5, pseudointron I4, or a splice junction thereof. In one

embodiment of the invention, the synthetic antisense oligonucleotide is targeted to the E4-I4 splice junction.

Preferably, but not limitatively, the antisense oligonucleotide is targeted to the common coding domain of AChE mRNA. The synthetic antisense oligonucleotide may be targeted to one or more exons of the common coding domain of AChE mRNA, or to a junction thereof. Preferably, the synthetic antisense oligonucleotide is targeted to one or more of exons 2, 3, or 4, or a junction thereof. More preferably, the synthetic antisense oligonucleotide is targeted to one or more of exon 2, and a splice junction of exons 2-3, 3-4, or 4-6. Further preferably, the synthetic antisense oligonucleotide is targeted to any one of exons 2, 3, or 4. In another preferred embodiment, the synthetic antisense oligonucleotide is targeted to exon 2 of the AChE gene. Preferably, the synthetic antisense oligonucleotide is targeted at a region near to or overlapping with the AChE ATG start codon.

The synthetic antisense oligonucleotide of the invention preferably comprises one or more of the nucleotide sequences selected from

5'-CTGCAATATTTTCTTGCACC-3' (AS3, SEQ ID No. 1);
5'-GGGAGAGGAGGAGGAAGAGG-3' (AS1, SEQ ID No. 2);
5' ACGCTTTCTTGAGGC (SEQ ID No. 3);
5' GGCACCCTGGGCAGC (SEQ ID No. 4);
5' GCCAGAGGAGGAGGAGAAGG (SEQ ID No. 5);
5' TAGCGTCTACCACCCCTGAC (SEQ ID No. 6);
5' CCACGTCCTCCTGCACCGTC (SEQ ID No. 7);
5' ATGAACTCGATCTCGTAGCC (SEQ ID No. 8);
5' TCTGTGTTATAGCCCAGCCC (SEQ ID No. 9);
5' GGCCTGTAACAGTTTATTT (SEQ ID No. 10);
5' AGAGGAGGGACAGGGCTAAG (SEQ ID No. 11);

5' TAGCATCCAACACTCCTGAC (SEQ ID No. 12); and
5' ATGAACTCGATTTCATAGCC (SEQ ID No. 13).

In a preferred embodiment, the synthetic oligonucleotide of the invention comprises one or more of the nucleotide sequences
5'-CTGCAATATTTTCTTGCACC-3' (AS3, SEQ ID No. 1) and
5'-GGGAGAGGAGGAGGAAGAGG-3' (AS1, SEQ ID No. 2).

The synthetic antisense oligonucleotide of the invention preferably leads to the specific destruction of I4 AChE mRNA.

The synthetic antisense oligonucleotide according to the invention is preferably used at a dosage of about 0.01 to 2.5 μg oligonucleotide per gram of body weight. More preferably, the dosage is about 0.2 to about 1.0 μg/g.

The antisense oligonucleotide of the invention is preferably a modified oligonucleotide comprising (a) partially unsaturated aliphatic hydrocarbon chain(s) and one or more polar or charged groups including carboxylic acid groups, ester groups, and alcohol groups.

In another embodiment of the invention, the antisense oligonucleotide is a modified oligonucleotide linked to peptide structures, including membranotropic peptides.

Further preferably, the antisense oligonucleotide is a modified oligonucleotide linked to one or more groups selected from Palmityl groups, Geraniol groups, and membranotropic peptides.

The pharmaceutical composition of the invention preferably further comprises a lipid agent. The lipid agent is preferably selected from

Lipofectin, Lipofectam, Transfectam, DOTAP, liposomes and virosomes.

The compositions of the invention may be administered in a variety of ways. By way of non-limiting example, the composition may be delivered by injection intravenously, intramuscularly, or intraperitoneally. Intravenous administration, for example, is advantageous. In another preferred alternative, the composition of the invention may administrate orally.

A further aspect the invention relates to a method for the diagnosis of a progressive neuromuscular disorder in a mammal, preferably in humans. This method comprises the steps of: obtaining a sample from the subject and detecting intensified expression of at least one of AChE variants in said sample, preferably, intensified expression of the AChE-R "readthrough" variant.

In a preferred embodiment, the diagnostic method of the invention is intended for the diagnosis of a progressive neuromuscular disorder that involves muscle distortion, muscle re-innervation or neuromuscular junction (NMJ) abnormalities. The neuromuscular disorder may be selected from *Myasthenia gravis*, Eaton-Lambert disease, Muscular dystrophy, amyotrophic lateral sclerosis, post-traumatic stress disorder (PTSD), multiple sclerosis, Dystonia, post-stroke sclerosis, post-injury muscle damage, excessive re-innervation, and post-exposure to AChE inhibitors. Particularly, the method is intended for diagnosing Myasthenia Gravis.

In a specifically preferred embodiment, the sample used by the diagnostic method of the invention is one of serum, bone marrow and cerebrospinal fluid sample. More preferably, the sample may be a serum sample.

Detection of the intensified expression of different AChE variants in the

serum sample may be carried out by different techniques such as immunoassay, RT-PCR and nondenaturing activity gel electrophoresis. In a preferred embodiment, detection of intensified expression of different AChE variants in the serum sample may carried out by the nondenaturing activity gel electrophoresis. Briefly, this technique comprises the steps of preparing a protein extract from said serum sample, separating the protein extract on polyacrylamide gel using nondenaturing conditions and detecting catalytically active AChE variants by the Karnovsky staining technique.

Brief description of the drawings

Figure 1 *Natural AChE splicing variants*

The three natural AChEmRNA variants produced by splicing in the coding region yield proteins with characteristic C-terminal sequences, encoded by the open reading frame, ORF, of pseudointron 14, exons E5 and E6, respectively. Each differs in its developmental and tissue distributions. Synaptic AChE-S is encodes by the transcript 3'-terminated with E6, AChE-S has a c-terminal peptide predicted by the peptide structure program of the GCG software package (University of Winsconsin) to have a helical amphipathic sequence before the final 23 residues. The hematopoietic AChE-E protein carried a 14-residue C-terminus that is covalently linked to a glycosylphosphoinositol (GPI) anchor. The readthrough variant, AChE-R, includes a hydrophilic 26-residue C-terminus with no predicted secondary structure and/or option for membrane anchor. Non-polar residues are in red. Abbreviations: soluble monomers (sol mono), alternative transcripts (alt trans), gene (ge), erythrocyte (ery), carboxyl terminus (car term), synaptic (syn), anchored (anc).

Figure 2 *MG serum displays higher variability in AChE activity levels*
AChE activity levels from MG serum were compared to control serum. The activity units expressed as nmole substrate hydrolyzed/min/ml. average marked with black line. Note that the variability of the values is higher in MG serum (standard deviation=52) as compared to control (standard deviation=14.8) although the average values are similar (171.1 and 165 nmole substrate hyd/min/ml respectively. Abbreviations: Activity units (Act u), control (ctrl).

Figure 3 *Alterations in seral AChE activity levels are independent of anti-AChR antibodies present*
Serum samples from MG patients were assayed for AChE catalytic activity (Act). Graph represents activity levels [percentage of controls (ctrl)] as function of anti-AChR antibodies concentration in the serum (pmole/ml). Notice that there is no significant correlation between the two parameters.

Figures 4A-4B *Rapid migrating active AChE-R protein accumulates in MG serum*
4A - Serum samples from MG patients are non-MG controls were run on non-denaturing gels and stained for catalytically active AChE using the Karnovsky staining technique. A representative 7.5% native gel is shown. Note that rapid migrating bands appeared in MG serum but not in control(ctrl) serum (healthy(heal) or irrelevant disease). Those bands run in parallel to plasma from transgenic mice overexpressing the AChE-R variant.
4B - Example of two lane profiles of healthy and MG samples measured by the Image-Pro software (6% native gel).
Abbreviations: mouse (mow), human (hum), signal intensity (sig inten), arbitrary units (arb U).

Figure 5 *Tetramer Analysis*

Linear sucrose gradient centrifugation was performed on serum samples from MG and non-MG patients. AChE catalytic activity (act) was assayed in all the fraction (frac) in the presence of BuChE inhibitor (50 μM ISO-OMPA) and expressed as mOD/min. Shown are the gradient profiles as AChE activity as function of the fraction number. Activity of AP (S=6.1) is marked by arrows. Note that the MG serum sample displays higher activity in fraction 23 most likely contains tetramers of AChE-S(S>11) while the non-MG sample displays higher activity in fraction 28 probably contains G2 dimers of AChE-S and AChE-E (S>7.5). In addition, the peak in fraction 31 of MG serum sample may represent G1 monomers of AChE-R.

Abbreviations: non (n).

Figures 6A-6B *Transgenic AChEs and DFP similarly inducing enhanced c-fos and AChE$_R$ mRNA production in muscle*

6A - The mouse AChE gene and alternative splicing products. Presented is a schematic illustration of coding exons 3, 4, 5 and 6 (shaded boxes) and intron 3 to 4 (white boxes) in the mouse AChE gene. The "synaptic" AChE$_S$ mRNA transcript (E6) results from splicing of exon 4 to 6; the "erythrocytic" AChE$_E$ mRNA transcript (E5) from splicing of exon 4 to 5; *Readthrough* AChE$_R$ mRNA retains intron I4 in a mature unspliced transcript. Arrowheads indicate PCR primer pairs detecting the individual mRNA transcripts.

6B - RT-PCR analyses. Presented are PCR products of AChE mRNA derived from tongue muscle of control (Crtl) or AChE transgenic (Tg) newborn mice or from control mice treated for 2 weeks with either DFP or vehicle (Veh).

Abbreviations: Relative band intensities (Rel ba inten), cycle (cyc).

Figure 7 *Overexpressed transgenic AChEs and DFP induce accumulation of endogenous AChER mRNA in both epithelium and muscle*

In situ hybridization was performed on 7 μm sections of tongue from newborn (P0) control (a, c) and AChE transgenic (b, d) mice, or 15-day-old (P15) control mice injected with either vehicle (e, g) or the AChE inhibitor DFP (f, h). Note enhanced and delocalized fluorescent labeling of $AChE_R$ but not $AChE_S$ mRNA in epithelial cells and muscle fibers from both transgenic and DFP-treated mice as compared to controls.

Figure 8 *AChE-Transgenic mice display pronounced non-junctional enzyme activity in muscle*

Tongue sections from one-month-old control and transgenic mice were stained for catalytically active AChE. At low magnification, diffuse, light staining of both epithelium (E) and muscle (M) layers was observed in sections from control mice (A). Higher magnification revealed strong AChE activity localized to motor endplates (B). In sections from transgenic mice, minimal levels of AChE are visible in the epithelium (C), while intense staining is evident in the muscle layer, widely distributed along muscle fibers (C, arrow; D).

Figure 9 *Comparison of tongue muscle from control DFP and ACHE transgenic mice*

The figure shows tongue muscle from P15 untreated control (Ctrl), chronic-DFP-treated control (DFP), or untreated ACHE transgenic (Tg) mice.

Figures 10A-10D *Both transgenic AChE and DFP induce neurite sprouting*

Silver-stained tongue-muscle neurites are shown in parallel sections from 15-day-old control (**A**), DFP-injected control (**B**) and AChE transgenic (**C**) mice. Both DFP-treated and transgenic mice displayed numerous small

(<200 μm^2) bundles of neurites as compared with untreated controls (black arrows). Shown are representative photomicrographs in an equivalent location beneath the tongue epithelium. White arrow indicates a representative large (>1000 μm^2) neurite bundle observed in all groups in similar numbers. Sections from vehicle-injected animals were indistinguishable from untreated controls (not shown). (**D**) Bar graph represents number of small neurites (neur) per mm^2 (average ±SEM) for at least 3 animals from each group. Asterisk indicates statistically significant difference compared to control ($p<0.05$).

Figures 11A-11D *AChE activity in diaphragm motor endplates*

The Figure shows AChE activity in diaphragm motor endplates (Fig. **11A-C**), and the correlation thereof to the number of synapses (Fig. **11D**), depending upon treatment with the AChE inhibitor DFP, AChE antisense oligonucleotide AS3, and transgenic overexpression of AChE. Abbreviations: Control (ctrl), activity (act).

Figures 12A–12E *The pronounced fatigue exhibited by transgenic diaphragmatic muscle*

12A-12B - graphically demonstrate the pronounced fatigue exhibited by transgenic diaphragmatic nerve.

12C-12D - graphically demonstrate the pronounced fatigue exhibited by transgenic diaphragmatic muscle showing the relative contributions of the muscle responses and nerve responses to the fatigue phenotype.

12E - shows schematic illustration of the response measurements.

Abbreviations: Nerv (N), stimulation (stim), Muscle (Mus) Normalized force (Norm Fc), Transgenic (Tg), control (ctrl).

Figures 13A-13C *Cellular behavior of neuromuscular transmission*

13A - demonstrates the cellular behavior of a control (ctrl) neuromuscular

transmission under physiological conditions, using a cut muscle preparation.

13B - demonstrates the cellular behavior of a Transgenic (Tg) neuromuscular transmission under physiological conditions.

13C - shows graphic comparison of both.

Abbreviations: Normalized (Norm), pulse number (pul num).

Figures 14A-14C *The post-synaptic properties of neuromuscular junctions for transgenic and control animals*

14A - graphically depicts the post-synaptic properties of neuromuscular junctions for both transgenic (Tg) and control (ctrl) animals. The bar graph show the effects of higher levels of AChE on: amplitude (Amp).

14B - the bar graph shows the effects of higher levels of AChE on rise time (Ri-T).

14C - the bar graph show the effects of higher levels of AChE on half-decay time (Ha dec T) of the post-synaptic response.

Abbreviations: No drug (N dr).

Figures 15A-15B *Quantal content and hypersensitivity to anticholinesterase of transgenic synapses*

15A - graphically demonstrates the increased quantal content of the transgenic synapses.

15B - graphically demonstrates the increased hypersensitivity to anti-cholinesterase of transgenic synapses.

Abbreviations: Quantal content (Quan con), Frequency of spontaneous release (Freq spont rel), No drug (N dr).

Figures 16A-16B *2'-O-Methyl AS-AChE displays a wide effective window at extremely low concentrations*

Varying concentrations of phosphorothioate (PS) and 2'-O-methyl (Me) AS1 or ASB were added to PC12 cells once daily for 2 days following 24 hr exposure to NGF and cells were analyzed for AChE catalytic activity or mRNA encoding AChE-S.

16A - AChE catalytic activity was measured using a colorimetric assay and acetylthiocholine as substrate. Results are expressed as percent of activity in control cells ± standard error of the mean (SEM) for 5-9 triplicate measurements for each point. Both oligonucleotides displayed decreasing efficacy at ODN concentrations above 2 nM. The non-relevant control AS-ODN ASB, in both the Me and PS forms had minimal effects on AChE activity at concentrations up to 100 nM (inset).

16B - *In situ* hybridization with an exon 6-specific AChE cRNA probe was employed to detect AChE-S mRNA in AS3-treated cells. Quantification of AChE-S mRNA levels was by confocal microscopy and computer-assisted image analysis. Shown are average ± SEM of AChE-S mRNA levels as a percentage of that observed in untreated cells. Inset depicts cells from the same experiment following cytohistochemical staining for catalytically active AChE. From left to right are representative cells from cultures treated with 0.2, 2, and 1000 micromolar AS3, respectively.

Abbreviations: Enzyme (Enzy), Activity (Act), control (ctrl) concentration (conc).

Figures 17A-17B *Reduction of AChE-SmRNA levels and AChE activity in PC12 cells by AS3*

17A - detection of AChE-S mRNA and its protein product. Color coding (right hand side scale) highlights cytoplasmic sites with greater AChE-S mRNA concentrations.

17B - sequence-specific changes in AChE-Sm RNA levels of PC12 cells. Shown are average values ± SEM of AChE-S mRNA levels in aggregates/cell (left scale) or percent of untreated cells (right scale).
Abbreviations: pixels/cell (pix/c), control (ctrl), concentration (conc).

Figures 18A-18B *Focal nuclear accumulation of AChE mRNA in AS-ODN treated PC12 cells*

18A - Presented are pseudocolored compound confocal images of representative PC12 cells following incubation with 2'-O-methyl RNA-protected AS3 and in situ hybridization with a probe detecting AChE-S mRNA. Color coding (upper right corner) correlates with intensity of fast red staining and therefore AChE-S mRNA levels. Punctuate nuclear staining for AChE-S mRNA is indicated by arrows.
18B - Graph depicts the percentage of total potential nuclear sites (assuming 2 per cell) labelled by in situ hybridization at the noted concentrations of AS3. Inset presents ethidium bromide stained products of RT-PCR performed on RNA extracted from PC12 cells treated with the noted concentrations of AS3. Abbreviations: control (ctrl), nuclear (nuc), concentration (conc), labeled (lab), sites (si), Unspliced (U-sp), spliced (sp).

Figures 19A-19B *AS3 uptake and its effect on cell volume*

19A - shows limited AS3 penetrance under increasing concentrations. Differentiated PC12 cells were incubated for 24 hrs with biotinylated AS3, treated with alkaline phosphatase-conjugated streptavidin, and subjected to Fast Red detection. Fast Red signals from 20 cells were quantified.
19B - shows that cytoplasmic but not nuclear volume changes under increasing concentrations of AS3.Total cellular and nuclear volumes of AS-ODN-treated PC12 cells were determined using confocal microscopy as described in Methods. Abbreviations: aggregates (aggr), per cell (p s),

concentration (conc), cyt (cytoplasm), nucl (nucleus).

Figure 20 *Saos2 cells display a decrease-increase curve of ACHE mRNA levels under increasing AS1 doses*

Top, confocal projections of Saos cells hybridized *in situ* with exon 6-specific AChE cRNA probe; Bottom, AChE-S mRNA levels.

Abbreviations: pixels/cell (pix/c), cell (c), antisense (α-sen), activity (act).

Figure 21 *AChE-R and AChE-SmRNA yield decrease-increase curves under distinct ODN ranges in CD34⁺ cells*

The top and bottom sections of the Figure show confocal projections of primary $CD34^+$ cells subjected to 24 hr treatment with the noted concentrations of AS_1 or ASB and then to *in situ* hybridization with AChE cRNA probes. The center of the Figure shows quantification of the noted AChE mRNA transcripts in aggregates x 10^{-3} per cell ± SEM for 20 cells in each point.

Abbreviations: pixels (pix).

Figures 22A-22C *The effect of AS3 AChE antisense oligonucleotide on electromyography response*

22A - The EMG ratio between first (I) and fifth (V) response (values shown in %).

22B - The ratio between first and fifth response (S5/S1, values shown in %) was recorded in control (ctrl) FVB/N mice (triangles), AChE E6 transgenic mice (Naive Tg, black squares), and AS3-treated AChE E6 transgenic mice (Tg+AS3, gray circles), at the indicated times. The number of animals used in each experiment is indicated in the Figure (Naive Tg, N=16, Tg+AS3, N=10, and control mice, N=5).

22C - shows the variance (Var)of S5/S1 values in control mice (Ctrl), 5 weeks old transgenic mice (Tg 5 wk), 5 month old transgenic mice (Tg 5 mo),

and in transgenic mice treated with AS3 (Tg+AS3).
Abbreviations: week muscle (w mus), normal (nor), depolarization (dep).

Figures 23A-23C *AChE-R Accummulation in Severe Experimental Myasthenia Gravis is Selectively Suppressed by AS3*

23A - Shown is *in situ* hybridization of paraffin embedded sections of front leg extensor muscle of severely ill EAMG or control Lewis rats.

23B - AChE-R immunolabelling of paraffin embedded sections of front leg extensor muscle of severely ill EAMG or control Lewis rats.

23C - ACh-R immunolabelling of paraffin embedded sections of front leg extensor muscle of severely ill EAMG or control Lewis rats.

Abbreviations: untreated (u-tr), protein (pr), nicotinic (ni), control (ctrl).

Figures 24A-24D *Lasting AS3 Improvement in Muscle Function of Myasthenic Rats*

24A - Electromyograph (EMG) recordings from gastrocnemius muscle of EAMG after single IV administration (sin adm) of AS3 in different concentrations, reversed 5'-3' nucleotide sequence of AS3 (Inv-AS3), or Mestinon. Shown are averages of at least 3 rats in each group.

24B - Duration of AS3 effects is dose-dependent from 24-96 hours following a single i.v. administration of different AS3 concentrations. Shown are EMG data collected from EAMG rats 24 hrs post-treatment.

24C - shows effective oral administration of AS3. AS3 (50 μg/kg body weight) was administered by a gastric feeding tube or by i.v. injection (25 μg/kg) to EAMG rats once daily for up to 4 days. EMG was performed after 1 and 5 hrs and then each 24 hrs, prior to the subsequent feeding. Graph compares oral administration ofAS3 (n=8) to oral administration of Mestinon (n=4) or inverse-AS3 (n=4).

24D - Graph depicts the equivalent improvement in muscle function elicited by oral (n=8) as compared to i.v. administration (n=4).

Abbreviations: post injection (po inj), ratio (Ra), Invers (Inv), hours (hr), Oral (Or) administration (Adm), Repeated (Rep), Doses (Do).

Figures 25A-25B *The decrement upon repetitive stimulation at 3 Hz in a rat with EAMG*

25A - shows baseline response with 12.4% decrement.

25B - shows response at 1 h after injection of 100 μg AS-3 with reversal of decrement (6.4% increment).

Abbreviations: myastenic (mya), untreated (untr), treated (trea), depolarization (dep), first (I), fifth (V).

Figure 26 *The difference between the muscle action potential amplitudes in control and EAMG rats*

A representative curve of the difference between the first and fifth muscle action potential amplitudes (values represent percent change from baseline) of healthy (heal) control rat (closed circles) and EAMG rats following injection of neostigmine (triangles) or AS-3 (squares).

Abbreviations: response (resp), untreated (utr), myastemic (mya), hours (hr), post-treatment (p-tr).

Figures 27A-27B *AS3 Improves Stamina in Myasthenic Rats*

27A - shows the treadmill.

27B - EAMG rats with varying severity of clinical symptoms [Severe (Se) n=11, Moderate (Mo) n=4, Mild (M) n=5] and control (ctrl) (n=7) (inset) Lewis rats were prodded to run on an electrically powered treadmill (Tread) (25 meter/min.) until visibly fatigued. The time each rat was able to run was recorded before, and 24 hrs following, i.v. administration of 250 μg/Kg AS3.

Abbreviations: improvement (imp).

Detailed description of the invention

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

Antisense oligonucleotide: A nucleotide comprising a sequence essentially reverse complementary 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.

Progressive neuromuscular disorder: A disorder or condition associated with excess AChE mRNA or protein production, characterized by changes in the morphology of the NMJ and impairment in neuromuscular transmission. The neuromuscular disorder may involve muscle distortion, muscle re-innervation or neuromuscular junction (NMJ) abnormalities. More preferably, the progressive neuromuscular disorder is *Myasthenia gravis*, Muscular Dystrophy, Multiple Sclerosis, Amyotrophic lateral sclerosis, post-traumatic stress disorder (PTSD), or Dystonia.

The invention relates to a pharmaceutical composition for the treatment and/or prevention of a progressive neuromuscular disorder, comprising as active ingredient at least one synthetic antisense oligonucleotide targeted to AChE mRNA.

In addition to the part of the sequence which is complementary to AChE sequence, the antisense oligonucleotide of the invention may also comprise RNA sequences with enzymatic nucleolytic activity, or may be linked to

such sequences. Preferred nucleolytic sequences are ribozyme sequences, which were shown to specifically interact with mRNA transcripts. They are ribonucleic acid sequences, including RNase active sites flanked by antisense oligonucleotides, see e.g., Haseloff and Gerlach, Nature 3, p. 585, 1988, Sarver *et al.*, Science 247, p. 1222, 1990. Preferred ribozymes are hammerhead ribozymes, see e.g., Conaty *et al.*, Nucleic Acids Res. 27, 2400-2407, 1999, and Xu *et al.*, Endocrinology, 140, 2134-44, 1999, and references therein. Another preferred ribozyme is the hairpin ribozyme structure, e.g., as derived from tobacco ringspot virus satellite RNA (see Perez-Ruiz, Antisense Nucleic Acid Drug Dev., 9, 33-42, 1999).

The antisense oligonucleotide of the invention is preferably between about 7 and about 300 nucleotides long, more preferably between about 10 and 80 nucleotides, most preferably between about 12 to about 25 nucleotides. These numbers do not include the optional ribozyme part as detailed above. A ribozyme including antisense sequence may comprise between 20 and 300, preferably between 30 and 100, more preferably about 40 nucleotides.

The antisense oligonucleotide preferably corresponds to the reverse complement of human AChE mRNA sequence. While the region targeted by the antisense oligonucleotide is preferably located within the coding domain of the AChE mRNA, the 5' or 3' non-coding regions, or their junctions with the coding sequence, may also be targeted. Prior work by the present inventors has demonstrated the usefulness of antisense oligonucleotide in the treatment of memory deficiency. In said prior work, a number of AChE antisense oligonucleotides have been disclosed. Said prior work further discloses desirable features of such antisense oligonucleotides and possible modifications thereof, such as nuclease resistance oligonucleotides, modifications to enhance membrane transport of oligonucleotides, and the like. Said prior work, which is WO 98/26026, is therefore incorporated

herein in its entirety by reference. In another publication, the present inventors describe the role of antisense oligonucleotides in the treatment of a variety of neurodegenerative diseases (Seidman, S. *et al.*, Antisense Res. Nucl. Acids Drug Devel. 9: 333-340, 1999).

WO 98/26062 discloses that the activity of AChE antisense oligonucleotides varies from one oligonucleotide sequence to another, but that there is no clear correlation of activity and targeted mRNA region. Some authors have suggested that antisense oligonucleotides should be targeted to the 5' region of a mRNA, preferably to the 5' noncoding region thereof, and more preferably to the junction between the 5' noncoding region and the coding region, including the ATG translation start codon. Others have reported good antisense effects with oligonucleotides targeting the 3' region of a mRNA.

In the case of AChE, WO 98/26062 teaches that oligonucleotides targeting the E6 exon of the AChEmRNA are less effective compared to oligonucleotides targeting the preceding exons.

The present invention preferably relates to AChE antisense oligonucleotides that target the I4 AChE mRNA isoform. Therefore, the antisense oligonucleotides must target an exon selected from exon 1, 2, 3, 4, I4, and 5. Of course, any splice junction between said exons may be targeted as well. The I4 mRNA is the only mRNA that contains the I4 pseudointron. However, in order to target the I4 mRNA, it is possible to target any of the exons therein, despite of the fact that other AChE mRNA isoforms will be targeted as well. Nevertheless, such oligonucleotides, when used according to the invention, will preferentially lead to destruction of the I4 mRNA, according to the teaching of the invention. Without wishing to be bound by theory, it is the inventor's belief that the reason for the preferential

destruction of I4 mRNA is the low stability of this AChE mRNA isoform, compared to the other isoforms.

An example for an antisense oligonucleotide directed at exon 5 of murine AChE is

5' AGAGGAGGGACAGGGCTAAG (SEQ ID No. 11)

Alternatively, antisense oligonucleotides may be targeted directly at I4-specific sequences, such as the E4-I4 junction, the I4 pseudointron, or the I4-E5 junction.

In a preferred embodiment of the invention, the antisense oligonucleotides are targeted at an exon selected from exons 2 and 3, and the splice junction thereof. Examples of such oligonucleotides targeted at murine AChE sequences are

mAS1 5' GGGAGAGGAGGAGGAAGAGG (SEQ ID No. 2)
mAS2 5' TAGCATCCAACACTCCTGAC (SEQ ID No. 12)
mAS3 5' CTGCAATATTTTCTTGCACC (SEQ ID No. 1)
mAS4 5' ATGAACTCGATTTCATAGCC (SEQ ID No. 13)

Examples of such oligonucleotides targeted at human AChE are

5' GCCAGAGGAGGAGGAGAAGG (SEQ ID No. 5)
5' TAGCGTCTACCACCCCTGAC (SEQ ID No. 6)
5' CCACGTCCTCCTGCACCGTC (SEQ ID No. 7)
5' ATGAACTCGATCTCGTAGCC (SEQ ID No. 8)

In a more preferred embodiment of the invention, the oligonucleotides are

directed against exon 2 of the AChE mRNA. In a further preferred embodiment of the invention, the antisense oligonucleotides are targeted against the region of the ATG translation start codon located in exon 2 of AChE. Examples for such oligonucleotides targeted at murine AChE are

mAS1 5' GGGAGAGGAGGAGGAAGAGG (SEQ ID No. 2)
mAS2 5' TAGCATCCAACACTCCTGAC (SEQ ID No. 12)

Examples for such oligonucleotides targeted at human AChE are
5' GCCAGAGGAGGAGGAGAAGG (SEQ ID No. 5)
5' TAGCGTCTACCACCCCTGAC (SEQ ID No. 6)

The antisense oligonucleotides of the invention are 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.

The present inventors have found that AChEmRNA may be up-regulated as a stress response in cells or tissues. Specifically, the inventors have found

this response in muscle and epithelium, e.g., diaphragm muscle, tongue muscle and epithelium, in retina tissue, and in umbilical chord blood stem cells.

In a preferred embodiment of the invention, the concentration of the antisense oligonucleotides of the invention lies within a treatment window. A treatment window is the concentration of antisense oligonucleotide where said antisense oligonucleotide will achieve its objective according to the invention, i.e., the antisense oligonucleotide will exert a beneficial treatment effect according to the invention. The beneficial treatment effect preferably results from preferential destruction of AChE I4 mRNA.

The cells on which the antisense oligonucleotide of the invention exerts its effects are preferably muscle cells and cells of the NMJ, including the nerve axons and endplate structures.

In another preferred embodiment, AChE I4 mRNA is measured as an indicator of antisense oligonucleotide concentrations that are outside of the preferred treatment window. Methods for extracting RNA from tissues or cells are well known in the art and are described e.g., in Ausubel *et al.* (eds), Current Protocols in Molecular Biology, Wiley Interscience. RNA levels may be quantified by Northern analysis, which is well known in the art and described e.g., in the Ausubel *et al.* reference. Alternatively, AChE mRNA levels may be quantified by quantitative RT-PCR. Also this technique is well known to the skilled person. An example of RT-PCR is described herein below in Example 2. An indication of AChE 14 mRNA levels is an indication for antisense oligonucleotide concentrations that are outside of the preferred treatment window.

Using the antisense oligonucleotides according to the invention, it is

expected that AChE-R amount and AChE I4 mRNA levels are reduced by at least about 30%, preferably by at least about 40%, and more preferably by at least about 50%.

In yet another embodiment of the invention, the preferred treatment window of candidate oligonucleotides is evaluated by in situ hybridization. The technique of *in situ* hybridization is well known to the man of skill in the art, and is described e.g., *In situ* Hybridization, Wilkinson, D.G. (Ed.) ISBN: 0199633274; *In situ* Hybridization for the Brain, Wisden W., Morris B.J. (Eds.), ISBN: 0127599207, PCR *in situ* Hybridization: A Practical Approach (Practical Approach Series 186), Herrington C.S., John O'Leary J., (Eds.) ISBN: 019963632X. Detailed protocols relating to in-situ hybridization using non-radioactively labeled probes are available from Boehringer Mannheim.

As probes for in-situ hybridization labeled I4 AChE cRNA sequences may be used. The ACHE cRNA probe preferably comprises I4 intron sequences.

In a preferred embodiment of the invention, the AChE mRNA determination is carried out by using in situ RT-PCR, which technique is described, e.g., in the above-mentioned references, see also PCR *in situ* hybridization: Protocols and Applications, 3rd ed., by Nuovo, G.J. Lippincott, Raven Press, New York (1996).

A concentration of antisense oligonucleotide that is outside of the preferred treatment window is indicated when AChE mRNA production is enhanced, compared to the control reaction without antisense oligonucleotide.

Phosphorothioate-modified oligonucleotides are generally regarded as safe and free of side effects. Peng *et al.* teach that undesired in vivo side effects of

phosphorothioate antisense oligonucleotides may be reduced when using a mixed phosphodiester-phosphorothioate backbone. 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 10μM. 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 anti-sense oligonucleotides protected in this way.

In contrast to the prior art teachings regarding the suitability of modified AChE antisense oligonucleotides, it has now been found that a preferred treatment window of antisense oligonucleotide that is outside of the preferred treatment window of the invention may lead to an increase in AChE activity and I4 AChE mRNA. This contravenes the desired effect of the antisense agent of the present invention and should therefore be avoided. The treatment window is generally located at concentration levels

of oligonucleotide about two orders of magnitude lower that the concentrations previously taught in the prior art to be preferred as safe and lacking side effects.

Therefore, according to the present invention, any nuclease-resistant oligonucleotide must be tested for its preferred treatment window, and care must be taken in choosing an appropriate modification so as to avoid the said above indications of the concentration of oligonucleotide being outside of the preferred treatment window.

The present inventors have found that 2-O-methyl modified oligonucleotides have a wide treatment window. In general, the antisense oligonucleotide of the invention should be effective in the nanomolar range, to enable its use at concentrations that are within the preferred treatment window.

In accordance with the invention, the dosage of the antisense oligonucleotide is about 0.01 to 2.5 μg oligonucleotide per gram of body weight of the treated animal. More preferably, the dosage is about 0.2 to about 1.0 μg/gr.

The antisense oligonucleotide of the invention is provided for use in the treatment of a disorder that involves excessive AChE mRNA production.

The disorder is preferably a disorder involving functional and morphological changes in the NMJ.

The progressive neuromuscular disorder preferably involves overexpression of I4 AChE mRNA.

More preferably, the disorder is selected from among Multiple Sclerosis,

PTSD, Myasthenia Gravis, Muscular Dystrophy, Amyotrophic lateral sclerosis, Dystonia, muscle distortion, muscle re-innervation or excessive muscle innervation.

The excessive muscle innervation is selected preferably from among excessive innervation after trauma, preferably after amputation.

Four methods for treatment of Myasthenia Gravis are currently in use: enhancement of neuromuscular transmission with anticholinesterase agents, surgical thymectomy, immunosuppression and short-term immunotherapies, including plasma exchange and intravenous immune globulin (Drachman, D., *ibid*, 1994). Most of these treatments point at serum constituents as potentially important modulators of the severity of MG symptoms. Anti-cholinesterases are the first line of treatment, mostly used is Pyridostigmine (Mestinon), but most patients require further help.

While the neuromuscular malfunctioning associated with MG can be transiently alleviated by chronic systemic administration of AChE inhibitors, e.g. pyridostigmine, it was recently found that pyridostigmine induces a feedback response leading to excess AChE accumulation (Friedman, A., *et al.*, Nat. Med. 2, 1382-1385, 1996; Kaufer. D., *et al.*, Nature 393, 373-7, 1998). In transgenic mice, chronic neuronal AChE excess was found to cause progressive neuromotor deterioration (Andres *et al.*, *ibid.*, 1997; Sternfeld *et al.*, J. Neurosci. 18, 1240-9, 1998). Yet more recently, it was demonstrated that transgenic expression of neuronal AChE alters pre-synaptic properties and intensifies anticholinesterase responses in mouse NMJs (Farchi *et.al*, unpublished, 1998).

Therefore, treatment of MG using the compositions of the present invention would be advantageous, mostly due to the low effective concentrations and

the particularly effective window.

A synthetic oligonucleotide may be used as antisense oligonucleotide. The oligonucleotide is preferably a DNA oligonucleotide. The length of the antisense oligonucleotide is preferably between 9 and 150, more preferably between 10 and 60, and most preferably between 12 and 40 nucleotides. The region covered by the antisense oligonucleotide comprises preferably the 3' untranslated region of the cDNA, more preferably it comprises the polyadenylation signal or the translation stop codon, or both.

It is to be understood that the definition of antisense oligonucleotide within the invention also comprises RNA nucleotides. For example, antisense RNA may be used in the context of the present invention. The mechanism of action of antisense RNA and the current sate of the art of use of antisense tools is reviewed in Kumar *et al.* Microbiol Mol Biol Rev. 62, p. 1415-1434, 1998.

Generally, antisense RNA may be encoded by an expression vector. Alternatively, a viral vector, e.g., a retroviral vector may be used. Examples for retroviral vectors are Adenovirus-derived vectors and Adenovirus-associated virus-derived vectors. Examples of Adenovirus vectors include the Ad CMV BA AP vector, which comprises a nuclear targeted LacZ epitope, the Ad CMVLacZ vector, which comprises the CMV early enhancer and promoter and the LacZ gene, the Ad deltaE1 vector, and the like. Such vectors are available e.g., from The Michigan University Vector Core project.

The AChE mRNA region targeted by the antisense mRNA generally corresponds to the regions discussed further above. Preferably, a region comprising exons 1, 2, 3, 4, I4, and/or 5 is targeted. More preferably, a

region comprising exons E2 and/or E3 is used. Most preferably, a region comprising the ATG translation start codon or a region located nearby is targeted.

The length of the antisense RNA is preferably from about 9 to about 3,00 nucleotides, more preferably from about 20 to about 1,000 nucleotides, most preferably from about 50 to about 500 nucleotides.

In order to be effective, the antisense oligonucleotides of the invention must travel across cell membranes. In general, antisense oligonucleotides have the ability to cross cell membranes, apparently by uptake via specific receptors. As the antisense oligonucleotides are single-stranded molecules, they are to a degree hydrophobic, which enhances passive diffusion through membranes. Modifications may be introduced to an antisense oligonucleotide to improve its ability to cross membranes. For instance, the oligonucleotide molecule may be linked to a group comprising optionally partially unsaturated aliphatic hydrocarbon chain and one or more polar or charged groups such as carboxylic acid groups, ester groups, and alcohol groups. Alternatively, oligonucleotides may be linked to peptide structures, which are preferably membranotropic peptides. Such modified oligonucleotides penetrate membranes more easily, which is critical for their function and may therefore significantly enhance their activity. Palmityl-linked oligonucleotides have been described by Gerster *et al.*, Anal. Biochem. 262, p. 177-84, 1998. Geraniol-linked oligonucleotides have been described by Shoji *et al.*, J. Drug Target 5, p. 261-73, 1998. Oligonucleotides linked to peptides, e.g., membranotropic peptides, and their preparation have been described by Soukchareun *et al.*, Bioconjug. Chem. 9, p. 466-75, 1998. Modifications of antisense molecules or other drugs that target the molecule to certain cells and enhance uptake of the oligonucleotide by said cells are described by Wang, J. Controlled Release 53, p. 39-48, 1998.

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

The pharmaceutical compositions of the invention generally comprise 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.

Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the subject to be treated. While formulations include those suitable for rectal, nasal, preferred formulations are intended for oral or parenteral administration, including intramuscular, intradermal, subcutaneous and specifically intravenous administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy.

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

A preferred buffering agent is phosphate-buffered saline solution (PBS), which solution is also adjusted for osmolarity.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents and the like. The use of such media and agents for pharmaceutical active

substances is well known in the art. Except as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic composition is contemplated.

A preferred pharmaceutical formulation is one lacking a carrier. Such formulations are preferably used for administration by injection, including intravenous injection.

The compositions of the invention may be administered in a variety of ways. By way of non-limiting example, the composition may be delivered by injection intravenously, intramuscularly, or intraperitonealy. Intravenous administration, for example, is advantageous.

The pharmaceutical forms suitable for injection use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glyol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases,

it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above.

In the case of sterile powders for the preparation of the sterile injectable solutions, the preferred method of preparation are vacuum-drying and freeze drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As described in Example 10, the inventors have surprisingly found that oral administration of AS was effective as intravenous injection. Therefore as a preferred alternative embodiment, the composition of the invention may administrated orally.

For oral administration, the composition of the invention may be mixed with nutritive feed material or water supplies for the subject to be treated. It is contemplated however that the effective composition can either be mixed with the nutritive feed material or water or fed to the subject separately.

The preparation of pharmaceutical compositions is well known in the art

and has been described in many articles and textbooks, see e.g., Remington's Pharmaceutical Sciences, Gennaro A.R. ed., Mack Publishing Company, Easton, Pennsylvania, 1990, and especially pages 1521-1712 therein.

Additives may also be designed to enhance uptake of the antisense oligonucleotide across cell membranes. Such agents are generally agents that will enhance cellular uptake of double-stranded DNA molecules. For instance, certain lipid molecules have been developed for this purpose, including the transfection reagents DOTAP (Boehringer Mannheim), Lipofectin, Lipofectam, and Transfectam, which are available commercially. For a comparison of several of these reagents in enhancing antisense oligonucleotide uptake see e.g., Quattrone *et al.*, Biochemica 1, 25, 1995 and Capaccioli *et al.*, Biochem. Biophys. Res. Comm. 197, 818, 1993. The antisense oligonucleotide of the invention may also be enclosed within liposomes. The preparation and use of liposomes, e.g., using the above mentioned transfection reagents, is well known in the art. Other methods of obtaining liposomes include the use of Sendai virus or of other viruses. Examples of publications disclosing oligonucleotide transfer into cells using the liposome technique are e.g., Meyer *et al.*, J. Biol. Chem. 273, 15621-7, 1998, Kita and Saito, Int. J. Cancer 80, 553-8, 1999, Nakamura *et al.*, Gene Ther. 5, 1455-61, 1998, Abe et al., Antivir. Chem. Chemother. 9, 253-62, 1998, Soni *et al.*, Hepatology, 28, 1402-10, 1998, Bai *et al.*, Ann. Thorac. Surg. 66, 814-9, 1998, see also discussion in the same Journal p. 819-20, Bochot *et al.*, Pharm. Res. 15, 1364-9, 1998, Noguchi *et al.*, FEBS Lett. 433, 169-73, 1998, Yang *et al.*, Circ. Res. 83, 552-9, 1998, Kanamaru *et al.*, J. Drug Target. 5, 235-46, 1998, and references therein. The use of Lipofectin in liposome-mediated oligonucleotide uptake is described in Sugawa *et al.*, J. Neurooncol. 39, 237-44, 1998. The use of fusogenic cationic-lipid-reconstituted influenza-virus envelopes (cationic virosomes) is described in

Waelti *et al.*, Int. J. Cancer, 77, 728-33, 1998.

The above-mentioned catioinic or nonionic lipid agents not only serve to enhance uptake of oligonucleotides into cells, but also improve the stability of oligonucleotides that have been taken up by the cell.

The invention also relates to a method for the treatment or prevention of a progressive neuromuscular disorder or other disease involving excessive production of AChE I4 mRNA, comprising administering a pharmaceutical composition of the invention or of any of the preferred embodiments thereof, to a patient in need thereof.

Neuromuscular junctions from MG patients show morphological changes, in particular simplification of the postsynaptic membrane folding and end-plate potentials which fail to trigger action potential in some fibers (Drachman, *ibid.*, 1994). The diagnosis is routinely made by searching for the anti-AChR antibody titer, which is positive in 80% to 90% of patients (Drachman, *ibid*, 1994; Vincent *et al.*, *ibid.*, 1998).

However, the clinical characteristics of seronegative patients do not differ substantially from those of patients with high antibody titers. Moreover, lack of serum antibodies does not preclude the clinical phenotype of muscle fatigue or favorable response to therapies like thymectomy or plasmapheresis (Soliven *et al.*, Neurology 38, 514-7, 1988).

Actually, these patients have circulating AChR antibodies that are not detected by radio-immunoassay. Passive transfer of their immunoglobulin to mice caused the loss of junctional AChRs (Drachman D., *et al.*, Neurology 37, 214, 1987). Together with results from cultured-muscle-cell assay systems, these findings suggest that the antibodies may be directed at

epitopes not present in soluble AChR extracts or may present affinity too low for detection in soluble assay systems (Drachman D., *et al.*, *ibid.*, 1994).

Three other sensitive tests are being used to diagnose MG: Anticholinesterases test, the repetitive nerve stimulation (RNS) test and the single fiber electromyography (SFEMG) (Drachman D., *et al.*, *ibid.*, 1994; Oh *et al.*, Muscle Nerve 15, 720-4, 1992). Edrophonium (Tensilon) is commonly used for the anticholinesterase test, because of the rapid onset (30 seconds) and short duration (about 5 minutes) of its effect. This drug, which inhibits the enzyme acetylcholinesterase, allows ACh that is released from the nerve to interact repeatedly with the limited number of junctional AChRs, resulting in enhanced strength of myasthenic muscle functioning. If there is unequivocal improvement in an objectively weak muscle, the test is considered positive. In RNS, electric shock is delivered to the nerve and action potentials are recorded from surface electrodes over the muscle. A rapid reduction in the amplitude of the evoked muscle action potential (decremental response of 15 percent) is considered a positive response. SFEMG detects delayed or failed neuromuscular transmission in pairs of muscle fibers supplied by branches of a single nerve fiber. The anticholinesterase and RNS tests are the least sensitive and specific of the tests, perhaps indicating that excess AChE also occurs in other diseases. A positive assay for AChR antibodies is specific for Myasthenia gravis but detectable in only about 85 percent of all patients. SFEMG is sometimes helpful in difficult diagnostic situations but its specificity is limited, with positive findings in other disorders of nerves, muscles or neuromuscular junctions (Drachman, D., *ibid.*, 1994).

This may reflect electrophysiological failure due to AChE excess that is unrelated to anti-AChR antibodies, similar to the situation in AChE transgenic mice (Andres. C., *et al.*, Proc. Natl. Acad. Sci. USA 94, 8173-8,

1997). Altogether, this raises the need for alternative diagnostic assay, preferably using the serum phase.

The inventors have found high variability of AChE specific activity in serum samples from myasthenics as compared to healthy individuals, alterations that appeared to be non-associated to anti-nAChR antibody levels and hence independent of the intensity of the autoimmune response. In non-denaturing gel electrophoresis, rapidly migrating "readthrough" AChE was observed in higher levels in myasthenics as compared with control serum, suggesting generally intensified AChE gene expression in MG patients.

The inventors hypothesize that in the presence of the nAChRs as part of the cholinergic system it is possible to find alternative splicing mechanism which produces more of the AChE variants. In muscle, cholinergic insults like the autoimmune response will disturb the cholinergic balance and initiate a transcriptional process, producing selectively more AChE-R, the stress related variant. In the case of AChE accumulation in myasthenic serum, the inventors presume that the protein is secreted from endothelial cells of blood vessels.

Thus, as a further aspect the invention relates to a method for the diagnosis of a progressive neuromuscular disorder in a mammal. This method comprising the steps of: obtaining a sample from said mammal and detecting intensified expression of at least one of AChE variants in said sample.

In a preferred embodiment the method of the invention is intended for the diagnosis of a progressive neuromuscular disorder that involves muscle distortion, muscle re-innervation or neuromuscular junction (NMJ) abnormalities. The neuromuscular disorder may be selected from

Myasthenia gravis, Eaton-Lambert disease, Muscular dystrophy, amyotrophic lateral sclerosis, post-traumatic stress disorder (PTSD), multiple sclerosis, Dystonia, post-stroke sclerosis, post-injury muscle damage, excessive re-innervation, and post-exposure to AChE inhibitors. Preferably, said disorder is Myasthenia gravis.

In a specifically preferred embodiment, the sample used by the method of the invention is one of serum, bone marrow and cerebrospinal fluid sample. More preferably, the sample may be a serum sample.

Detection of the intensified expression of different AChE variants in the serum sample may be carried out by different techniques that are well known to the man of skill in the art. For example immunoassay, RT-PCR and nondenaturing activity gel electrophoresis.

In a preferred embodiment detection of intensified expression of different AChE variants in the serum sample may carried out by the nondenaturing activity gel electrophoresis, essentially as described by Kaufer *et al* (Kaufer. D., *et al.*, Nature 393, 373-7,1998).. Briefly, this technique comprising the steps of preparing a protein extract from said serum sample, separating the protein extract on polyacrylamide gel using nondenaturing conditions and detecting catalytically active AChE variants by the Karnovsky staining technique.

As described in the Examples, AChE accumulation has several important implications: It was shown that transgenic overexpression of neuronal AChE-S causes progressive neuromotor deterioration and simplification of the post-synaptic fold, similarly to myasthenic symptoms (Andres. C., *et al.*, Proc Natl Acad Sci U S A 94, 8173-8, 1997, Sternfeld. M., *et al.*, J Neurosci 18, 1240-9, 1998). Furthermore, as was mentioned herein before, excess of

AChE-R was found in the myasthenic serum, and both Transgenic overexpression of AChE-R and exposure to AChE inhibitors lead to formation of new neuromuscular junctions. As described in Example 3, transgenic mice overexpressing the AChE-R variant and mice injected with anti-AChE displayed increase in the density of NMJ per mm2 in the diaphragm muscle as compared to control mice or mice over expressing the AChE-S variant. Using the antisense oligonucleotide directed against AChE mRNA, this NMJ formation was prevented, suggesting involvement of AChE-R in this process. The findings that AChE-R accumulates in MG serum raise the possibility that AChE-R carries similar capacities in myasthenic patients.

Therefore, as a specifically preferred embodiment, detection of enhanced expression of the AChE "readthrough" variant may be a promising method for the diagnosis of MG disease.

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

Experimental Procedures

MG patients serum experiments

Serum samples:

A total of 28 serum samples were investigated. Of these, 18 were taken from myasthenic patients hospitalized in Bulgaria and 5 from myasthenic patients at the Haddassa hospital, Jerusalem, Israel. Serum was also taken from 5 healthy control individuals or controls with other diseases at the Haddassa hospital. Assays were performed on the supernatant removed from those serum samples.

AChE activity assays:

Serum samples from healthy and MG patients were analyzed for enzymatic activity with a standard colorimetric assay adapted to a 96-well microtiter plate (Seidman. S., *et al.*, Mol Cell Biol 15, 2993-3002, 1995). Assays were performed in 0.1M phosphate buffer (pH=7.4), 0.5mM dithio-bis-nitrobenzoic acid (DTNB) and 1mM acetylthiocholine substrate added after 30 min of incubation at room temperature. The substrate is

degraded to acetate and thiocholine that reacts with DTNB, producing a visible yellow color. 50mM Iso-OMPA (tetraisopropylpyrophosphoramide) was used to block BChE in the serum. Optical density at 405nm was monitored for 40 min at 1min intervals. Activity units were expressed in nmole substrate hydrolyzed/min/ml.

Sucrose gradient analysis:

Serum samples were applied to 10ml 5-20% linear sucrose density gradients and centrifuged 18 hrs at 4°C. Fractions were collected and assayed for AChE catalytic activity as described above. Sedimentation of alkaline phosphatase was also analyzed as a marker (S=6.1), using pNPP (p-Nitroophenyl phosphate tablet, Sigma) as a substrate and optical density was monitored at 405nm.

Radio-immunoassay of antibodies to acetylecholine receptor:

AChR extract prepared from muscles of male rats, was incubated with [125I]αBgT (7.5 mole/ml) for 2 hr at room temperature and this mixture was incubated with 0.5-2 µl of the test serum. After incubation for 2 hr at room temperature and overnight at 4°C, the putative antigen-antibody complex was precipitated, centrifuged and washed as described by Brenner *et al.* The amount of radioactivity in the precipitants was determined in an autogamma scintillation counter. The results were expressed as pmole of bat bound per ml of serum (Breeder. T., *et al.*, Sir J Ed Sci 14, 986-9, 1978).

Nondenaturing activity gel electrophoresis:

Electrophoresis was performed in 6% or 7.5% polyacrylamide gels. Serum samples including 120µg protein were prepared with 1% Triton x-100. Gels were run for 2 hr. at 4°C and were stained for several hours to detect catalytically active AChE using the Karnovsky staining technique, essentially as described by Kaufer *et al* (Kaufer. D., *et al.*, Nature 393,

373-7,1998).

The incubation medium in this procedure consists of acetylthiocholine as substrate, potassium ferricyanide, copper sulfate, and citrate to complex the cupric ions, thereby attenuating inhibition of cholinesterase and reducing the formation of copper ferricyanide.

The following major reaction sequence is postulated: enzymatically released thiocholine reduces the ferricyanide to ferrocyanide *in situ*. Cupric ions then react with the ferrocyanide to form, at sites of enzyme activity copper ferrocyanide, an insoluble russet-colored precipitate known as Hatchett's brown (Karnovsky and Roots, J. Histochem. Cytochem. 12, 219-221, 1964). In addition to this reaction, it has been suggested that a secondary reaction take place, identical to that occurring in indirect coloring thiocholinester procedures. That is, a cuprous thiocholine iodide complex is formed by the reduction of Cu^{2+} to Cu^{+} by enzymatically released thiocholine and the reaction of Cu^{+} with iodide-complexed thiocholine. A brownish red deposit of cuprous ferricyanide is then postulated. The solution contained 5 mg thiocholine substrate in a mixture of 6.5ml 0.1 M pH 6.0 phosphate buffer, 0.5ml 0.1M sodium citrate, 1.0 ml 30 mM cupric sulphate, 1.0ml water (double distilled) and 1.0 ml 5mM potassium ferricyanide.

Transgenic Animals

FVB/N control mice or transgenic mice expressing human AChE were used. (for references regarding transgenic and control mice, see Beeri *et al.*, Curr. Biol. 5, 1063-1071, 1995, Beeri *et al.*, J. Neurochem. 69, 2441-2451, 1997, Andres *et al.*, Proc. Natl. Acad. Sci. USA 94, 8173-78, 1997, Andres *et al.*, Neurochem. Int. 32, 449-456, 1998, Sternfeld *et al.*, J. Physiol. Paris 92, 249-55, 1998). Transgenic mice expressing functional hu AChE E6 mRNA ($AChE_S$) in spinal cord motoneurons are described in the above Andres et al.

Tissue preparations for experiments with transgenic mouse work

Mouse tongue preparation

FVB/N control mice or transgenic mice, aged postnatal day (P) 0, 15, 30 and 4 months (M) were sacrificed and their tongues removed into liquid nitrogen for PCR and biochemical analyses. For *in situ* hybridization and silver staining, $2mm^3$ cubes of tongue tissue were incubated in 3.7% formaldehyde overnight at room temperature and then paraffin embedded. Sections were cut (5μm) and placed on 3-aminopropyltriethoxysilane treated slides, dried at 37°C overnight and kept at 4°C until use. For cytochemistry, cryostat cuts (20μm) of fresh-frozen tongues were mounted on gelatin-coated slides, fixed for 1hr in 4% paraformaldehyde at 22°C, rinsed twice in phosphate buffered saline (PBS), air dried and kept at -20°C until use. Cytochemical AChE activity staining was as described (Andres *et al.*, Proc. Natl. Acad. Sci. USA 94, 8173-8178, 1997).

Mouse diaphragm preparation

Mouse diaphragmatic muscle was obtained in the following way, and used to investigate the relative contributions of nerve and muscle activity to the fatigue phenotype. FVB/N control mice or transgenic mice aged 3-5 months were sacrificed and their diaphragmatic muscles removed. Each diaphragm was fixed with a pin on one of its edges, and a transducer brought into contact with the opposite side. A train of 200 stimuli at a frequency of 66 Hz was given, and the contraction response was recorded. A complete recording consisted of a series of 15 such trains, each of which were administered at 10 second intervals.

Chronic DFP treatment

Mouse pups were housed with the dam in a light- and temperature-controlled room. Animals were injected subcutaneously once daily with

either 1.0 mg/Kg DFP (Aldrich Chemical Co., Milwaukee, WI) dissolved in corn-oil or with corn oil alone during the first 2 postnatal weeks. All pups were pretreated with 10 mg/kg (i.p.) atropine sulfate (Sigma Chemical Co., St. Louis, MO) in saline 15 min before injection. At P15, about 4 h after the last injection, pups were sacrificed and their tongues removed.

RT-PCR analysis

RNA from tongue samples was extracted by RNA Clean (PeqLAb, Heidelberg, Germany) according to manufacturer's instructions. RT-PCR reactions were performed as previously described (Kaufer *et al.*, Nature 393, 373-377, 1998), using a common upstream (+) primer and downstream (-) primers selective for each of the alternative AChE mRNA exons (Fig. 1A):

E3: 1361(+) {5'-CCGGGTCTATGCCTACATCTTTGAA-3'}, SEQ ID No. 14
E6: 1844(-){5'CACAGGTCTGAGCAGCGCTCCTGCTTG-CTA-3'}, SEQ ID No. 15
E5: 240(-) {5'-AAGGAAGAAGAGGAGGGA-CAGGGCTAAG-3'}, SEQ ID No. 16
I4: 74(-) {5'-TTGCCGCCTTGTGCATTCCCT-3'}, SEQ ID No. 17

To detect *c-Fos* mRNA the inventors used the primer pair 1604(+)/2306(-) as described by Friedman and coworkers in Nat. Med. 2, 1382-1385, 1996. PCR products sampled every third cycle from cycles 24-36 for the AChE and *c-Fos* mRNAs, and from cycles 18-24 for β-actin mRNA were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

In situ hybridization

2'-O-methylated, 5'-biotinylated cRNA (custom-made by Microsynth, 9436 Balgach, Switzerland) probes selectively recognizing alternative mouse (m) AChE mRNAs were detected using alkaline phosphatase-conjugated

streptavidin and ELF detection kit (Molecular Probes). The probes had the following sequences:

mI4 (SEQ ID No. 18): (-79)
5'-AACCCUUGCCGCCUUGUGCAUUCCCUGCUCCCCCCACUCCAUGCG
CCUAC-3'(-29);
mE6 (SEQ ID No. 19): (209)
5'-CCCCUAGUGGGAGGAAGUCGGGGAGGAGUGGACAGGGCCUGGGGG
CUCGG-3' (258); mE5 (SEQ ID No 20): (-249)
5'-GAGGAGGAAAAGGAAGAAGAGGAGGGACAGGGCUAAGUCCGGCCC
GGGC-3'(-200);

Paraffin embedded tongue sections were deparaffinized and dehydrated in a methanol/PBT (PBS, 0.1% Tween-20) series. Hybridization included preclearing in H_2O_2 (6% in PBT, 30 min), proteinase treatment, glycine wash and refixation (4% paraformaldehyde, 20 min), all essentially as described (Andres *et al.*, Proc. Natl. Acad. Sci. USA 94, 8173- 8178, 1997; Lev Lehman *et al.*, Blood 89, 3644-3653, 1997), except that 1% SDS was added to the hybridization buffer and to solution 1 and 0.1% Tween-20 was added to solution 2.

Neurites silver stain

Paraffin embedded tongue muscle sections were stained for neuronal fibers basically according to Gros-Bielschowski (see e.g., Slayter, Vet. Pathol. 35, 150-3, 1998) using silver nitrate (20%, 60 min at 37°C), distilled water washes and incubation in ammonium hydroxide-silver nitrate solution (60 min). Color was developed for 24 to 36 h. For fixation, slides were dipped in sodium thiosulfate (2 sec), washed in water and dehydrated.

Tissue culture experiments

All cells were grown in a fully humidified atmosphere at 37°C and 5% CO_2. All tissue culture reagents were purchased from Biological Industries (Beth Ha-Emek, Israel).

PC12 rat pheochromocytoma cells were grown in Dulbecco modified Eagle medium (DMEM) containing 8% fetal calf serum (FCS) and 8% horse serum (HS). For the induction of differentiation, 50 ng/ml NGF (Alomone, Jerusalem) was added to the medium with 1% FCS and 1% HS. Tissue culture plates or cover slips were coated with 10 μg/ml collagen type IV (Sigma, St. Louis, MO).

Human osteosarcoma Saos-2 cells were maintained in F-10 HAM growth media containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.12% bicarbonate (w/v) and were passaged once a week. For antisense manipulations, 20,000 cells/well, plated in 96-well flat bottomed plates were washed twice with phosphate-buffered saline (PBS) and maintained in RPMI-1640 medium without phenol-red and with a serum substitute, Biogro-1 (Beit-Haemek, Israel).

Umbilical cord blood (UCB) was collected, following informed consent of the parents and with the approval of the Sourasky Medical Center Ethics Committee. Following 1:1 (v/v) dilution in Iscove's modified Dulbecco medium (IMDM, Beit Haemek, Israel), mononuclear cells were separated using 3% gelatin (Difco, Detroit, MI) and Ficoll-Hypaque gradients (<1.077 g/ml; Pharmacia, Uppsala, Sweden). $CD34^+$ cells were enriched using CD34 immunoglobulin-coated magnetic beads (CD34 progenitor cell selection system, Dynal, Norway). $CD34^+$ cell analysis was performed by flow cytometry (Becton Dickinson Immunocytochemistry System Inc., San Jose,

CA). CD34-PE (Becton Dickinson Immunocytometry System, Inc.) and CD45-FITC (Dako, Glostrup, Denmark) monoclonal antibodies confirmed ca.90% $CD34^+$ purity. Liquid cultures were grown at a concentration of 10^5 cells mL in IMDM, containing 10% autologous plasma, 2 mM L-glutamine (Sigma Chemical Co., St Louis, MO), penicillin and streptomycin (100 mg/mL), amphotericin B (2×10^{-5} M) (Sigma Chemical Co.), and heparin (20 IU/mL, Gibco, Grand Island, NY).

For antisense oligonucleotide experiments, 2'-O-methylated or phosphorothioated 15- and 20-mer antisense (AS) oligonucleotides, were targeted against the common sequence domain in human AChE mRNA and were used as detailed herein. Oligonucleotides targeted against butyrylcholinesterase (BChE) mRNA served for control (Grisaru et al., Mol Cell Biol 19, 788-795, 1999). All ODNs were chemically modified at their three 3' terminal nucleotides by either 2'-O-methyl groups or phosphorothioate groups, for protection against nuclease degradation, whereas the rest of the chain remained phosphodiester.

For in situ hybridization (as in example 3, 5'-digoxigenin labeled or 5'-biotinylated, fully 2'-O-methylated AChE cRNA probes complementary to 3'-alternative human ACHE exons were employed:
S ("synaptic" form):
(5402) SEQ ID No. 21.
5'-CCGGGGGACGUCGGGGUGGGGUGGGGAUGGGCAGAGUCUGGGGC
UCGUCU-3' (5352);
R ("readthrough" form):
(4397) SEQ ID No. 22.
5'-CUAGGGGGAGAAGAGAGGGGUUACACUGGCGG
GCUCCCACUCCCCUCCUC-3'
(4349)

Numbers denote nucleotide positions in the GenBank deposited sequence (accession no. M55040). Digoxignin labeling was employed in PC12 cells, which contain endogenous biotin. Other cell types were hybridized with biotinylated probes.

For ODN labeling studies, 5'-digoxigenin labeled ODN probes were incubated with cultured cells at the noted concentrations. Their cellular distribution was determined by decorating intracellular ODNs using anti-digoxigenin antibodies and a second alkaline phosphatase conjugated antibody, followed by fast red production by the conjugated enzyme and confocal microscopy localization of the corresponding red aggregates.

The laser scanning confocal microscope (LCSM) consisted of a Biorad MRC-1024 scanhead coupled to a Zeiss Axiovert 135M inverted microscope equipped with a 63 x 1104 objective.

Fast-Red precipitate procedures. Fast-Red fluorescence was excited with the 488 nm line of an argon-ion laser, while the emission was detected after passing through a 580df32 (580 nm ± 16 nm) interference filter. Confocal quantification of *in situ* hybridization data takes into consideration the cell volumes involved, reveals the sites of accumulation of specific mRNA transcripts, and enables comparison between mRNA and protein levels with high resolution and precision. Sections were acquired every 0.42 μm, and a maximum value projection was created from these sections. A commercially available software package (Image-Pro 3.0; Media Cybernetics, Silver Spring, MD, USA) was used to mask the cells. Following masking, the background signal was eliminated and the cells were then unmasked. Only pixels in blocks of five or more with a pre-defined threshold value fluorescence intensity were counted. Inter-experiment standardization was achieved by selecting the fluorescence intensity threshold such that control

cells in all preparation have identical levels of labeling. The fluorescence intensity images were pseudocolored in order to visualize more easily focal sites of intensified AChEmRNA labeling.

Nuclear and cytoplasmic volume calculations were based on average values of nuclear and cell diameter as measured in the imaged sections. The measured cells and their nuclei were assumed spherical, and cytoplasmic volume was taken to be equivalent to cell volume minus nuclear volume.

Cytochemical staining for AChE catalytic activity was performed on non-fixed cells grown on glass slides as described previously by Grisaru *et al.*, Mol. Cell. Biol. 19, 788-795, 1999. Staining was performed in the presence of 10^{-5} M iso-OMPA (ISO) or BW284C51 (BW), selective inhibitors of BuChE and AChE, respectively. Staining times were 24, 48 and 72 hr for PC12, $Saos_2$ and $CD34^+$ cells.

Animals used for EAMG

Experimental autoimmune Myasthenia gravis (EAMG) was induced in female Lewis rats (120-180 g) purchased from Jackson Laboratory (Bar Harbor, ME), and housed in the animal facility at the Hebrew University Medical School in accordance with NIH guidelines. A total of 45 rats were induced in the study group: EAMG was induced in 35 and 10 naive rats served as controls.

Preparation of Torpedo AChR (T-AChR)

T-AChR was purified from the electric organ of *Torpedo california* by affinity chromatography on neurotoxin-Sepharose resin, as previously described (Brenner *et al.*, Tumor Biology 5, 263-274, 1984).

Induction of EAMG

EAMG was induced by immunizing the rats with 40μg of purified T-AChR emulsified in complete Freund's adjuvant supplemented with 1 m^2 mg of Mycobacterium tuberculosis H37Ra (Difco). The animals were injected subcutaneously in the hind footpads and a booster injection of the same amount was given after 30 days. A third injection was employed in animals that did not develop the disease after the second injection. Animals were weighed and inspected weekly during the first month and daily after the booster immunization, for evaluation of muscle weakness.

Electromyographic evaluation

Animals (e.g., rats or mice) anesthetized by intraperitoneal (i.p.) injection of pentobarbital (Nembutal, Sanofi, France) were immobilized and subjected to repetitive sciatic nerve stimulation using a pair of concentric needle electrodes at 3 and 5 Hz. Baseline muscle action potentials were recorded by a concentric needle electrode placed in the gastrocnemius muscle, following a train of repetitive nerve stimulation's at supramaximal intensity. Decrement values (percent difference between the first and fifth muscle action potential amplitudes) were determined in two sets of repetitive nerve stimulation's for each animal. A reduction of 7% or more was considered indicative of neuromuscular transmission dysfunction.

AS administration

AS injection- Venous access was gained through the right jugular vein for sampling of blood and injection of AS, other drugs or vehicle (PBS).

For oral administration, a special gastric needle was used. Following administration of AS, a train of repetitive stimuli at 3 and 5 Hz was performed at 1, 5, 24, 48 and 72 hrs post injection (some rats were also tested 7 and 14 days post AS injection).

Treatment with AChE inhibitors neostigmine or pyridostigmine (mestinon)
The effect of AS treatment was compared with that of the approved AChE inhibitor neostigmine (75 μg/kg) or mestinon (1mg/kg). Following neostigmine injection, a train of repetitive stimuli at 3 and 5 Hz was performed at 15, 30, 60, 90, 120, 150 min. and 5 hrs. For mestinon treatment following injection or oral administration, a train of repetitive stimuli at 3 Hz was performed at 30, 60, 300 min and 24h.

Exercise training on the treadmill, EAMG rats and control rats were prodded to run on an electrically powered treadmill, at a moderate effort of 25 meter/min. until visibly fatigued. The amount of time the rats were able to run was recorded before and after AS or mestinon treatment.

Determination of anti-AChR antibodies
Serum samples were assayed by direct radioimmunoassay, using ^{125}I-labeled Bungarotoxin bound to T-AChR, as described by Brenner *et al.* (1984).

Example 1

Involvement of AChE variants in the cholinergic imbalance, and the use of anti-AChE antibodies as a surrogate marker for MG.

Myasthenia gravis (MG) is an antibody-mediated autoimmune attack directed against the nicotinic acetylcholine receptor, nAChR at neuromuscular junctions. The primary characteristics of MG include decreases in the density of nAChRs at neuromuscular junctions, morphological changes at the postsynaptic membrane and a failure to trigger action potentials in part of the fibers. Current diagnosis is based on anti-AChR antibody titers, which are positive in 80% to 90% of patients. However, seronegative patients display similar clinical symptoms and

response to therapies. Three other tests are being used to diagnose MG, yet none of them selectively detects the autoimmune response against the acetylcholine receptor. It was recently found that the anticholinesterase carbamate pyridostigmine, the first line of drugs used for MG treatment, induces a feedback response leading to excess AChE accumulation. Moreover, both transgenic overexpression of AChE in neuromuscular junctions and anticholinesterase exposure, inducing such overexpression, were shown to cause progressive neuromuscular dysfunctioning in laboratory animals. Based on these findings, the inventors hypothesized that AChE may serve as a potential modulator of MG and hence a promising surrogate marker in MG diagnosis. To this end, a study aimed at characterizing the AChE variants in blood samples from MG patients was initiated.

Alterations in AChE catalytic activity levels in serum from MG patients

To explore the possibility that either the endothelial cells lining blood vessels or the muscle tissue from MG patients secret catalytically active AChE to the blood, The inventors first measured enzyme specific activities in serum samples from MG patients as compared with healthy individuals or patients with other diseases (together considered as non-MG controls). Activity values were calculated as a percentage from a mean value of 5 of those controls, based on previous reports demonstrating average population differences of 15-20% variation in red blood cells AChE activity (Shapira, M., *et al.*, Hum. Mol. Genet. 9, 1273-1281, 2000). In general, activity mean values were similar in myasthenic and control sera, but the myasthenic sera displayed considerably higher variability in serum AChE activities as compared to controls (Fig. 2). Six out of the 23 tested MG serum samples displayed an increase in AChE catalytic activity (>115%) as compared to healthy controls and patients with irrelevant diseases (Table 1). Seven

samples displayed significant decreases in serum AChE specific activities (< 85%). Interestingly, no correlation was found between AChE activity levels and the levels of anti-acetylcholine receptor (AchR) antibodies detected in the sera (Table 1 and Fig. 3).

Table 1
AChE catalytic activity levels and anti-AchR antibodies of MG serum VS control serum

MG serum Sample	AChE activity (% of Ctrl)	AchR Ab (pmol/ml)
1	67.5	13.2
2	67.9	11.5
3	69.2	48.3
4	73.4	38.7
5	79.8	38.2
6	84.3	58.9
7	84.5	36.3
8	88.4	5.3
9	89.9	0.3
10	90.1	32.0
11	93.0	17.5
12	95.8	31.5
13	98.1	0.1
14	98.9	24.4
15	100.1	3.5
16	104.4	26.4
17	112.9	31.0
18	122.4	7.7
19	129.9	12.6
20	132.7	15.4
21	144.3	29.8
22	170.2	28.9
23	187.1	0.1
Average±SD	103.7±31.5	22.2±16.2

Presented are AChE catalytic activity levels (percent of control) of myasthenic serum and anti-AChR antibodies detected in those serum

sample (pmole/ml). presents of activity units were calculated from the mean value based on 5 non-MG control samples and its value was 165 nmole substrate hyd/min/ml (100%).

The high variability of AChE activity in MG serum may be explained by the different drugs used for treatment and the time duration between drug administration and the blood test. Patients with lower activities may represent Short-term muscle AChE inhibition if they were treated with anti-AChE just before their blood was sampled and in contrast, patients with higher activities may possibly represent long-term up-regulation of muscle or endothelial AChE secreted to the blood.

If AChE increases are solely dependent of the disease process, one should expect that serum AChE specific activities will increase as function of the severity of the disease or as function of the intensity of the autoimmune response. If, however, the individual's capacity to respond to the stress insults involved is the cause, no correlation should necessarily exist between AChE activity levels and the levels of anti-acetylcholine receptor (AChR) antibodies detected in the sera. This indeed was the apparent situation. (Table 1 and Fig. 3). These findings suggest that the alterations in AChE catalytic activity are independent of the intensity of the autoimmune response. Moreover, they potentially indicate causal involvement of AChE overexpression with the disease process itself.

Catalytically active rapidly migrating AChE-R protein accumulates in myasthenic sera.

The Ellman's assay for AChE activity is not a variant specific measurement. To distinguish between the different AChE variants, the AChE catalytic activity was measured on non-denaturing activity gels, which are

separating between the different AChE variants. The gels were stained for catalytically active AChE using the Karnovsky staining technique (see Materials and Methods). A slowly migrating band, with apparent size of 440 KDa band was common to all serum samples. This complex band most likely reflects tetramers of the BuChE G4 isoform, which is the primary cholinesterase present in normal serum and AChE-S G4 tetramers if overexpression indeed exists. Additional bands with rapid migration appeared only in MG sera (Figs. 4A and B). These most likely represent lighter isoforms, possibly AChE-E dimers, released from red blood cells and AChE-R monomers. Fig. 4B shows lane profiles of two representative control and MG samples, which together emphasize the different migrations on the gel. The rapidly migrating bands appeared also in plasma from transgenic mice overexpressing the AChE-R variant, but not in plasma from FVB/N control mice (Fig. 4A), suggesting that at least part of those bands constitute active AChE-R monomers, accumulating in myasthenic serum.

AChE-S G4 tetramers accumulate in MG serum.

Serum samples from MG patients and non-MG controls were applied to 5-20% linear sucrose density gradients. Fractions were assayed for AChE catalytic activity in the presence of the BuChE inhibitor iso-OMPA. Fig. 5 shows AChE activity levels (mOD/min) as function of the fraction in the gradient. Both gradients contained fractions migrating as globular dimers of AChE (G2), most likely AChE-E, as well as a heavier form (S=8.3). Both also contained monomers of AChE G1, or fragments thereof. (S=4.3 in fraction 32 of non-MG sample and 3.7 in fraction 31 of MG serum sample). The lighter of these isoforms sediments similarly to E4 AChE monomers, whereas the heavier co-sediments with AChE-R monomers. MG serum samples displayed higher activity in the tetrameric fraction as compared to high activity in the dimeric fraction of non-MG controls. Because AChE-R can only form monomers, this suggested additional AChE-S accumulation in

myasthenic serum. Interestingly, the non-MG samples were not taken from healthy individuals but from patients with other irrelevant diseases. This implies that the gradient profile shown in Fig. 5 reflects a pattern that is highly specific for MG.

These findings point at the causal involvement of AChE variants in the cholinergic imbalance initiated by autoimmune responses and suggest the use of isoform-specific anti-AChE antibodies to detect AChE secreted to the blood, which may serve as a surrogate marker for MG.

Example 2

Chronic cholinesterase blockade and transgenic overexpression of synaptic neuronal AChE similarly promote *c-Fos* and *readthrough* ACHE mRNA increments in muscle

Reverse-transcription (RT) PCR was performed on mRNA extracted from tongue of two-week-old (P15) mice injected daily, from birth, with 1 mg/kg DFP. This dose of DFP blocked approximately 80% of muscle AChE activity, but did not elicit overt symptoms of cholinergic poisoning. In parallel, RT-PCR was performed on tongue RNA from transgenic mice overexpressing $AChE_S$ in spinal cord motoneurons (Andres *et al.*, Proc. Natl. Acad. Sci. USA 94, 8173-8178, 1997). Fig. 6B shows PCR reactions sampled every third cycle from cycle 24 for AChE and c-fos, and from cycle 18 for β-actin. One of 4 experiments for each mRNA, using different RNA preparations is presented. Bar graphs represent the relative band intensity for each mRNA in the presented PCR images as determined by densitometric analysis. Quantification was performed at cycle 33 for AChE and c-fos mRNAs and at cycle 24 for β-actin mRNA, within the exponential phase of product accumulation. The semi-quantitative analyses performed with primers detecting mouse mRNAs encoding either *c-fos* or $AChE_R$ revealed over 2-fold elevated levels of both transcripts following either

chronic inhibition or congenital overexpression of AChE (Fig. 6B). In contrast, neither endogenous $AChE_S$- nor $AChE_H$-mRNA levels were detectably affected by either DFP or transgenic AChE. β-actin mRNA was similar among all groups, indicating equal starting amounts of RNA in all reactions (Fig. 6B). These data indicated that both chronic neuronal AChE-S overproduction and suppressed catalytic activity of AChE stimulate selective *de novo* transcription of $AChE_R$ in muscle.

Readthrough AChE mRNA accumulates in muscle and epithelium under cholinesterase blockade and transgenic AChE overexpression

To determine the localization of induced $AChE_R$ mRNA in tongue, high resolution, fluorescent *in situ* hybridization was employed. Fluorescent hybridization signals obtained using an $AChE_S$ mRNA-specific probe as described further above in "Experimental Procedures" exhibited similar moderate intensities and bandwidth in epithelium of newborn control and transgenic mice (Figs. 7 a,b). $AChE_H$ mRNA was detected at low levels in the epithelium of both control and transgenic mice (not shown). $AChE_R$ mRNA was barely detectable in sections from control mice, consistent with the PCR data. In contrast, pronounced expression of $AChE_R$ mRNA was observed in tongue epithelium of newborn transgenic mice (Figs. 7 c,d). With DFP, no significant differences were observed in the expression of $AChE_S$ mRNA in treated- versus vehicle-injected mice (Figs. 7 e,f). However, fifteen-day-old DFP-treated, but not control FVB/N pups exhibited high levels of $AChE_R$ mRNA across the entire width of the tongue epithelium, extending into the muscle (Figs. 7 g,h). In general, hybridization with the $AChE_S$ mRNA probe gave moderate and somewhat punctuated staining, consistent with the localization of this message around junctional nuclei (Jasmin *et al.*, Neuron 11, 467-477, 1993). In contrast, staining with the $AChE_R$ mRNA-specific probe yielded a more diffuse staining pattern

especially following DFP treatment, suggesting extrajunctional synthesis. These data demonstrated that both transgenic overexpression and inhibitor-mediated blockade of AChE promote a specific induction of $AChE_R$ mRNA that takes place in both muscle and epidermis.

Example 3

Transgenic mice display delocalized overexpression of catalytically active AChE in muscle.

High-salt/detergent extracts of tongue revealed a developmental increase in AChE enzyme activity in control mice (12±2 nmol substrate hydrolyzed/min/mg protein at P7 versus 24±4 at P15; N=8). Two-fold increased levels of catalytically active AChE were observed in tongue homogenates from transgenic over control mice at P7, but only 25% at P15. These findings suggested that adjustments in the feedback response take place over time and/or during development. To localize overexpressed AChE in the tongue, the inventors performed cytohistochemical staining for AChE on sections from one-month-old control and transgenic mice. In control mice, activity staining was pale except for intense, highly localized staining observed at motor endplates (Figs. 8 A,B). In contrast, transgenic mice displayed overall darker staining of the muscle layers, particularly near the submucosal epithelium (Fig. 8C). In transgenic mice, intense staining was observed along muscle fibers, not restricted to endplate regions (Fig. 8D). The relative contributions of endogenous $AChE_R$ and transgenic $AChE_S$ isoforms to this overexpression pattern were not discernible in this experiment. Nevertheless, the pronounced overexpression of $AChE_R$ mRNA in epithelium of transgenic mice contrasted the accumulation of catalytically active protein primarily in the muscle.

Transgenic and DFP-induced AChE excesses associated with similar muscle pathologies

To determine if the ability of cholinesterase inhibition to promote deterioration of muscle is correlated with overexpressed $AChE_R$, gross morphological features of tongue muscle were studied. Fig. 9 shows tongue muscle from P15 untreated control (Ctrl), chronic-DFP-treated control (DFP), or untreated AChE transgenic (Tg) mice stained with hematoxyllin-eosin and evaluated for gross morphological features. Muscle tissue of control mice displayed a high degree of organization, with fibers closely aligned in regular parallel arrays. In control mice injected daily with DFP, and in untreated transgenic mice, the corresponding tissues appeared distorted, with apparently atrophic, disorganized muscle fibers. Upper panels present low magnification photomicrographs that include epithelial and muscle layers. Lower panels display high magnification of the muscle layer alone. At higher magnification, severely atrophic, vacuolated muscles could be observed in both experimental systems. Therefore, without wishing to be bound by theory, it is the inventors' belief that *readthrough* AChE exerts morphogenic activities on muscles.

Both transgenic and anti-AChE insults cause excessive muscle reinnervation

Bielshovskey-based silver staining was used to characterize the distribution of motor axons in tongue muscle from DFP-treated FVB/N and untreated AChE transgenic mice as compared to untreated control mice. Large bundles of neurites (Fig. 10A) were observed in muscles of all three groups in similar numbers, suggesting similar primary nerve input to the tongue in all mice. However, both DFP-injected and AChE transgenic mice displayed 2-fold increases in the number of small (<200 μm^2), apparently unbundled neurites (24.2 ±3.7 and 21.5±1.5 vs. 11.3±0.6 per mm^2, respectively) (Figs. 10B,C) as compared to both untreated and vehicle-injected controls ($p<0.05$).

These results indicated axon branching in $AChE_R$ overexpressing muscles, and suggested a process of denervation-reinnervation in muscles of both DFP-treated and ACHE transgenic mice.

Transgenic AChE and DFP promote proliferation of motor endplates

The observed increase in small silver-stained neurites in transgenic and DFP-treated mice suggested that these cholinergic insults may be associated with reinnervation processes and the formation of new endplates. To examine this possibility, histochemical staining for catalytically active AChE was used to mark motor endplates in intact diaphragms from adult control and transgenic mice, and from control mice one month following a course of four daily i.p. injections of DFP (1mg/kg). Diaphragm motor endplates were visualized by staining for catalytically active AChE in control mice versus DFP-treated mice and AChE-transgenic mice. Mice were four month old at the start of the experiments.

In addition, AChE enzymatic activity was determined in high salt/detergent extracts and calculated as nanomoles acetylcholine hydrolized per min and per mg tissue, as described in Seidman *et al.*, Mol. Cell. Biol. 15, 2993-3002, 1995.

Fig. 11 shows micrographs of diaphragm motor endplates of control mice (A), control mice treated with DFP (B), and AChE-transgenic mice (C). In diaphragms from DFP-treated mice, a 2-fold increase over controls in the number of endplates per mm^2 as measured along the length of the innervating nerve was observed. Moreover, these endplates appeared smaller than endplates from control mice. In diaphragms from transgenic mice, a 50% increase in the density of motor endplates compared to controls

could be demonstrated. In muscles from both DFP-treated and transgenic mice abnormal, elongated endplates were often observed. Together, these data indicate that both DFP and transgenic overexpression of AChE in motoneurons lead to feedback overproduction of *readthrough* AChE in mammalian muscle that is spatio-temporally correlated with muscle degeneration, reinnervation and NMJ genesis.

The experiments demonstrate that in both DFP-treated and AChE-transgenic mice, the number of small synapses is increased, as compared to control mice. This indicates proliferation of motor endplates under these conditions.

The number of synapses and AChE activity appeared to be positively correlated when control mice and DFP-mice are compared. The ratio between synapse numbers and AChE activity is within the linear correlation for DFP-treated mice that received AS3 (Fig. 11D). Thus, when AS3 was administered to DFP-treated mice, it reduced AChE catalytic activity and synapse number by about the same degree (Fig. 11D, "AS3+DFP"). In contrast, when AS3 was administered to control mice that had not received DFP, it clearly lowered their synapse number, but slightly increased enzymatic activity of AChE (Fig. 11D, "AS3 alone").

Example 4

Muscle from transgenic mice displays increased fatigue

Diaphragmatic muscle was obtained from AChE transgenic and control mice (age 3-5 months) and prepared as described above in "Experimental procedures" (see Fig. 12E). Stimulation of the nerve resulted in a complex picture (Fig. 12A). For the first few contractions, the level of force generated remained constant. Thereafter, fatigue was observed during the contraction phase, which in turn led to fatigue in the initial force achieved in the

following contraction. This phenomenon continued until a constant low-level contraction force was achieved. It was found that the fatigue level carried over from one contraction to the next is less pronounced in the control subjects than in the muscle taken from the transgenic animals.

The differences between AChE-S TG and control with respect to the initial force achieved following nerve stimulation, from the 1st to the 10th contraction are shown in Fig. 12A. The results plotted in this graph are mean values obtained from three animals from each genotype, with five sessions being recorded for each muscle. While the control muscle demonstrated a 35% decrease in normalized force, the corresponding parameter for the transgenic muscle decreased by approximately 60% (Fig. 12B).

Muscle fatigue following muscle stimulation was also investigated. The graph of Fig. 12C demonstrates that in control muscle, the fatigue developed between contractions, with no fatigue being observed during the contractions themselves. In contrast, intra-contraction fatigue was observed when the corresponding measurements were made on the transgenic muscle preparation (Fig. 12C, 12D). When all of the above results are taken together, it is seen that about 25% of the fatigue in control nerve, and about 40% of the fatigue in transgenic muscle originates from muscle-related factors alone (Fig. 12D). This implies that a major part of the fatigue in nerve stimulation contraction is due to muscle mechanisms. It therefore appears that elevation of neuronal AChE levels is able to affect endogenous muscle contraction mechanisms.

In conclusion, both muscle and nerve properties contribute towards the greater fatigue seen in the muscles of mice having the transgenic phenotype.

Example 5

Neuromuscular transmission under conditions of high release probability

To characterize the cellular behavior of neuromuscular transmission under physiological conditions a cut-muscle preparation was used, in which muscle action potential generation was prevented, while allowing high probability of release conditions.

Six trains of 15 stimuli at 50 Hz were given at 10-sec intervals. Amplitudes were measured and normalized to the first stimulus in the train. The decrease in amplitude was somewhat more pronounced in the transgenic animal (Fig. 13B) than in the control preparation (Fig. 13A). Each plot represents the averaged results from 4 muscle preparations.

It may be seen from Fig. 13C that control mouse fibers show a 25% fatigue level, while those fibers taken from transgenic animals demonstrated a 33% decrease. This difference between the transgenic and control groups is small, but statistically significant.

Example 6

Release characteristics of transgenic neuromuscular junctions

The possibility that transgenic neuromuscular junctions exhibit different release characteristics from those present in control tissue, and whether such differences occur at pre- or post-synaptic sites, was investigated. The experimental model chosen for this study was one in which there was a low probability of release, in view of the low calcium concentrations and paralysis of muscle contraction.

It was predicted that one of the immediate consequences of higher AChE

levels would be to reduce the measured quantal size, especially with respect to the half decay time. However, it was found that quantal responses were not significantly different, neither in amplitude (1.004mV control and 1.05mV transgenic- Fig. 14A) nor in rise time (0.89ms control and 0.97ms transgenic (Fig. 14B). In contrast, it was surprisingly found that transgenic half decay time (1.29ms control and 1.69ms transgenic - Fig. 14C) was significantly larger than in the control group. Even if the amplitude and rise time behavior are interpreted as being part of a compensatory mechanism in response to excess amounts of the enzyme, the longer decay time is still quite surprising. In order to investigate whether changes in cholinergic neurotransmission are involved AChE catalytic activity was blocked with a high concentration (10mM) of the AChE blocker, physostigmine. Following treatment, the quantal size increased for all three parameters, and for both groups of animals. A significantly larger increase in amplitude was seen in the transgenic animal group (190%) than that of the controls (150%). Rise time and decay time of both control and transgenic mice changed in a similar manner (for rise time 141% and 132%, for decay time 175% and 162%, respectively). The distinct amplitude response may reflect a compensation process, showing that the "real" amplitude is higher in the transgenic mice. Since the decay time is already longer in transgenic animals than in control and this relation does not change after blocking AChE, it is suggested that this phenomenon results from post-synaptic changes.

Inhibition of the catalytic activity of AChE therefore reversed compensation in the post-synaptic response under conditions of AChE overexpression, thus resulting in a reversion of the single quanta to their standard dimensions.

Example 7

Pre-synaptic behavior of transgenic neuromuscular junctions

In order to analyze pre-synaptic behavior, the quantal content of NMJs was measured in transgenic tissue (Fig. 15). Two fold higher values of both quantal content (Fig. 15A) and frequency (Fig. 15B) of spontaneous release were recorded in transgenic NMJs than in control tissue. (Quantal content 0.13 in transgenic, as compared with 0.07 in control; frequency of spontaneous release 0.295/sec in Transgenic, compared with 0.152/sec in control). The higher probability of release in the transgenic animals probably reflects a direct compensatory response to the hypo-cholinergic condition at the synapse.

Treatment with physostigmine caused a surprising 40% decrease in the quantal content in transgenic mice, while the control animals were not significantly affected. Even more surprising was that the frequency of spontaneous release was not affected by physostigmine treatment. This implies that physostigmine, either via AChE blockade or by some other intrinsic property, preferentially inhibits evoked release in transgenic mice. Therefore, treatment with physostigmine, and possibly other anti-acetylcholinesterases, may be expected to be effective in individuals with excess AChE.

Example 8

The AS-ODN treatment window

It has now been found that the preferred treatment window of AS-ODN agents in the context of the present invention is located at lower doses of oligonucleotide that previously assumed. Detailed dose-response curves of protected AS-ODNs (including 3 3'-terminal phosphorothioate internucleotidic linkage groups for protection against nucleolytic degradation) were performed in PC12, Saos2 and $CD34^+$ cells. Different

concentrations of phosphorothioate- and 2'-O-methyl-modified AS1-AChE (AS_1) and AS-BCHE (ASB) ODNs were added to PC12 cells once daily for 48 h after treatment with NGF for 24 h. AChE activity was measured as described elsewhere (Grifman *et al.*, PNAS 95, 13935-13940, 1998). AS-BCHE is described in Ehrlich *et al.*, Antisense Res Dev. 4, 173-83, 1994. This oligonucleotide targeted against butyrylcholinesterase (BCHE) is used herein as a control. BCHE is an AChE-related gene, whose protein product is 50% identical to AChE. The sequence homology at the mRNA level, however, is very low. Most AChE inhibitors also inhibit BCHE, but antisense ODNs to AChE mRNA would not hybridize to BCHE mRNA.

Results are expressed as percent of activity in control cells ± standard evaluation of the mean (SEM) for 5-9 triplicate measurements for each point (Fig. 16). Both PS- and Me- capped AS1 suppressed AChE catalytic activity in nerve-growth factor (NGF)-stimulated PC12 cells to about 50% of controls in a dose and sequence-dependent manner within 48 hrs. AS1-Me displayed significant effects at an extracellular concentration of 0.05 nM, and maximum suppression of AChE activity was observed at 2 nM AS-ODN (Fig. 16A). In contrast, 2nM AS1-PS was required to exert prominent suppression of AChE activity in PC12 cells. Sequence-specificity was shown by the insignificant effects exerted by either PS or Me-protected AS-ODN targeting butyrylcholinesterase (BChE) (Fig. 16). Curiously, increasing the concentrations of AS1 above 2 nM reduced AS-ODN efficacy to the extent that PC12 cells exposed to 1 μM AS1, -PS or -Me, displayed only minor loss of AChE activity. Above 10 nM AS1, PC12 cells treated with either ODN regained most of their AChE activity with approximately similar dose dependence. Therefore, 2'-O-methyl modification afforded a considerably wider effective window in the ODN concentration between 0.05-10 nM for AChE suppression than phosphorothioate modification.

AChE suppression reflects modulated AChEmRNA levels under different AS-ODNs

To confirm the observation that increasing concentrations of oligonucleotide neutralize the potency of AS-ODN targeted to AChE mRNA, the inventors used *in situ* hybridization (ISH) to label AChE-S mRNA in cells treated with AS3-Me for 48 hours. Generally, AChE-S mRNA appeared concentrated in one or two cytoplasmic areas close to the nuclear margin, possibly in the Golgi apparatus where AChE would be translated, folded and rendered catalytically active. To quantify ISH signals detecting cytoplasmic AChE mRNA, confocal microscopy and computerized image-analysis were applied. This analysis revealed a "U-shaped" dose-response curve for AS3 that closely paralleled the curve obtained with AS1 using AChE catalytic activity as the measure of antisense potency (compare Fig. 16B to 16A). Dose-dependent increases and decreases in AChE-R mRNA levels were matched by corresponding changes in cell-associated AChE activity as determined by cytohistochemical staining (Fig 16B, inset). Nevertheless, AChE mRNA appeared more sensitive than AChE protein to the higher AS-ODN concentration.

To further explore whether similar curves would be observed with different AS-AChE ODNs, AS3 was used in a similar experiment as described above. Like AS1, AS3 is a 20-mer sequence targeted towards the common domain in AChE mRNA. AS3 has a GC content of 40%, with a predicted folding energy of –4.7 kcal/Mole, compared to 65% in AS1 with no predicted folded structure. This tentatively predicts more stable hybrids between AS1 and AChE mRNA. Hybridization *in situ* was used to label the major AChE-S-mRNA transcripts within AS3-treated cells. PC12 cells were treated with increasing doses of AS_3 as detailed above. *In situ* hybridization with an exon 6-selective AChE cRNA probe was employed to detect AChE-SmRNA in the treated cells (Fig. 17A). Color coding (right hand side

scale) was used to highlight cytoplasmic sites with greater AChE-SmRNA concentrations. Cytochemical staining of catalytically active AChE displayed parallel changes in the cell-associated enzyme molecules (Fig. 17B).

For analysis of sequence-specific changes in AChE-SmRNA levels of PC12 cells, AChE-SmRNA levels were quantified by confocal analysis as described (Grisaru *et al.*, Mol Cell Biol 19, 788-795, 1999). Fig. 17B, shows average values ± SEM of AChE-SmRNA levels in aggregates/cell (left scale) or percent of untreated cells (right scale).

Graphical presentation of these data yielded an essentially parallel curve to that observed for enzyme activity measurements in AS1-treated PC12 cells. Also, cytochemical labeling of AChE activity revealed less intense staining in cells with lower AChE-SmRNA labeling, suggesting that AS3-mediated destruction of AChE-S-mRNA was the primary cause for the suppressed AChE activity in both AS1 and AS3-treated PC12 cells (Fig 17). The data also show that the increased AChE activity under higher AS3 doses reflected parallel increases in AChE-S-mRNA.

These findings demonstrated effective suppression of AChE expression by two independent AS-ODNs targeted to AChE mRNA, demonstrated the enhanced potency and wide effective window conferred by 2'-O-Me modification of AChE AS-ODNs, and raised the question of why increasing concentrations of AS-ODN fail to elicit reliable suppression of AChE activity in these cells.

AS-ODN treatment stimulates nuclear accumulation of AChE mRNA

Control PC12 cell never displayed intranuclear staining following ISH. However, at low concentrations of AS3-Me (0.2-2 nM), cells often displayed

one or two small intra-nuclear foci of ISH signal (Fig. 18A,B). At higher concentrations of 100-1000 nM AS3-Me, intense nuclear staining of AChE-S mRNA was commonly observed in up to 2 highly-defined focal points.

These observations suggested a direct association between focal nuclear staining and the number of transcription sites, and hinted at *de novo* accumulation of hn-AChE mRNA in AS-ODN treated cells. To further examine AChE mRNA levels in antisense-treated PC12 cells, A reverse transcription and polymerase chain reaction (RT-PCR) on RNA extracted from cultures treated with AS3 for 48 hours following NGF-stimulated differentiation, were performed. RT-PCR revealed both a short mRNA representing mature 3'-spliced AChE-S mRNA, and a longer, unspliced transcript presumably representing heteronuclear (hn) AChE in differentiating PC12 cells (Fig. 18B, inset). Both the long and the short transcript were noted to be about 2-fold higher in cells treated with 100 nM as compared to 10 nM AS3. The nuclear accumulation of AChE mRNA under AS-ODN treatment suggests that the reduced potency of >2nM AChE AS-ODN in PC12 cells reflects feedback upregulation of the ACHE gene in response to highly effective primary antisense effects, and/or to the presence of excess AS-ODN or their degradation products. Moreover, it advances the notion that antisense, like pharmacological, inhibition of AChE below a certain threshold initiates feedback upregulation of AChE expression in multiple cell types. Together, these data point to the need to strike a balance in antisense studies between antisense effects and dose-dependent compensatory host cell responses.

Cellular uptake of AS-ODN

The role of AS-ODN uptake in host cell feedback responses was evaluated using 5'-digoxigenin (DIG)-tagged AS3-Me and the image analysis strategy of ISH. To estimate the sensitivity of the confocal approach, a 1 nM solution

of DIG-tagged AS-ODN was mixed into a PC12 cell extract, dried a 50 μl drop onto a microscope slide, and image analysis (N. Galyam, M.Sc. Thesis, 1999) was performed. In AS-ODN treated PC12 cells, this quantification procedure revealed concentration-dependent increases in intracellular oligonucleotide (Fig. 19A). However, increasing extracellular concentrations between 20 pM and 1 μM AS3-Me resulted in exceedingly limited increases in intracellular ODN (approximately 6-fold for the 5000-fold increased external concentration), both for AS1-Me and AS3-Me (Fig. 19A). The inventors calculated 10-100 AS-ODN molecules per 10 pL cell volume under 0.02 nM treatment conditions, close to the external concentration of oligonucleotide.

Therefore, 20pM external concentration of AS3 sufficed to introduce AS3 into PC12 cells at a concentration similar to that of its complementary AChE mRNA. From 0.02 nM, 10-fold increases in external ODN concentration resulted in non-proportional increases in intracellular concentration. Nevertheless, these data indicate that low external concentrations of AS-ODN elicited dose-dependent antisense effects in PC12 cells which are approximately proportional to internal AS-ODN concentration (compare to Fig. 16). At higher concentrations, however, non-specific cellular effects may be result from extracellular effects of oligonucleotides and/or their degradation products.

Excess AS-ODN mediates sequence-independent increases in cytoplasmic volume

In search of mechanism(s) controlling the increased AChE gene activity observed at AS-ODN concentrations above 2 nM, the inventors used the confocal microscope to measure cell volume. This analysis revealed >25% increases in cytoplasmic, but not nuclear, volume of PC12 cells exposed to AS-ODN over a range of 10-500 nM (Fig. 19B). The changes in cytoplasmic

volume were sequence independent, as they were common to cells treated with either antisense or inverse AChE AS-ODNs. Sustained nuclear volume under these conditions attested to the fact that no apoptotic changes took place. Therefore, increased cytoplasmic volume in this effective range of ODN concentrations may include a host cell response to the extra-and/or intracellular presence of > 2 nM ODNs and/or their degradation products.

AChE activity and mRNA levels were also measured in Saos-2 cells. Saos cells were treated for 24 hrs with various concentrations of ODN and then subjected to *in situ* hybridization with an exon 6-specific AChEcRNA probe. Quantification of AChE-SmRNA levels by confocal analysis was carried out as described (Grisaru *et al.*, Mol Cell Biol 19:788-795, 1999). Fig. 20 shows confocal projections of Saos cells subjected to *in situ* hybridization with an exon 6-specific AChEcRNA probe following 24 hr incubation with the noted concentrations of the noted ODNs (Fig. 20, top). The right hand side micrographs in Fig. 20 show the catalytically active AChE protein in single cells subjected to 2 nM of AS_1 (top row) or AS-BCHE (ASB, bottom row). Fig. 20, bottom shows AChE-SmRNA levels in $Saos_2$ cells treated with increasing concentrations of AS_1 or ASB as indicated.

AChE-SmRNA labeling in untreated Saos2 cells was approximately 2-fold higher than PC12 cells. However, AS1 reduced AChE-S-mRNA levels by 35% in Saos-2 cells at the range of 0.02 to 2 nM (Fig. 20 bottom), lower than the 0.05-10 nM range of PC12 cells. In contrast, AS-BChE displayed a shallow linear decrease of 15%, similar to the non-sequence dependent reduction observed in PC12 cells. Above 2 nM, AChE-S-mRNA levels increased in AS1-treated Saos-2 cells to reach 100% activity at 200 nM (data not shown). Thus, AS1 yielded a parallel, left-shifted curve of AChE-S-mRNA suppression in Saos-2 as compared to that of PC12 cells, yet with a width of effective-dose quite similar to that of PC12 cells (Fig. 20 and data

for PC 12 not shown).

To find out if the feedback response to antisense inhibition of ACHE gene expression is universal or whether it is limited to tumor cell lines, primary hematopoietic stem cells enriched by $CD34^+$ immunoreaction were also examined. These cells were exposed to increasing AS1 doses. The unique exons in their 3' alternative AChE-S or AChE-RmRNA transcripts were labeled by *in situ* hybridization, and the very low levels of these mRNAs were quantified by confocal microscopy. Fig. 21, top and bottom show confocal projections of primary $CD34^+$ cells subjected to 24 hr treatment with the indicated concentrations of AS_1 or ASB and then to *in situ* hybridization with AChEcRNA probes selective for exon 6, unique to AChE-SmRNA (top two rows). Fig. 21, center shows the levels of AChE-S and AChE-R mRNA in CD34+ cells treated with the indicated amounts of AS1 or ASB. Shown are average labeling intensities in aggregates x 10^{-3} per cell ± SEM for 20 cells in each point.

The experiments shown in Fig. 21 demonstrate that parallel doses of AS-BCHE had no apparent effect on either transcript. In contrast, AS1 treatment resulted in overlapping curves of the two AChEmRNAs. AChE-RmRNA levels were effectively reduced by 75% with 20 pM AS1 and returned to above control levels at 2 nM AS1, whereas AChE-SmRNA was only marginally reduced (by about 10%) at 2 nM and its level was fully recovered at 200 nM AS1 (not shown). The patterns for the two AChE mRNAs labeling were similar, with cytoplasmic labeling of both transcripts presenting an extranuclear ring. Nuclear labeling was only slight at the exceedingly low levels of AChE mRNA that are characteristic of hematopoietic stem cells.

Thus, the inventors have now found that the "readthrough" isoform of AChE

mRNA is particularly vulnerable to antisense-mediated nucleolytic degradation, compared to the other isoforms of the AChE mRNA, despite of the fact that the antisense oligonucleotides used are directed against the common coding domain of all AChE isoforms.

Without wishing to be bound by theory, it is the inventor's belief that the reason for this differential susceptibility to antisense-mediated nucleolytic degradation stems from the different length and nucleotide composition of the two AChE mRNA transcripts. E6AChEmRNA includes 219 bp at its 3'-non-translated region, with 66% G+C content. In contrast, I4AChEmRNA contains a 1,029 bp long 3' non-translated domain, with only 62% G+C (Ben Aziz et al., 1993). The inventors further believe that the distinction at the 3' domain - both because of reduced stacking forces between G-C pairs and the 5-fold longer region - confers general instability over the I4 transcript. This instability may be caused by reduced ability of the 3' domain of the I4 mRNA to interact with a 3' poly(A) binding protein.

Analysis of *in situ* hybridization results by confocal microscopy showed that AChE mRNA production was enhanced, implicating transcriptional activation of the AChE gene, when micromolar concentration amounts of AS-ODNs were used. Therefore, two measures should be taken when using AChE AS-ODNs. Firstly, the AS-ODN levels should be reduced to be within the treatment window. Secondly, the possible effect of the chemical protecting groups on the treatment window should be taken into account.

The above experiments suggest that AS-ODN-mediated destruction of mRNA may initiate, at least in certain cases, a cascade of feedback events initiating from post-transcriptional responses and proceeding with enhanced activation of the ACHE gene and/or facilitating retrieval of this target molecule within the host cell. Surprisingly, it was found that these complex

response patterns are common to a variety of mammalian cell types with highly variable AChEmRNA levels.

Assuming approximately $1x10^8$ cells/ml (or 10 pL per cell) and 1-100 molecules of a non-abundant target mRNA within a cell yields target concentrations ranging at 0.1-10 molecules per pL, or 0.2 to 10 pM. Therefore the effective windows of AS1 and AS3 concentrations of this invention demonstrate that these AS-ODNs should be present in treated cells in excess large enough to destroy all of the AChEmRNA molecules even in neurons, where AChE levels are highest. 2'-O-methyl AS1 suppresses AChE activity in PC12 cells at lower concentrations than its corresponding phosphorothioated ODN.

Further surprisingly is the recovery of AChE activity under higher ODN doses. This was sequence dependent, as it did not occur under AS-BChE. Moreover, it was largely independent of chemical protection, as it took place both with phosphorothioated and 2'-O-methylated AS1; this suggested that it involved a cellular response triggered by the AS-ODN at its extracellular and/or intracellular location.

Also surprising is the finding that catalytically active AChE molecules are localized in the nucleus under high AS-ODN concentrations. As AChE is produced in the cytoplasm, this implies that it is transported back into the nucleus in its fully active form. However, the AChE sequence does not include any obvious nuclear localization signal (NLS) motifs. This suggests the existence of a transporter protein(s) facilitating the nuclear transfer of AChE molecules.

The nuclear localization of AChE points at this protein as potentially involved in regulating ACHE gene expression.

In conclusion, it was found that when using AS-ODN concentrations between 50 pM and 20 nM, AChE activity was suppressed by 50 % and sequence-dependent increases in nuclear labeling suggest transcriptional activation of the target gene *ACHE*. When the AS-ODN concentration was between 10 nM and 50 nM, the cytoplasmic volume increased by 25 %, reflecting a sequence-dependent host cell response. Finally, at an AS-ODN concentration above 50 nM, penetrance of 2-O-methyl AS-ODNs into the cells plateaued and AChE activity was fully recovered.

Example 9

AS treatment stabilizes EMG and improves stamina in EAMG

AS3 stabilizes EMG in AChE transgenic mice

Transgenic mice overexpressing AChEs (AChE E6 mRNA) in spinal cord motoneurons are described above in "Experimental Procedures" and in prior work by the inventors (Andres *et al.*, Proc. Natl. Acad. Sci. USA 94, 8173-8178, 1997). Electromyography was carried out as described above under "Experimental Procedures", see also the above Andres et al., 1997. For measurement of electromyography response (EMG), Hp AChE E6 transgenic mice were used. The Hp (human promoter) AChE E6 transgenic mice carry a transgene comprising the human AChE E6 cDNA linked to the 600 upstream bp of the human ACHE promoter as a minimal promoter. This promoter has been shown to be approximately 20-fold weaker as compared to the CMV promoter in Xenopus (see Ben Aziz-Aloya *et al*, Proc. Natl. Acad. Sci. U.S.A., 90, 2471-2475, 1993). Adult (5 months) Hp AChE E6 transgenic mice were subjected to repetitive nerve stimulation (3 Hz), and the evoked response monitored at the gastrocnemius muscle. The ratio of fifth to first response (S5/S1) was determined and considered to represent a measure of muscle fatigue. The effect of the AChE antisense oligonucleotide

AS3 (see hereinabove) on the electromyographic response was evaluated.

The electromyograf recordingsin Fig. 22A, show significant decrement between the first and the fifth depolarizations of the muscle. Fig. 22B shows S5/S1 as a function of repeated measurements made on the noted time points with or without a single i.v. administration of 8 μg (320 μg/kg) AS3. EMG data of naive transgenic mice are represented by black circles, of AS3-treated transgenic mice by gray circles, and of control mice by triangles.

The naive transgenic mice present clearly lowered S5/S1 rations consistently under 100%, compared to control mice. Notably, AS3 administration elevates the S5/S1 ratio of transgenic mice, resulting in S5/S1 values statistically indistinguishable from those of control mice.
Fig. 22C shows variance data of S5/S1 measurements. Notably, the untreated control mice return to S5/S1 values close to 100% with minimal variance (Fig. 22B) between measurements. In contrast, naive transgenic mice display an age-dependent increase in intra-animal variability (Fig. 22C, Tg 5 wk –5 weeks, Tg 5 mo – 5 months). Administration of AS3 clearly reduces the variance in transgenic mice to variance levels comparable to that of control mice (Fig. 22C, Tg+AS3).

Thus, both the low S5/S1 values and the variability were largely corrected by treatment with AS3.

The response to RNS of 3 Hz of adult (5-month-old) AChE-S (E6) transgenic mice revealed a decremental response and instability, that was not found in young (5-8-week-old) mice (These findings are in accordance with previous demonstrations of age-related neuromuscular deterioration of Tg mice). Single i.v. administration of 320μg/kg AS-3 reversed and stabilized the

response of the adult mice (variance analysis).

Example 10

Outcome of AS treatment of EAMG

Clinical and electrophysiological examination of EAMG rats

The idea of using ODN therapy to treat MG has already been raised (Lee and Sullenger, Nature biotech. 15, 41-45, 1997). However, this approach involves the development of chemically protected RNA aptamers capable of blocking the nicotinic AChR autoantibodies. While innovative, this methodology has several drawbacks in that the RNA aptamers do not have the amplification power characteristic of the RNAseH-inducing antisense agent (Seidman *et al.*, Antisense and Nucleic Acid Drug Development, 9, 333-340, 1999) and the problem of potential feedback responses in MG is not addressed.

To determine whether the excess AChE production is contributes to the MG phenotype, the inventors initiated the *in vivo* use of antisense oligodeoxynucleotides (AS-ACHE-ODNs) suppressing AChE synthesis in rats with experimental autoimmune MG (EAMG).

Female Lewis rats (120-180 g) were immunized as described above in "experimental procedures". All the immunized rats had high titers of anti-n-AChR (nicotinic Acetylcholine Receptor) antibodies. Seventy-five percent of them exhibited clinical signs of muscle weakness, weight loss and a decremental EMG (electromyography) response (25% showed a typical myasthenic weakness after the first booster, 50% after the second booster and 75% after the third). Animals showing a reduction of 7% or more in the EMG decremental response were treated with either AChE AS oligonucleotides or neostigmine.

AChE-R accumulation in EAMG and suppression by AS treatment

In situ hybridization and AChE-R or ACh-R immunolabeling of paraffin embedded sections of front leg extensor muscle of severely ill EAMG or control Lewis rats. AChE-R immunolabeling of EAMG muscle revealed elevated levels of AChE mRNA and protein, as compared with those in control muscle. Almost complete suppression of this expression was found following a single i.v. treatment of 50μg/kg AS-3, (Fig. 23). However, AChE-S or AChR were not affected by AS-3 treatment.

Therapeutic effect of AS-3 treatment on the response to RNS in EAMG rats

In response to RNS of 3 and 5 Hz, a dose-dependent effect of AS-3 injection was elicited. The EAMG rats exhibited a baseline decremental response to RNS, ranging from 7 % to 47 % (mean 13 %). After intravenous injection of AS-3, the decremental effect in all rats rebound to the normal baseline level (100 %), (Fig. 24A), and was associated with marked clinical improvement.

The effect was also sequence-dependent, since comparable amounts of another AS-ODN with inverse sequence (INV-1) targeted at AChE did not improve the decremental response to RNS or the clinical outcome (Fig. 24B and 24C).

Figs. 25A and B show the decrement upon repetitive stimulation at 3 Hz in a rat with EAMG: (A) baseline response with 12.5% decrement, (B) response at 1 hr after injection of 100μg of AS-3, reversal of the decrement (there is an increment of 6.5%). Fig. 26 shows a representative curve of the percent difference between the first and fifth muscle action potential amplitudes (% ratio change from baseline) of an EAMG rat and a control rat following injection of 100μg of AS-3 (stimulation with 5 Hz). Table 2 summarizes the mean ± SD percent ratio change in repetitive stimulation of 5 EAMG and 5 controls stimulated with 5 Hz.

The beneficial effect of AS-3 treatment appears to be dose dependent, i.e. although smaller amounts of AS-3 (50, 10 and 2μg) elicited reversal of the myasthenic decremental response, the effect prevailed for shorter periods of 24 hrs. Therefore, it is assumed that amounts of AS-3 lower than 10μg/kg would suffice on a daily basis to sustain normal EMG. A similar beneficial effect on the clinical and electrophysiological responses in EAMG rats was observed following injection of 100μg of AS-1, but no effect was observed with AS-BCHE.

Table 2
The effect of AS-3 on the percentage ratio decremental response in EAMG and control rats

Time after AS-3 injection	EAMG	Control
Baseline	86.3 ± 4.9	101.0 ± 2.6
1 hr	103.6 ± 1.4	107.9 ± 5.1
5 hr	95.4 ± 7.3	102.9 ± 1.6
24 hr	105.3 ± 1.0	104.3 ± 3.8
48 hr	104.6 ± 4.3	103.8 ± 3.2
72 hr	102.5 ± 2.6	107.0 ± 5.9

The results are mean ± standard deviation of repetitive stimulation at 5 Hz.

Comparison of the effect of AS-AChE treatment with that of the AChE inhibitor pyridostigmine (Mestinon) or neostigmine on neuromuscular action in EAMG shows that pyridostigmine reversed the effect, starting 30 min after injection. However, the duration of the response did not last more than 2-4 hours. Following treatment with AS-AChE, improvement was observed 1 hour after injection and there was a normal response for a longer period, with a parallel clinical improvement (Fig.24B).

Antisense oligonucleotide improves stamina of EAMG rats

EAMG rats with varying severity of clinical symptoms and control Lewis rats were prodded to run on an electrically powered treadmill (25 meter/min.) until visibly fatigued. Control animals ran easily for about 20 min. without signs of fatigue. Myasthenic animals placed on the treadmill were able to run between 10 sec to 4.5 min (mean, 2 min 20 s ± 26 s) before any signs of fatigue appeared. The amount of time the rats were able to run was recorded before, and 25 hrs following, i.v. administration of 50 μg AS3 per rat. EAMG rats run considerably less time than controls (compare inset in Fig. 27, which shows running time of control rats, with "mild", "moderate", and "severe" phenotype of EAMG in Fig. 27). With AChE antisense oligonucleotide administration (black bars in Fig. 27), the running time is considerably enhanced. This effect is most pronounced in rats with moderate EMAG phenotype, but is also observed in mild and severe phenotype of EMAG. In addition, the effect is also demonstrated in healthy rats (Fig. 27, inset).

Comparison of the effect of AChE AS treatment with the effect of the AChE inhibitor neostigmine on neuromuscular action in EAMG.

Repair of the defect in neuromuscular transmission occurred following i.p. administration of neostigmine, improvement beginning 15 min after injection. The duration of the response was variable, but did not last more than 2 hrs. The results of neostigmine treatment in 4 EAMG rats are summarized in Table 3. Injection of similar doses of neostigmine into 4 normal controls caused mild fluctuations in EMG, ranging from 97% to 105%.

Table 3. The effect of neostigmine on the percentage ratio decremental response in EAMG rats

EAMG rats – time after injection	Mean % ratio
Baseline (0)	84.9 ±5.6
15 min	101.3 ± 5.9
30 min	103.7 ± 2.3
60 min	103.4 ± 0.8
90 min	101.2 ± 1.2
120 min	90.1 ± 10.3
150 min	87.6± 16.6
5 hr	87.3 ± 7.6

The results are mean±standard deviation of repetitive stimulation at 5 Hz.

Having now fully described the invention, it is to be understood that many variations in the treatment schedule, oligonucleotide sequence, oligonucleotide modification, the number and kind of the components of the pharmaceutical composition, or the like may be carried out, all within the scope of the invention, which is defined not by the above illustrative examples, but by the claims appended thereto.

Comparison between repeated oral versus i.v. AS-3 treatment

In addition, the response of EAMG animals to repeated injections of AS-3, and to oral administration of AS was evaluated. Oral administration of 50μg/kg AS-3 improved muscle activity and clinical EAMG symptoms similarly to the i.v. treatment (Figs. 24A and B). Comparison of oral administration of AS-3 with oral administration of mestinon or with INV-1 showed that 24 h following administration of mestinon or INV-1, no residual activity on RNS was observed, whereas, AS-mediated correction of the

response to RNS was still evident.

An equal improvement in muscle function was elicited by oral and i.v. administration of low dose (50μg/kg) AS-3. The rats were examined over a period of 5 days, showed no decremental responses to RNS of 3Hz during the tested period. Thus, oral administration of AS was as effective as i. v.

Claims:

1. A pharmaceutical composition for the treatment and/or prevention of a progressive neuromuscular disorder, for improving stamina and/or for use in decreasing muscle fatigue, comprising as active ingredient an antisense oligonucleotide targeted to AChE mRNA.

2. A pharmaceutical composition of claim 1 for the treatment and/or prevention of a progressive neuromuscular disorder, comprising as active ingredient an antisense oligonucleotide targeted to AChE mRNA.

3. The pharmaceutical composition of claim 1, characterized in that the antisense oligonucleotide causes preferential destruction of I4 AChE mRNA.

4. The pharmaceutical composition of claim 1, characterized in that the antisense oligonucleotide is a hammerhead or a hairpin ribozyme.

5. The pharmaceutical composition of claim 1, which is for a once daily use by a patient and comprises between about 0.1 and about 175 mg of active ingredient.

6. The pharmaceutical composition of claim 5, which is for once daily use by a patient and comprises between about 1 and about 70 mg of active ingredient.

7. The pharmaceutical composition of claim 6, which is for once a day use by a patient and comprises between about 15 and about 50 mg of active ingredient.

8. The pharmaceutical composition of claim 1, wherein the treatment and/or prevention comprises a dosage of active ingredient of about 0.01 to about 2.5 mg/kg.

9. The pharmaceutical composition of claim 8, wherein the treatment and/or prevention comprises a dosage of active ingredient of about 0.1 to about 1.5 mg/kg.

10. The pharmaceutical composition of claim 9, wherein the treatment and/or prevention comprises a dosage of active ingredient of about 0.25 to about 0.75 mg/kg.

11. The pharmaceutical composition of claim 2 for use in treating or preventing a progressive neuromuscular disorder, wherein said disorder is associated with an excess of AChE mRNA or protein.

12. The pharmaceutical composition of claim 11 for use in treating or preventing a progressive neuromuscular disorder, wherein said disorder is associated with an excess of I4 AChE mRNA.

13. The pharmaceutical composition of claim 12 for use in treating or preventing a progressive neuromuscular disorder, wherein said excess of I4 AChE mRNA is caused by enhanced transcription.

14. The pharmaceutical composition of claim 13 for use in treating or preventing a progressive neuromuscular disorder, wherein said enhanced transcription of I4 AChE mRNA involves the activity of an early immediate gene.

15. The pharmaceutical composition of claim 14 for use in treating or preventing a progressive neuromuscular disorder, wherein said enhanced transcription of I4 AChE mRNA involves the activity of the *fos* gene.

16. The pharmaceutical composition of claim 2 for use in treating or preventing a progressive neuromuscular disorder, wherein said disorder is associated with impairment of cholinergic transmission.

17. The pharmaceutical composition of claim 16 for use in treating or preventing a progressive neuromuscular disorder, wherein said disorder involves muscle distortion, muscle re-innervation or neuromuscular junction (NMJ) abnormalities.

18. The pharmaceutical composition of claim 17 for use in treating or preventing a progressive neuromuscular disorder, wherein said disorder involves changes in intracellular Ca^{++} levels.

19. The pharmaceutical composition of any one of claims 16-18 for use in treating or preventing a progressive neuromuscular disorder, wherein said disorder is selected from *Myasthenia gravis*, Eaton-Lambert disease, Muscular dystrophy, amyotrophic lateral sclerosis, post-traumatic stress disorder (PTSD), multiple sclerosis, Dystonia, post-stroke sclerosis, post-injury muscle damage, excessive re-innervation, and post-exposure to AChE inhibitors.

20. The pharmaceutical composition of claim 1 wherein the antisense oligonucleotide is targeted to one or more of exons 1, 2, 3, 4, 5, pseudointron I4, or a splice junction thereof.

21. The pharmaceutical composition of claim 20 wherein the antisense oligonucleotide is targeted to the E4-I4 splice junction.

22. The pharmaceutical composition of claim 20 wherein the antisense oligonucleotide is targeted to the common coding domain of AChE mRNA.

23. The pharmaceutical composition of claim 20 wherein the antisense oligonucleotide is targeted to one or more of AChE mRNA exons 2, 3, or 4, or a junction thereof.

24. The pharmaceutical composition of claim 20 wherein the antisense oligonucleotide is targeted to one or more of exon 2 or 3, and a splice junction of exons 2-3, 3-4, or 4-6.

25. The pharmaceutical composition of claim 24 wherein the antisense oligonucleotide is targeted to any one of exons 2, 3, or 4.

26. The pharmaceutical composition of claim 25 wherein the antisense oligonucleotide is targeted to exon 2 of AChE mRNA.

27. The pharmaceutical composition of claim 20 wherein the antisense oligonucleotide is targeted at a region near to or overlapping with the AChE ATG start codon.

28. The pharmaceutical composition of claim 20 wherein the antisense oligonucleotide sequence comprises a sequence selected from

5'-CTGCAATATTTTCTTGCACC-3' (AS3, SEQ ID No. 1));
5'-GGGAGAGGAGGAGGAAGAGG-3' (AS1 SEQ ID No. 2);

5' ACGCTTTCTTGAGGC (SEQ ID No. 3);
5' GGCACCCTGGGCAGC (SEQ ID No. 4);
5' GCCAGAGGAGGAGGAGAAGG (SEQ ID No. 5);
5' TAGCGTCTACCACCCCTGAC (SEQ ID No. 6);
5' CCACGTCCTCCTGCACCGTC (SEQ ID No. 7);
5' ATGAACTCGATCTCGTAGCC (SEQ ID No. 8);
5' TCTGTGTTATAGCCCAGCCC (SEQ ID No. 9);
5' GGCCTGTAACAGTTTATTT (SEQ ID No. 10);
5' AGAGGAGGGACAGGGCTAAG (SEQ ID No. 11);
5' TAGCATCCAACACTCCTGAC (SEQ ID No. 12); and
5' ATGAACTCGATTTCATAGCC (SEQ ID No. 13).

29. The pharmaceutical composition of claim 20 wherein the antisense oligonucleotide sequence comprises a sequence selected from

5'-CTGCAATATTTTCTTGCACC-3' (AS3, SEQ ID No. 1) and
5'-GGGAGAGGAGGAGGAAGAGG-3' (AS1, SEQ ID No. 2).

30. The pharmaceutical composition of any one of claims 1 and 20 to 29, wherein the antisense oligonucleotide is a modified oligonucleotide comprising partially unsaturated aliphatic hydrocarbon chain and one or more polar or charged groups including carboxylic acid groups, ester groups, and alcohol groups.

31. The pharmaceutical composition of claim 30, wherein at least one of the three 3' nucleotide bases is 2-O-methylated.

32. The pharmaceutical composition of any one of claims 1 and 20 to 29, wherein the antisense oligonucleotide is a modified oligonucleotide linked to peptide structures, including membranotropic peptides.

33. The pharmaceutical composition of any one of claims 30 to 32 wherein the antisense oligonucleotide is a modified oligonucleotide linked to one or more groups selected from Palmityl groups, Geraniol groups, and membranotropic peptides.

34. The pharmaceutical composition of claim 1 further comprising a lipid agent.

35. The pharmaceutical composition of claim 34 wherein the lipid agent is selected from Lipofectin, Lipofectam, Transfectam, DOTAP, liposomes and virosomes.

36. The pharmaceutical composition of any one of claims 1 to 10 and 20 to 35, wherein administration of said composition is by a single route of administration.

37. The pharmaceutical composition of any one of claims 1 to 10 and 20 to 35, wherein administration of said composition is by at least two different routes of administration.

38. The pharmaceutical composition of any one of claims 36 and 37 wherein said composition is administered by a route selected from intravenous, parenteral, transdermal, subcutaneous, intraperitonal, intramuscular, oral, sublingual and topical administration and any combinations thereof.

39. The pharmaceutical composition of claim 38 wherein said composition is administered intravenously.

40. The pharmaceutical composition of claim 38 wherein said composition

is administered orally.

41. The pharmaceutical composition as defined in any one of claims 1 to 10 or 20 to 40, for use in improving stamina in physical exercise or in decreasing muscle fatigue.

42. Use of an antisense oligonucleotide targeted to AChE mRNA in the preparation of a pharmaceutical composition according to any one of the preceding claims.

43. An antisense oligonucleotide targeted to AChE mRNA for use in treating or preventing a progressive neuromuscular disorder or for improving stamina in physical exercise or for use in decreasing muscle fatigue, substantially as described and illustrated and with particular reference to the Examples and Figures.

44. A method for the diagnosis of a progressive neuromuscular disorder in a mammal, comprising the steps of: obtaining a sample from said mammal and detecting intensified expression of at least one of AChE variants in said sample.

45. The method of claim 44, wherein said progressive neuromuscular disorder involves muscle distortion, muscle re-innervation or neuromuscular junction (NMJ) abnormalities.

46. The method of claim 45, wherein said disorder is selected from Myasthenia gravis, Eaton-Lambert disease, Muscular dystrophy, amyotrophic lateral sclerosis, post-traumatic stress disorder (PTSD), multiple sclerosis, Dystonia, post-stroke sclerosis, post-injury muscle damage, excessive re-innervation, and post-exposure to AChE

inhibitors.

47. The method of claim 46, wherein said disorder is Myasthenia gravis.

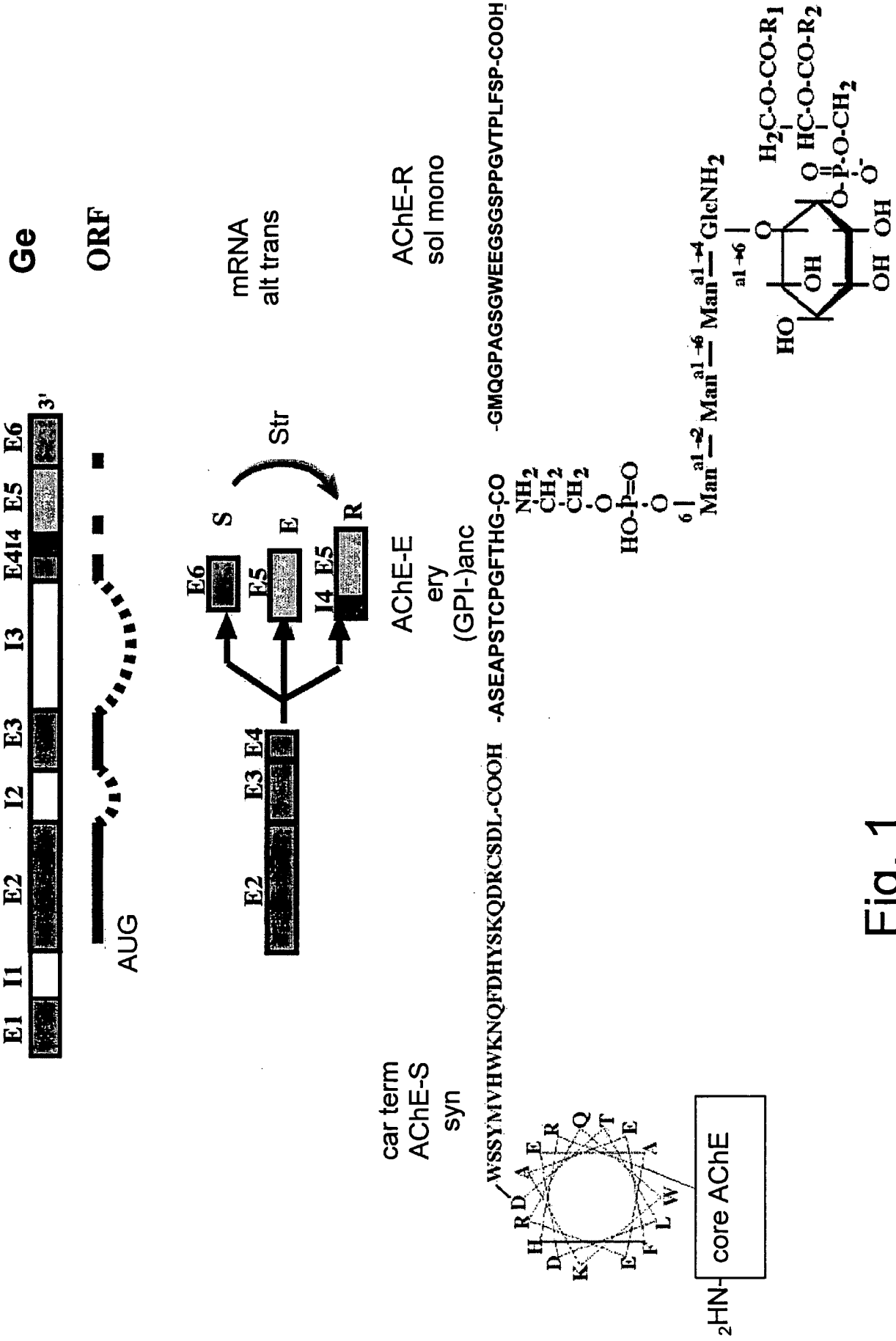
48. The method of any one claims 44 to 47, wherein said sample is one of serum, bone marrow and cerebrospinal fluid sample.

49. The method of claim 48, wherein said sample is serum.

50. The method of claim 44, wherein detecting the intensified expression of different AChE variants in said sample is by any one of immunoassay, RT-PCR and nondenaturing activity gel electrophoresis.

51. The method according to claim 50, wherein the nondenaturing activity gel electrophoresis comprises:
    a. preparing a protein extract from said serum sample;
    b. separating said protein extract on polyacrylamide gel using nondenaturing conditions; and
    c. detecting catalytically active AChE variants by the Karnovsky staining technique.

52. The method of claim 51, wherein the AChE variant is the AChE "readthrough" variant.


Ge
ORF
mRNA
alt trans
AChE-R
sol mono
AChE-E
ery
(GPI-)anc
car term
AChE-S
syn
E1 I1 E2 I2 E3 I3 E4I4 E5 E6 3'
AUG
E2 E3 E4
E6 E5 I4 E5
S Str E R
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NH2
CH2
CH2
O
HO-P=O
O
6
Man a1-2 Man a1-6 Man a1-4 GlcNH2
a1-6
O
HO
OH
OH OH
H2C-O-CO-R1
HC-O-CO-R2
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O
2HN-
core AChE

Fig. 1

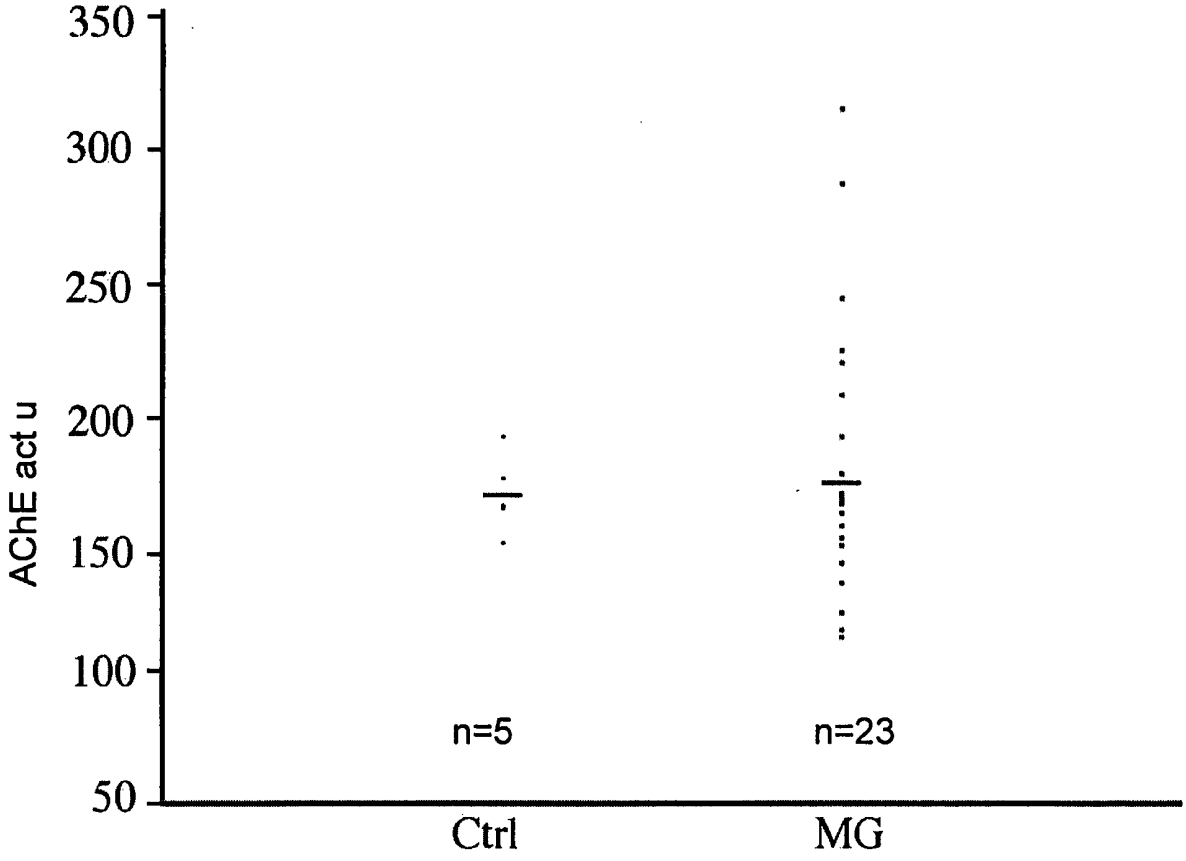
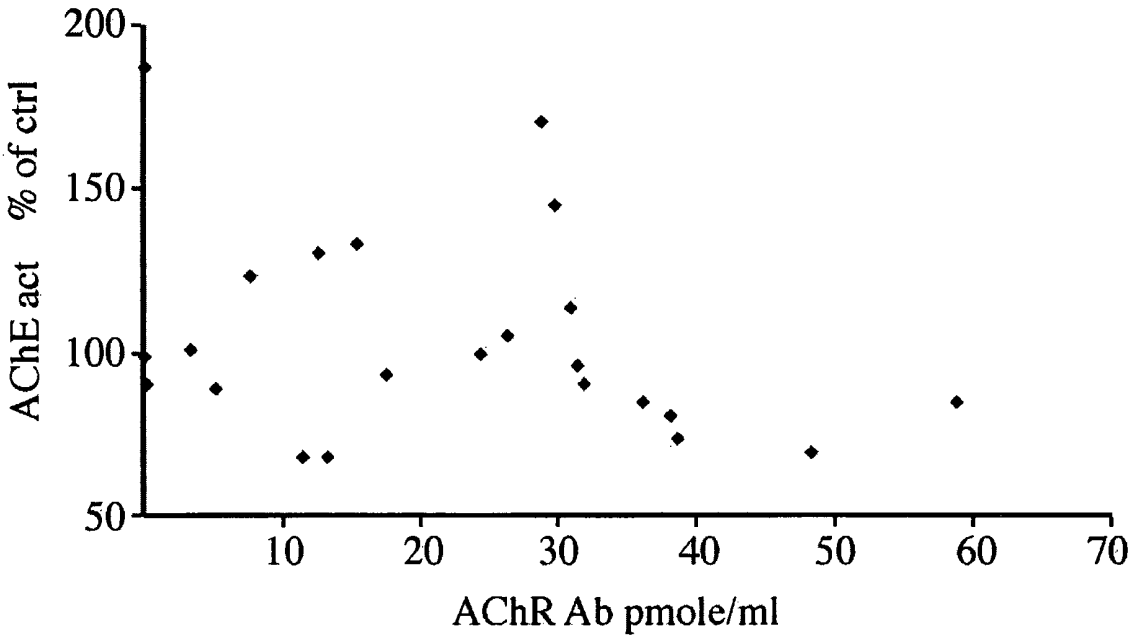


Fig. 2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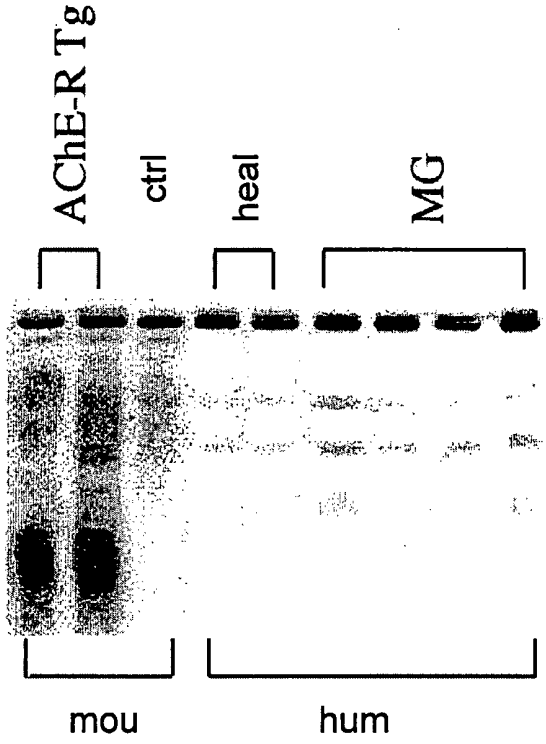
150
100
50
AChE act % of ctrl
10
20
30
40
50
60
70
AChR Ab pmole/ml

Fig. 3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ctrl
heal
MG
mou
hum

Fig. 4A

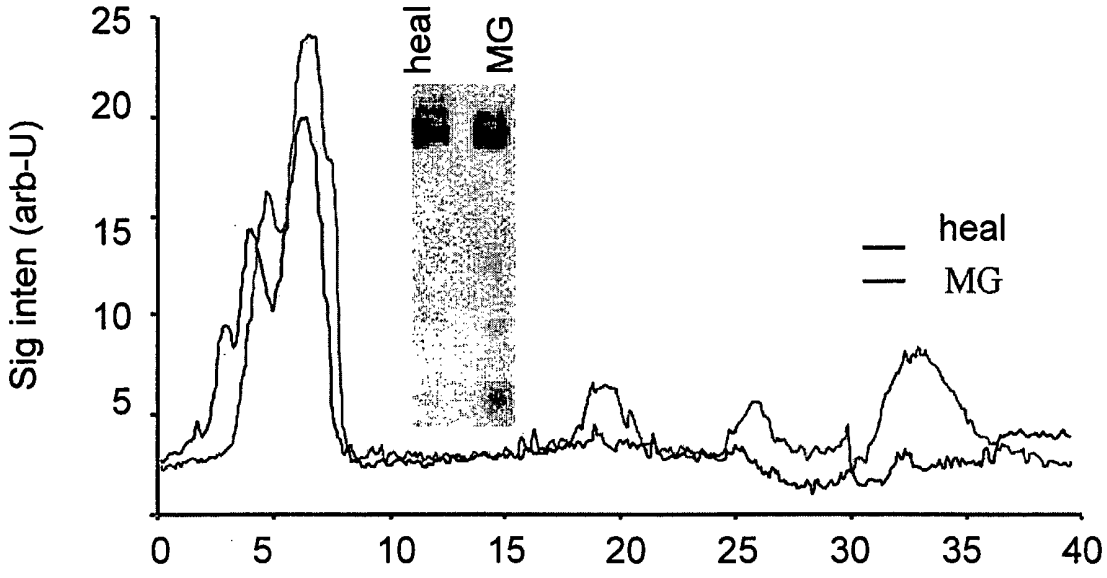

Sig inten (arb-U)
25
20
15
10
5
0
5
10
15
20
25
30
35
40
heal
MG
heal
MG

Fig. 4B

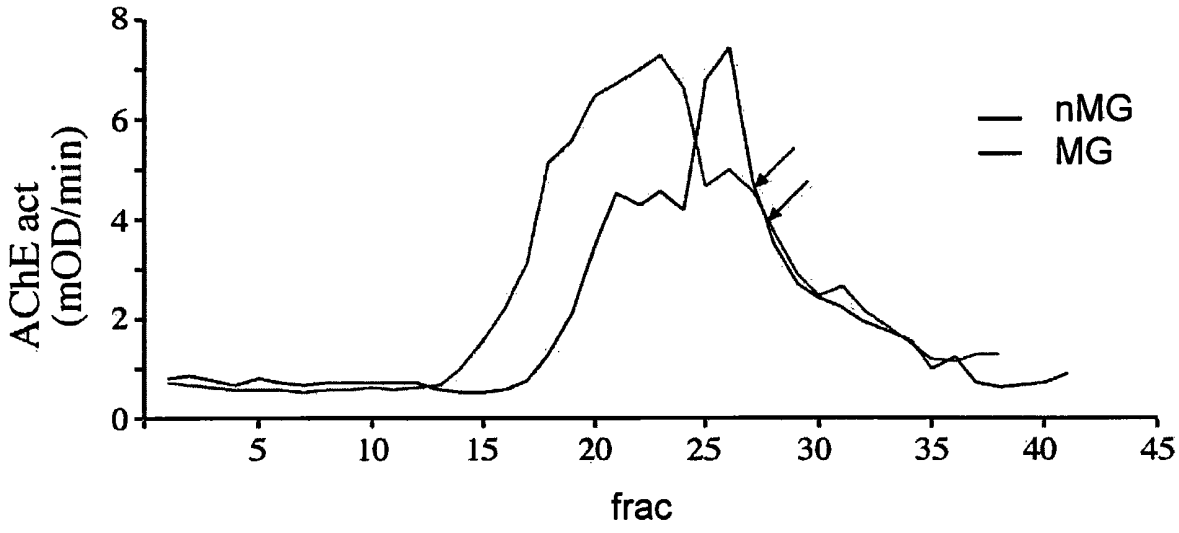


Fig. 5

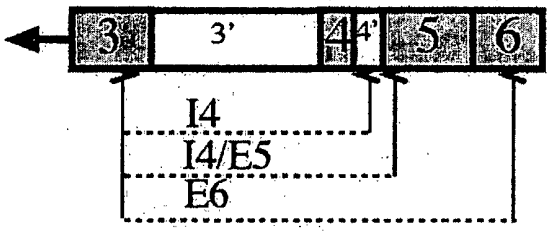


Fig. 6A

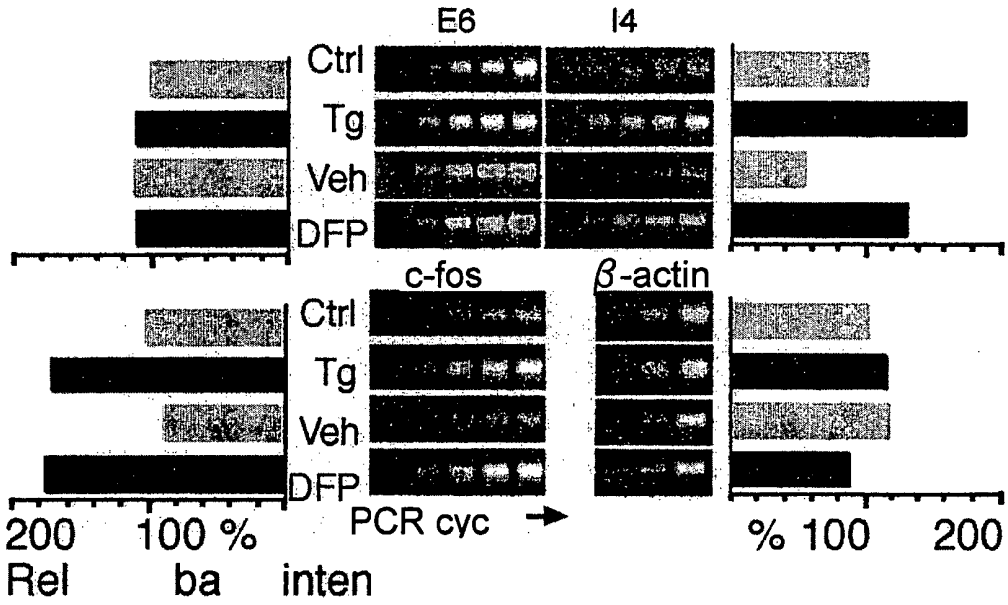


Fig. 6B

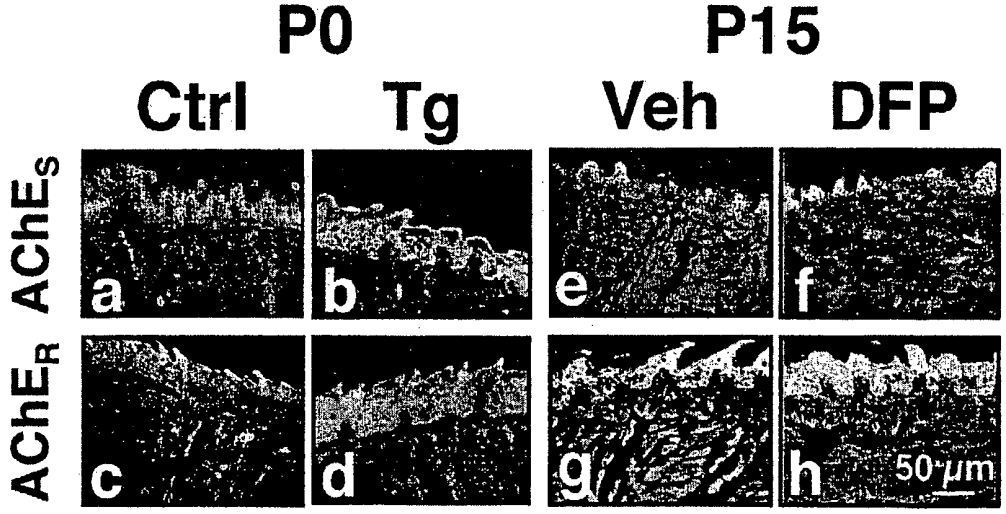


Fig. 7

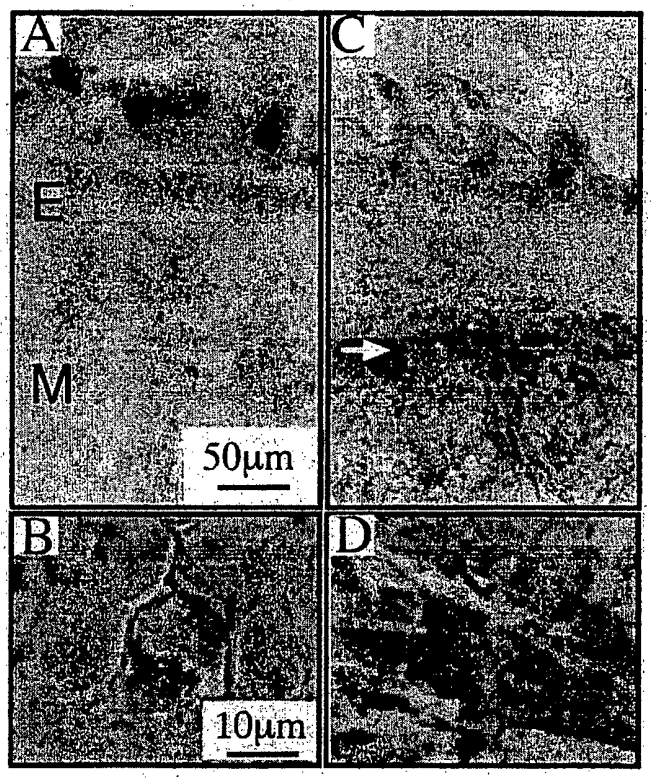


Fig. 8

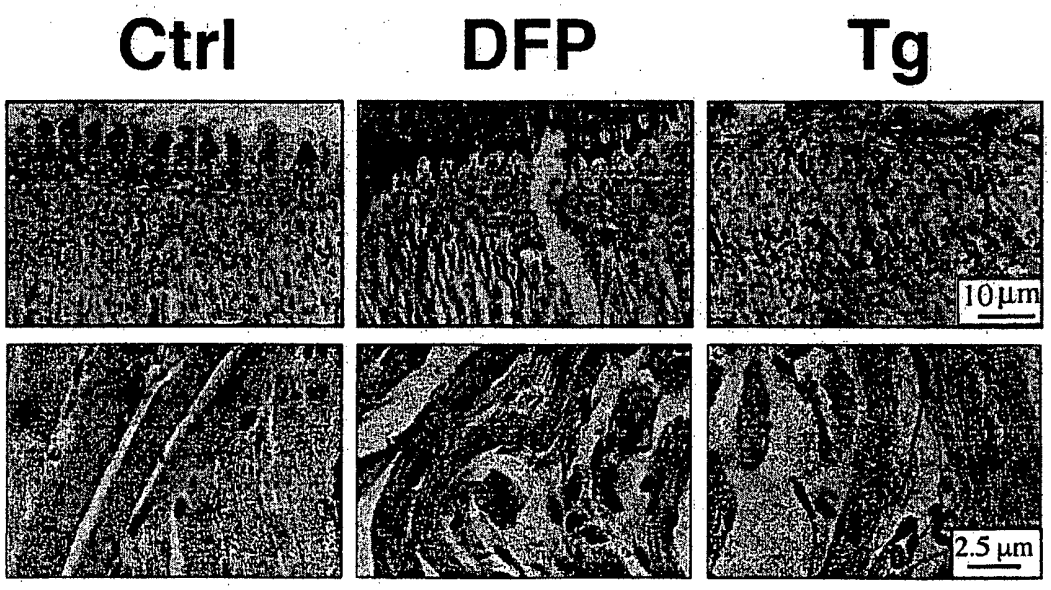


Fig. 9

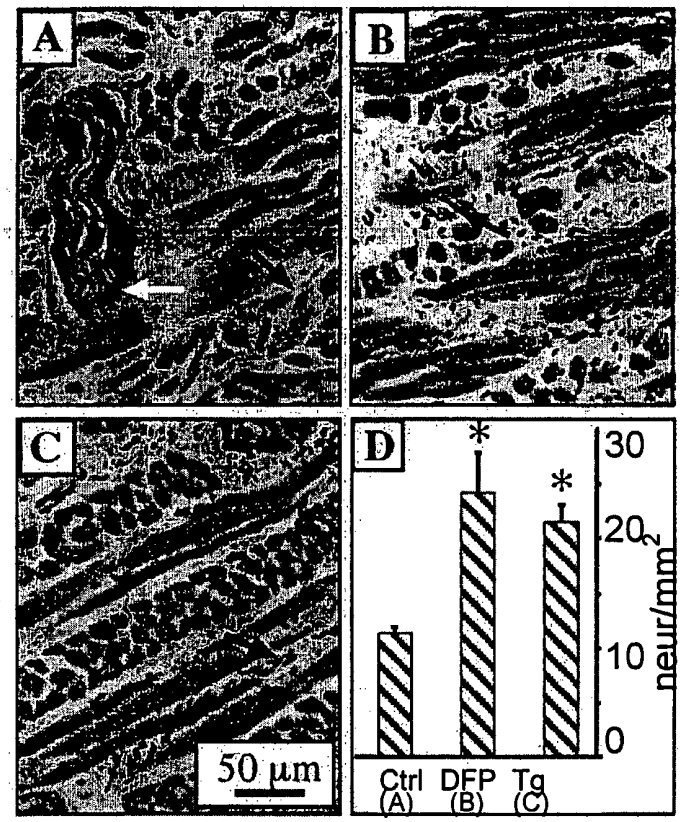
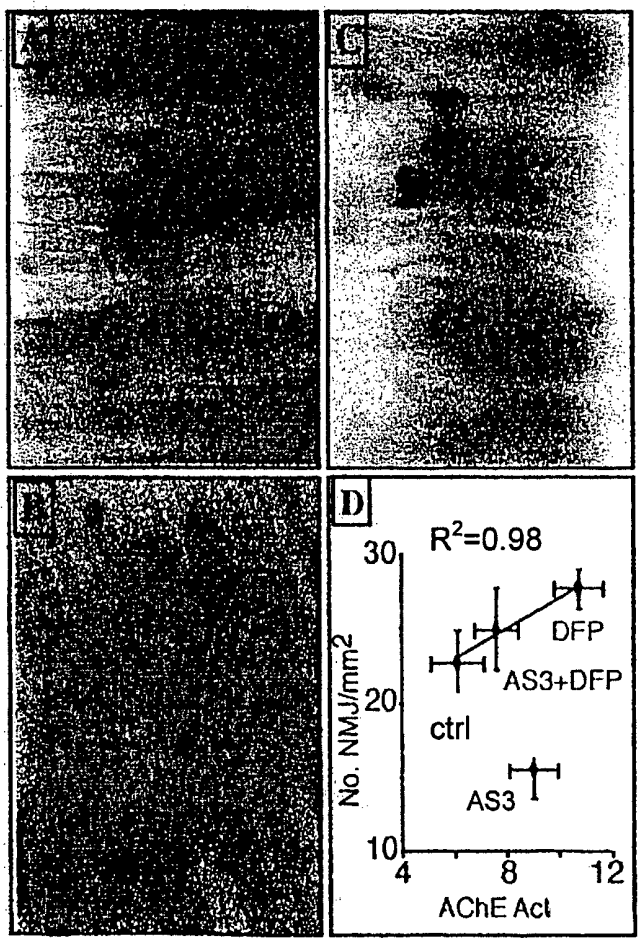

A
B
C
D
50 µm
30
20
10
0
neur/mm2
Ctrl (A)
DFP (B)
Tg (C)

Fig. 10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C
B
D
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20
10
No. NMJ/mm2
DFP
AS3+DFP
ctrl
AS3
4
8
12
AChE Act

Fig. 11

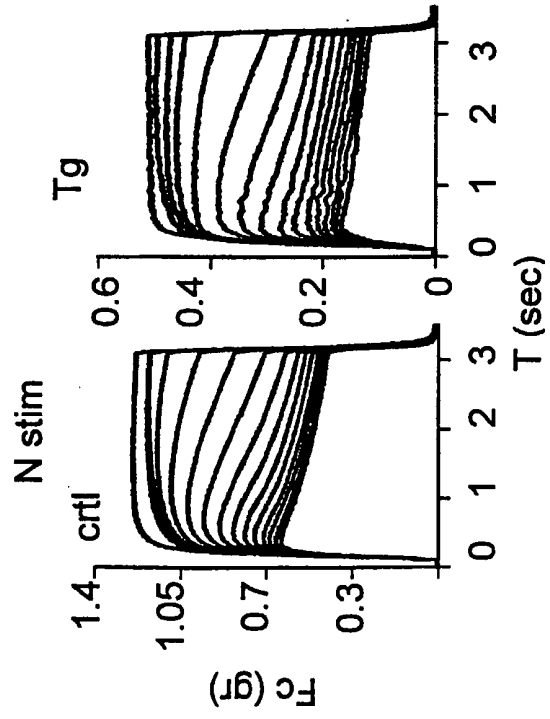


Fig. 12A

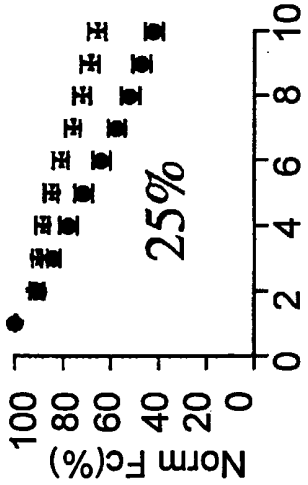


Fig. 12B

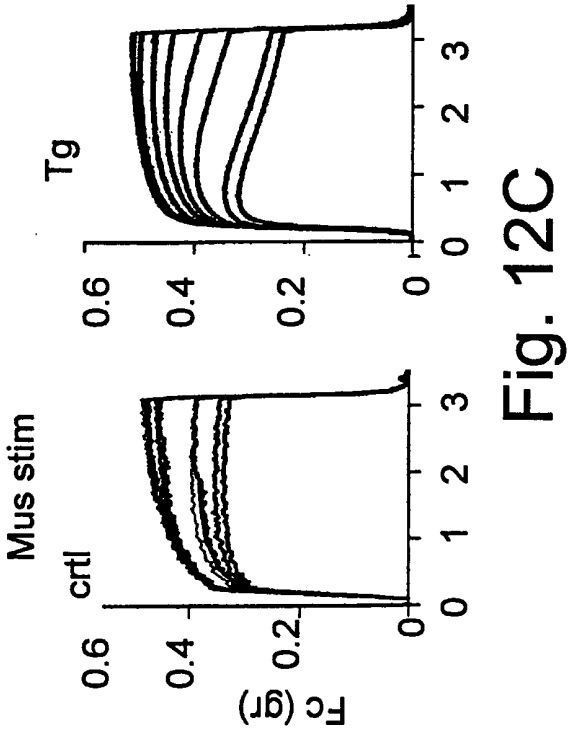


Fig. 12C

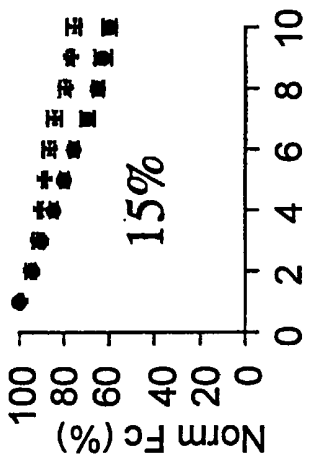


Fig. 12D

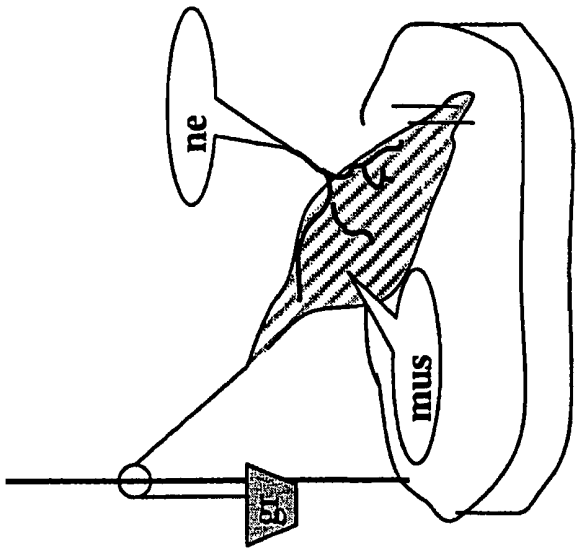
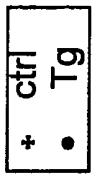


Fig. 12E



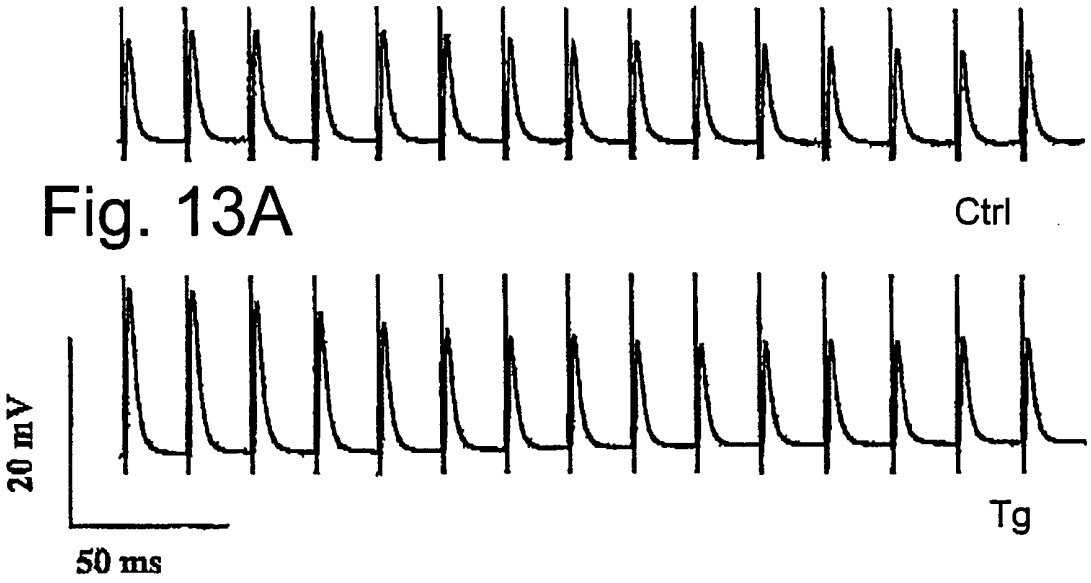


Fig. 13A

Fig. 13B

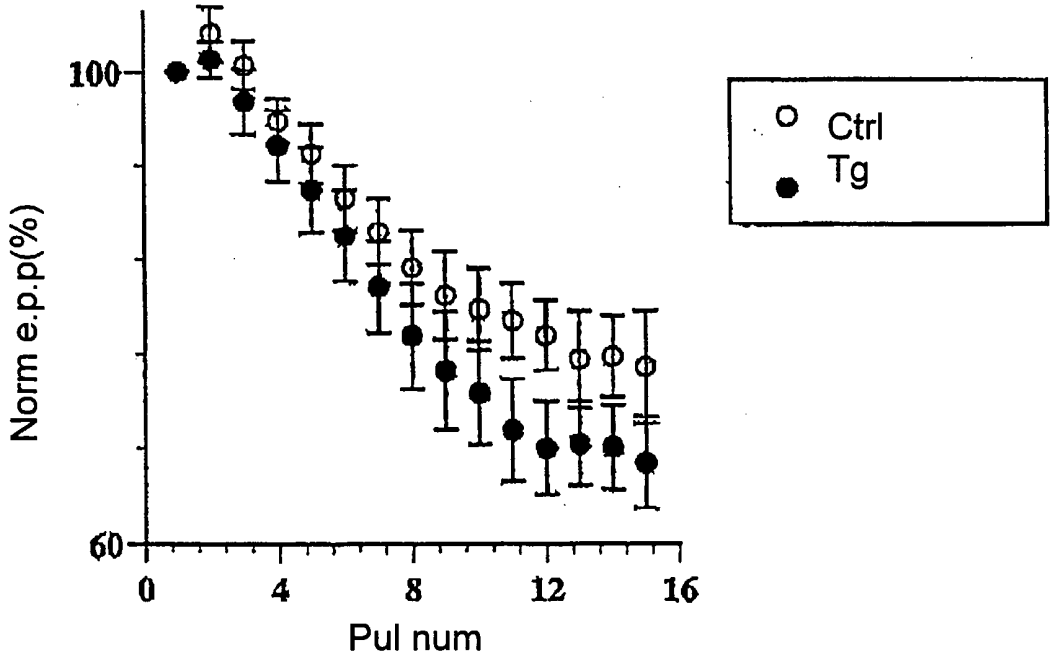


Fig. 13C

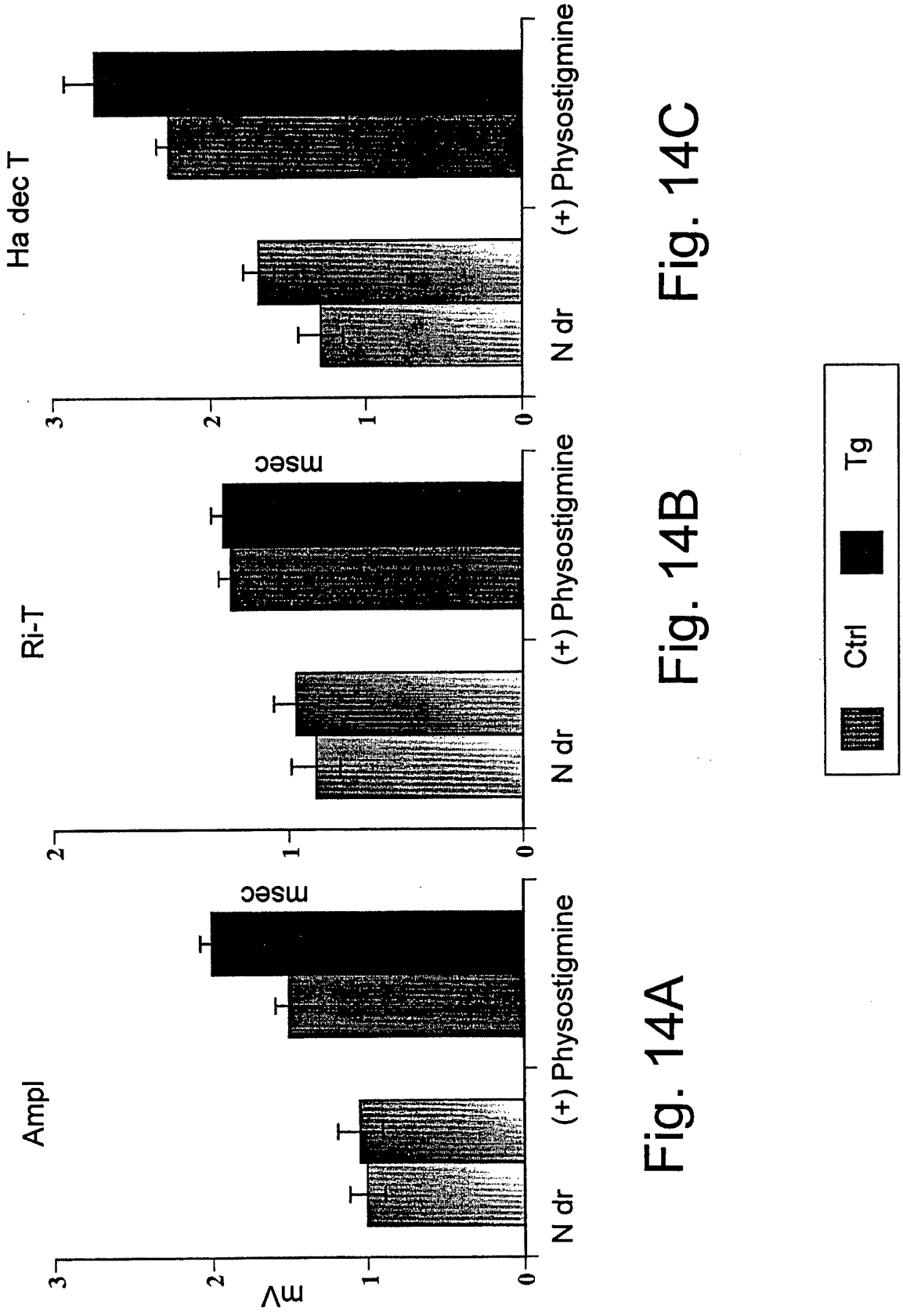


Fig. 14A

Fig. 14B

Fig. 14C

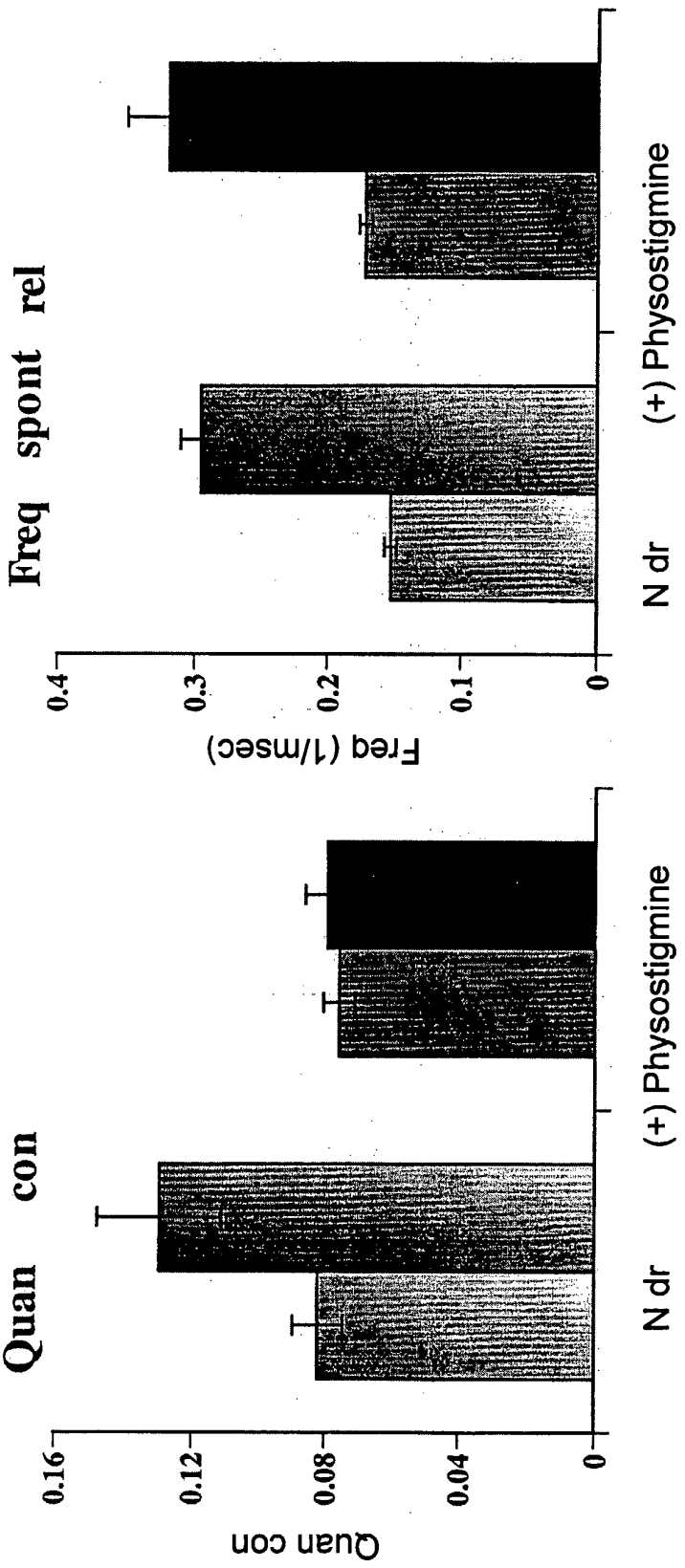


Fig. 15A

Fig. 15B

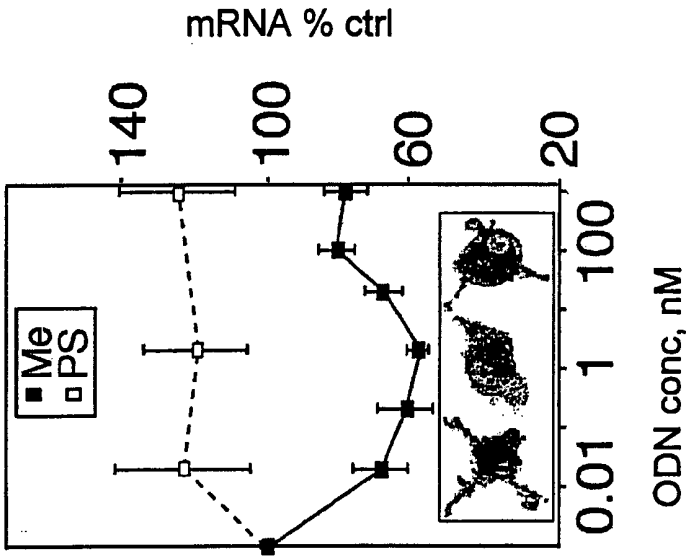


Fig. 16B

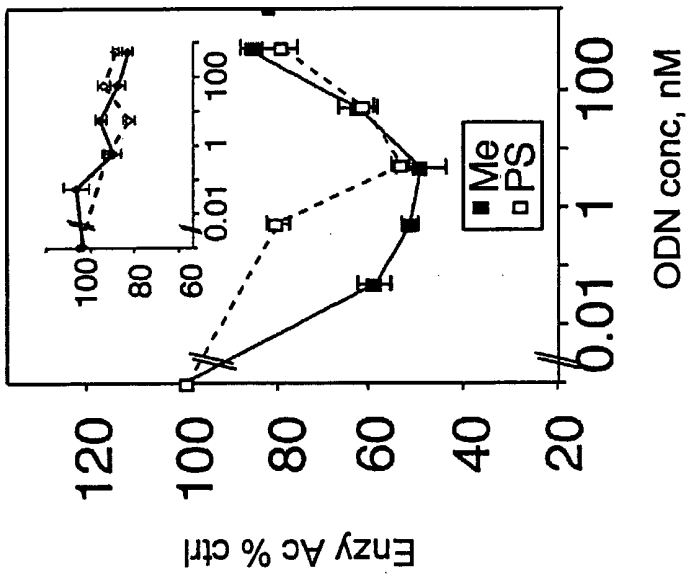


Fig. 16A

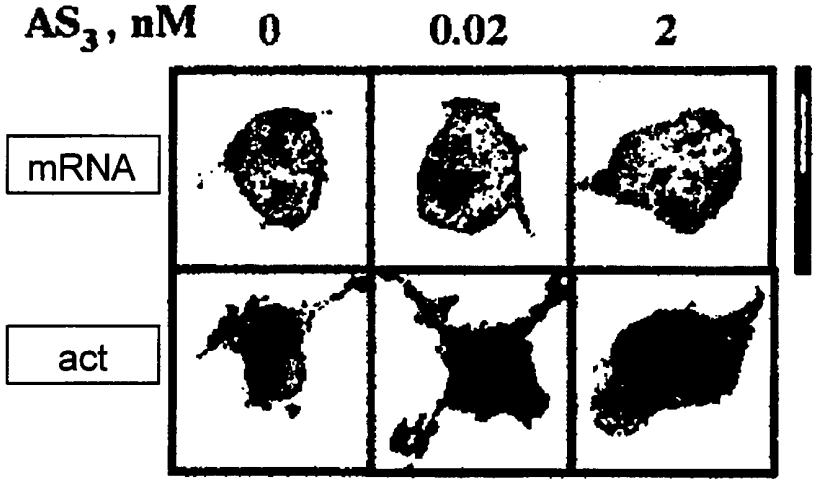


Fig. 17A

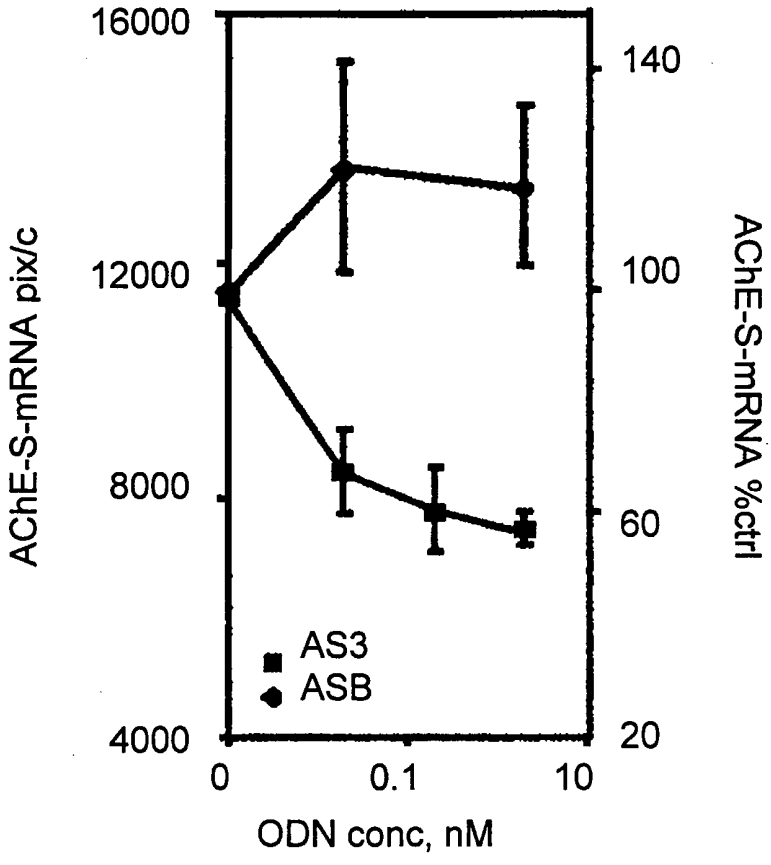


Fig. 17B

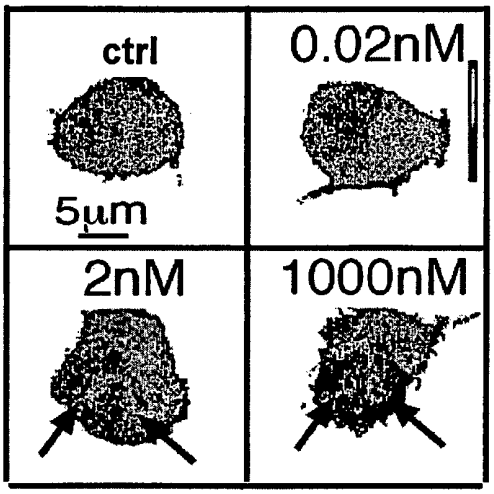


Fig. 18A

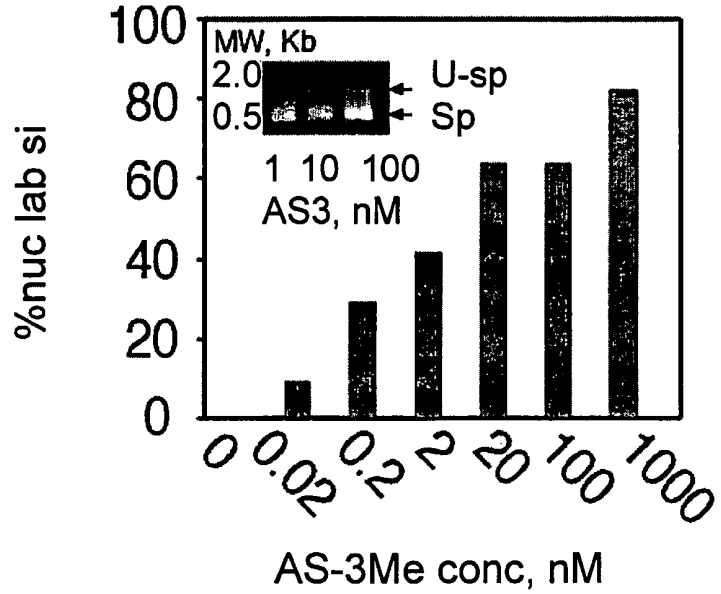


Fig. 18B

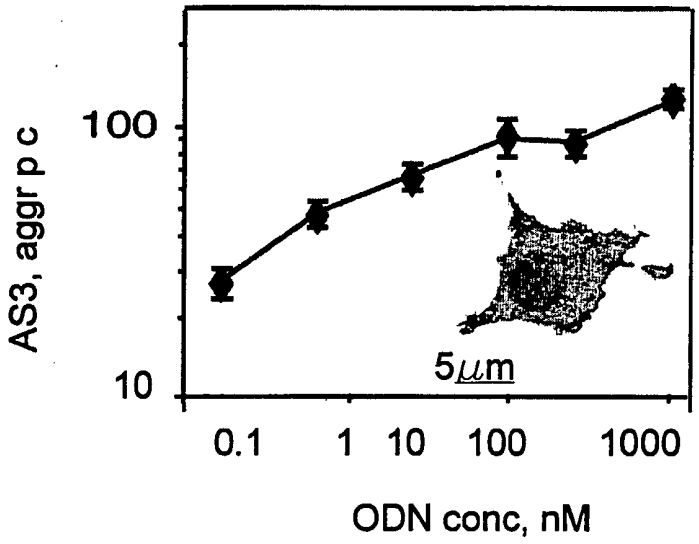


Fig. 19A

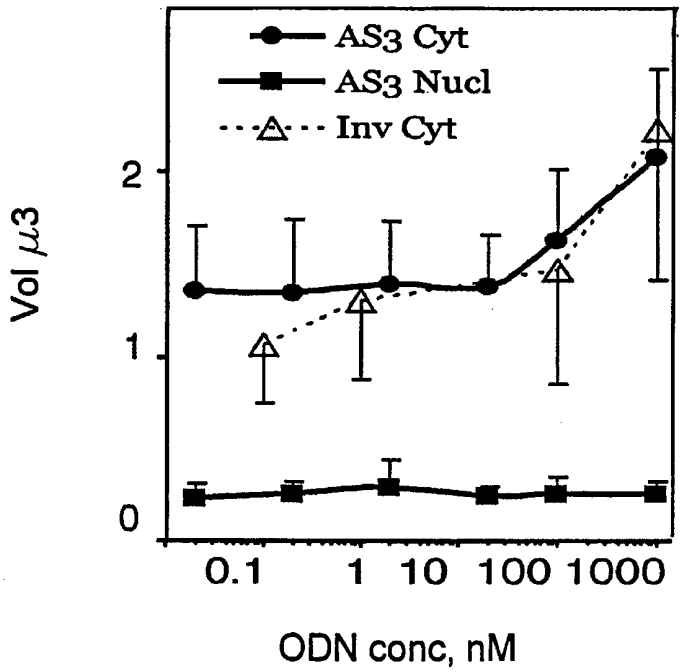
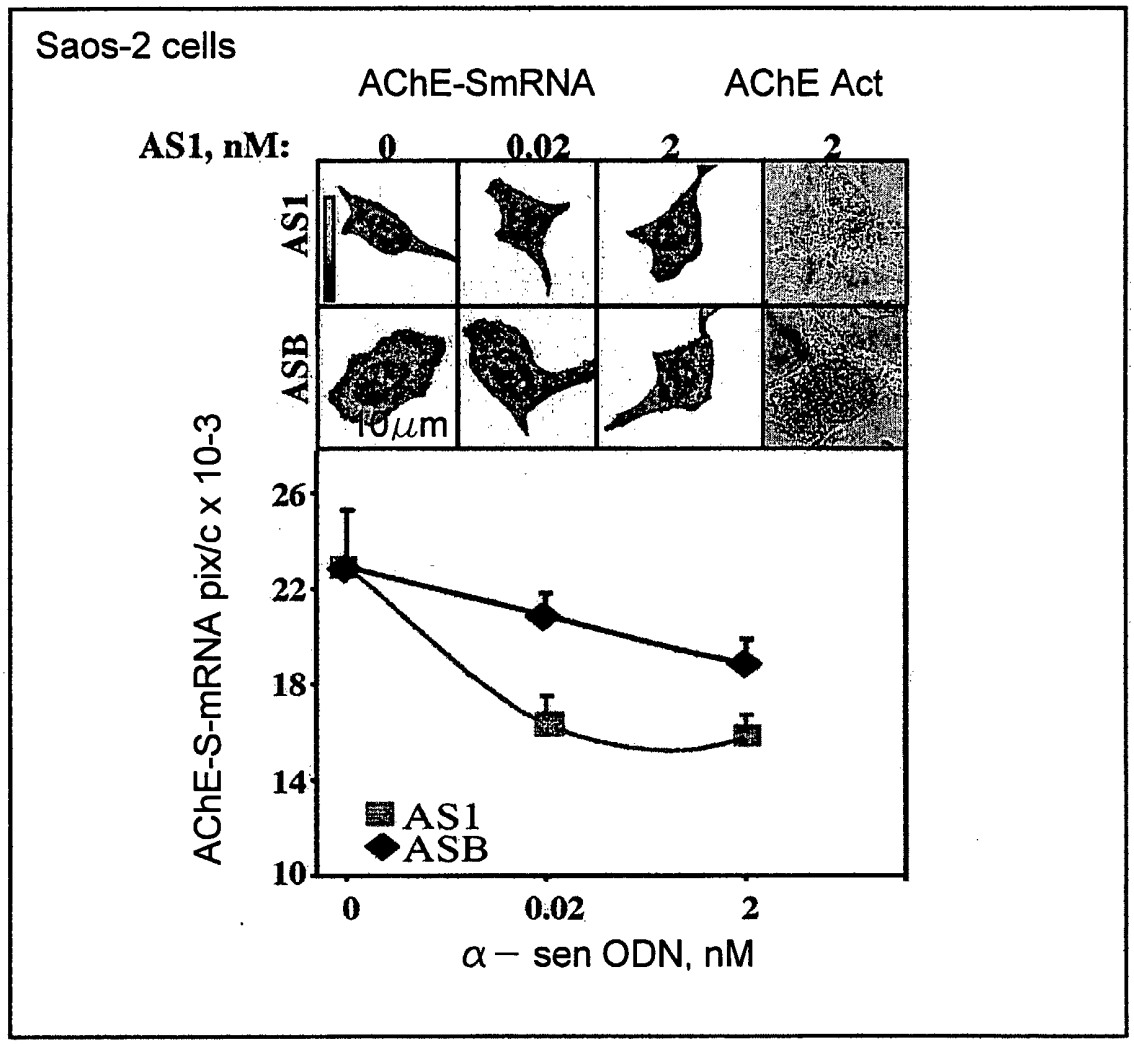


Fig. 19B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AChE Act
AS1, nM:
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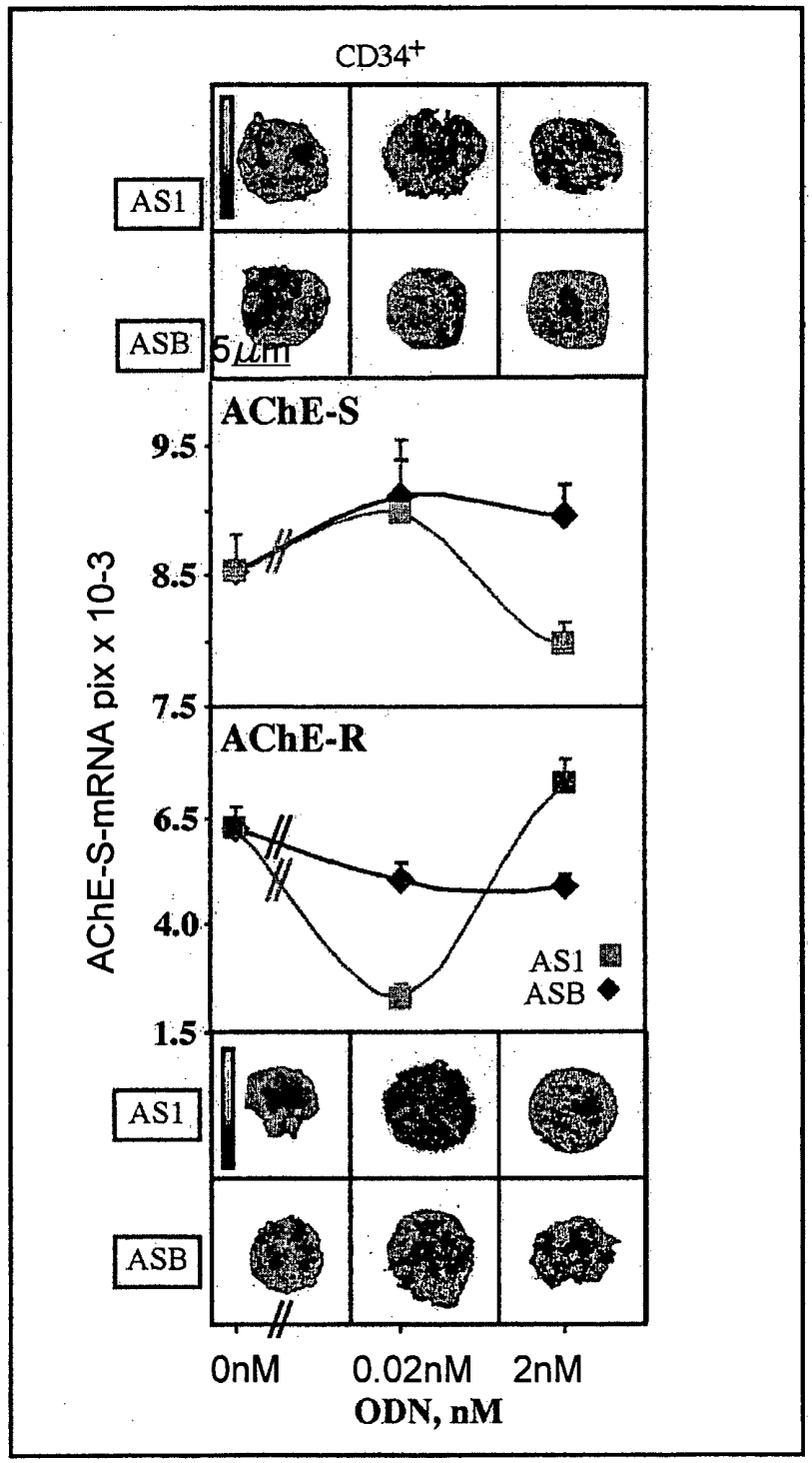
0.02
2
2
AS1
ASB
10μm
AChE-S-mRNA pix/c x 10-3
26
22
18
14
10
AS1
ASB
0
0.02
2
α – sen ODN, nM

Fig. 20


CD34+
AS1
ASB
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AChE-S
9.5
8.5
7.5
AChE-R
6.5
4.0
1.5
AChE-S-mRNA pix x 10-3
AS1
ASB
AS1
ASB
0nM
0.02nM
2nM
ODN, nM

Fig. 21

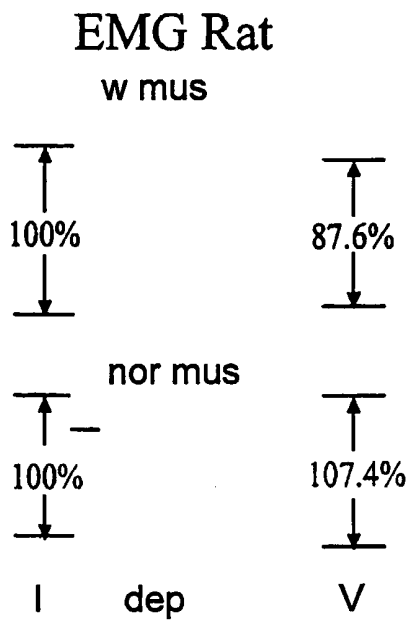


Fig. 22A

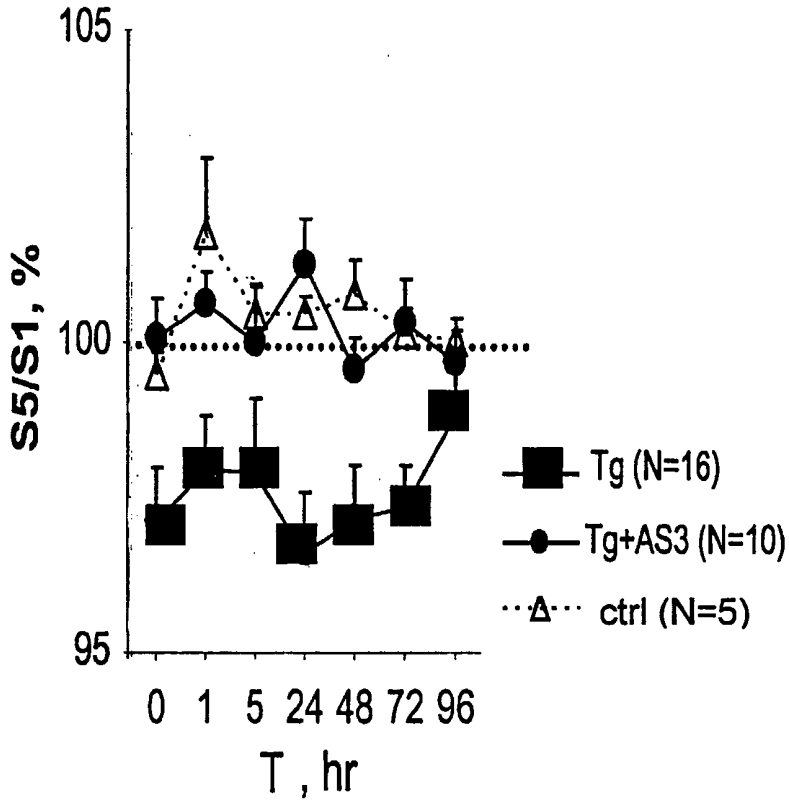


Fig. 22B

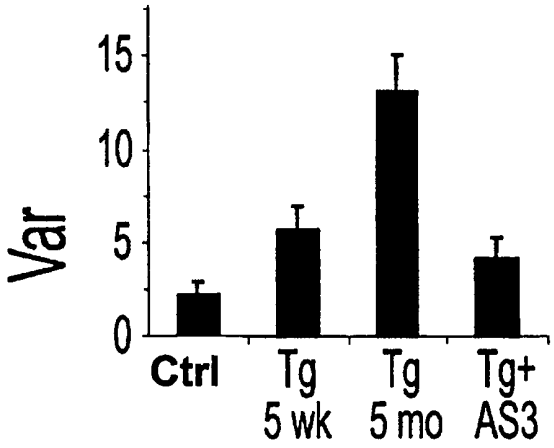


Fig. 22C

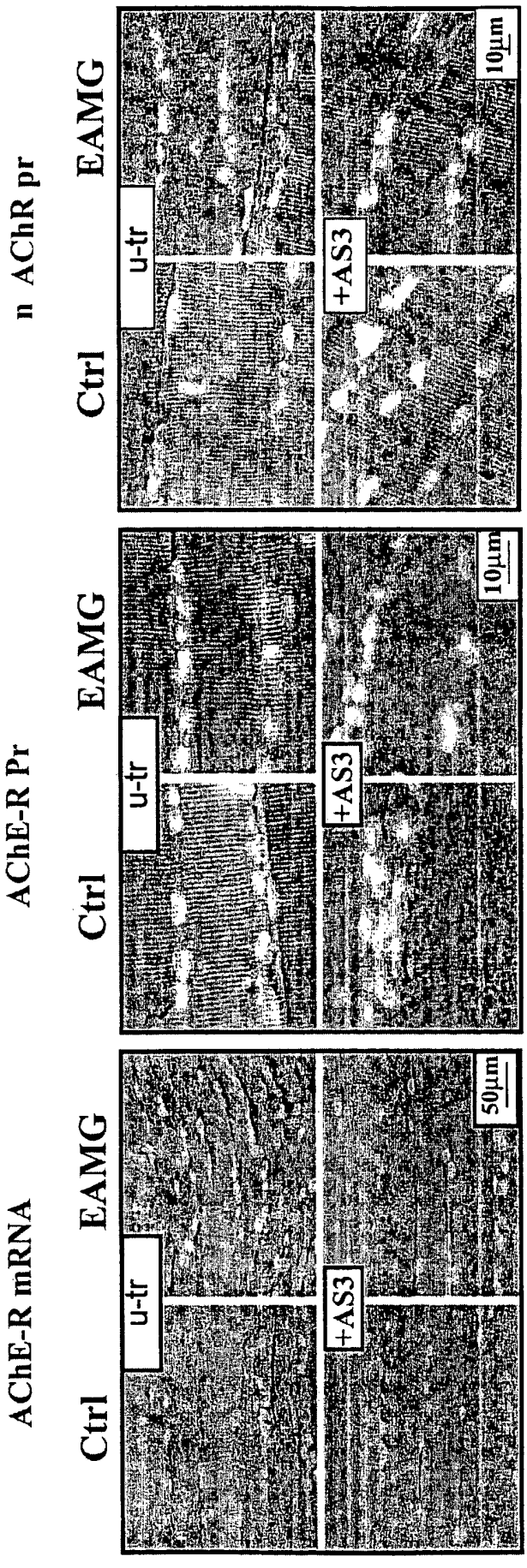


Fig. 23A

Fig. 23B

Fig. 23C

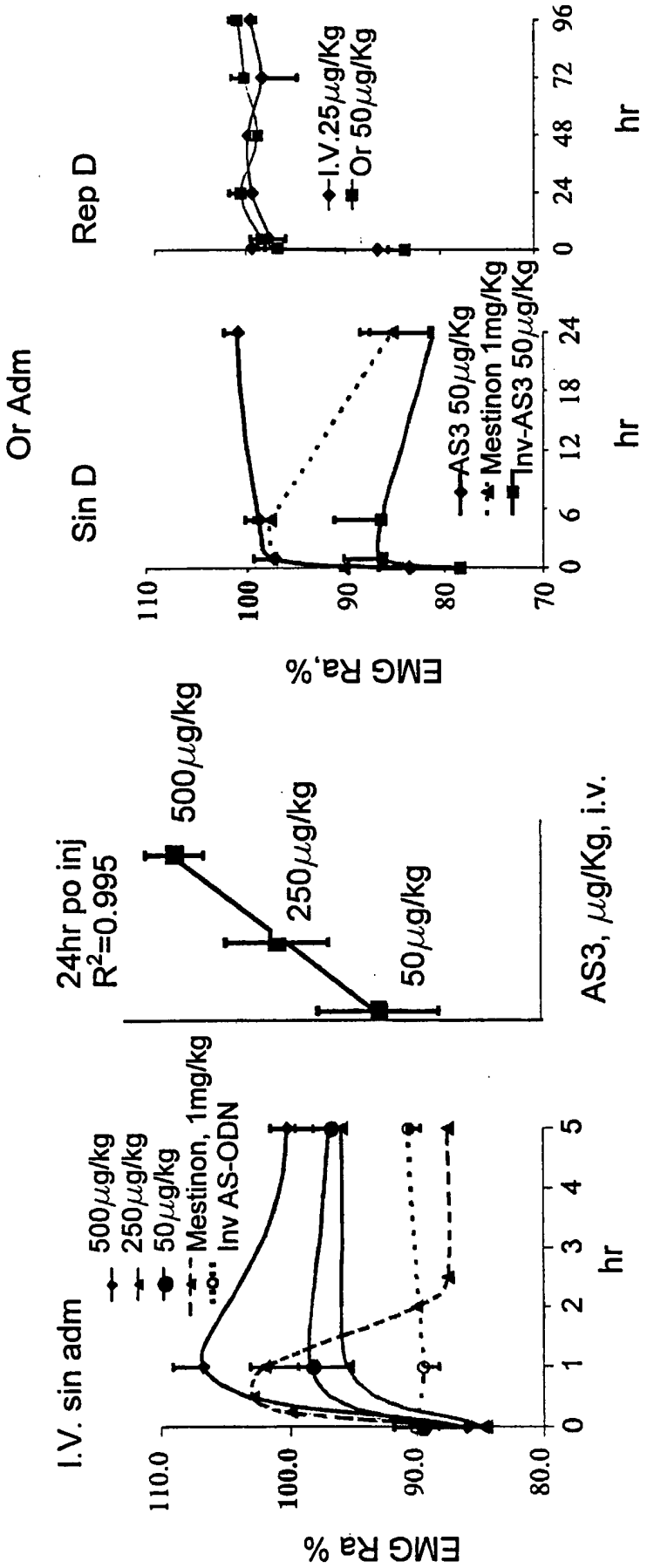


Fig. 24A Fig. 24B Fig. 24C Fig. 24D

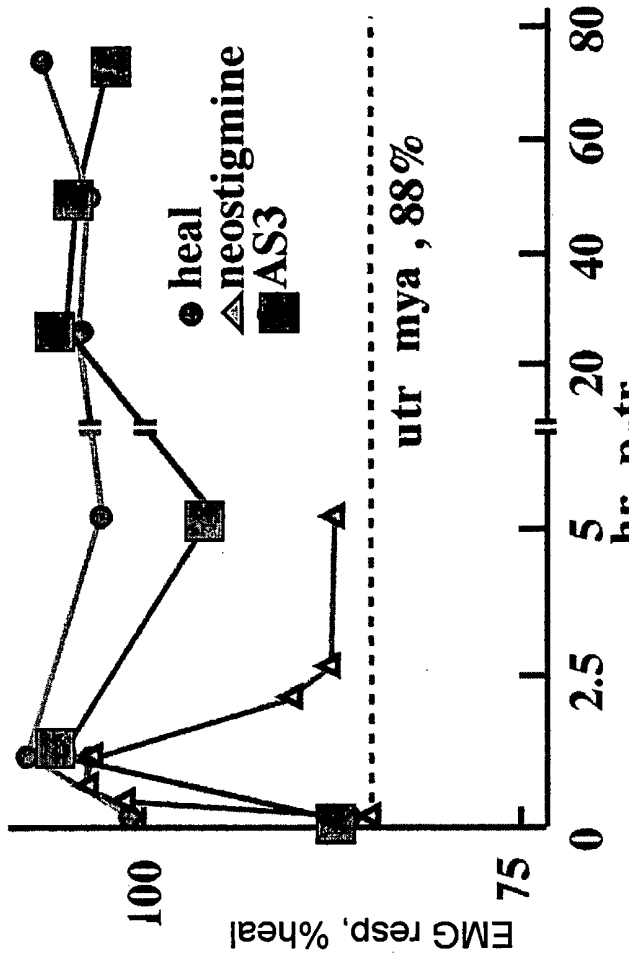
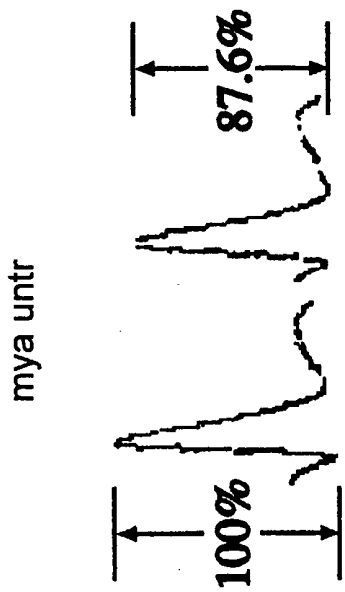


Fig. 26



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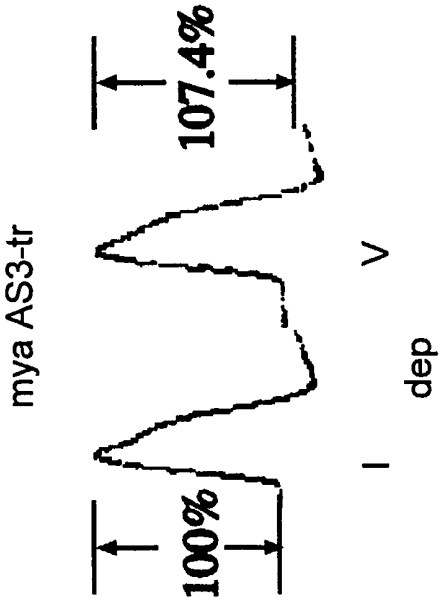

Fig. 25B

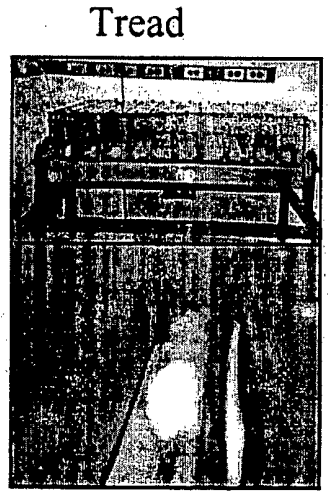

Fig. 27A

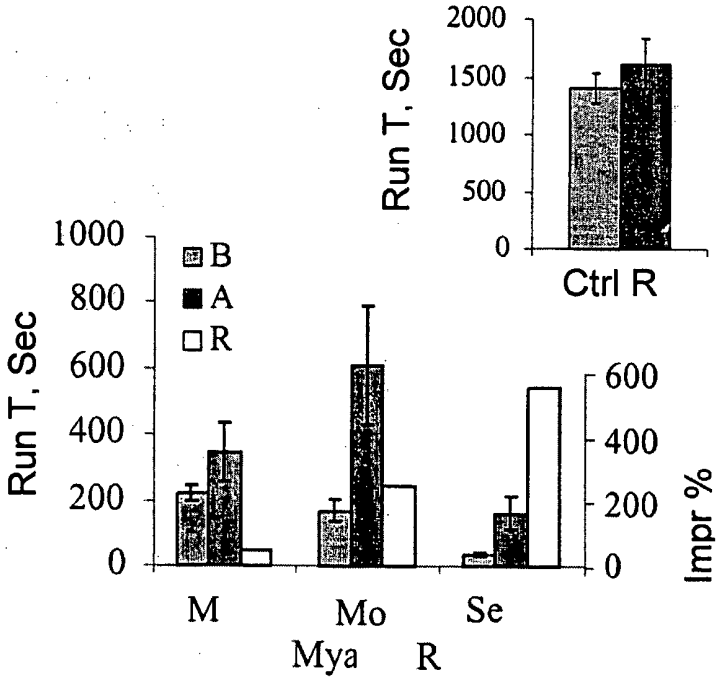

Fig. 27B

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