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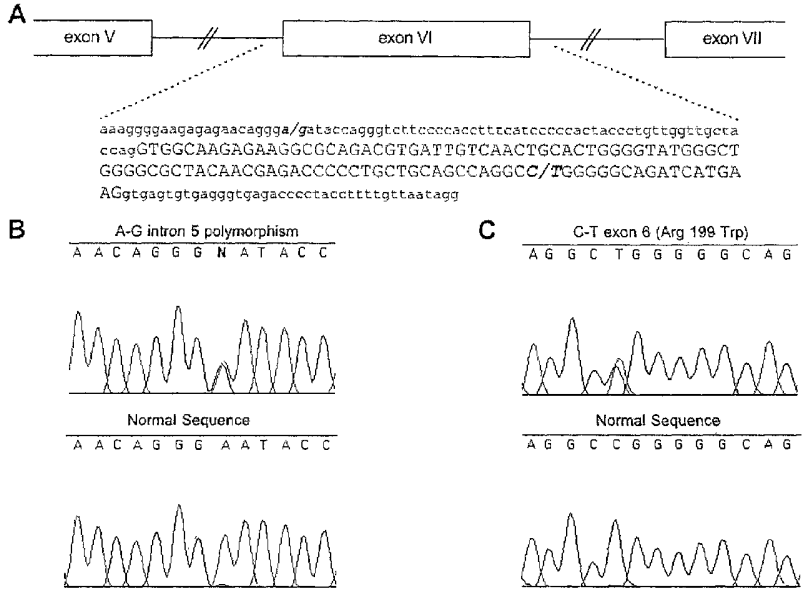
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(54) Title: DISEASE-ASSOCIATED GENE



(57) Abstract: A method of determining an increased risk of a late-onset neurodegenerative disease to a patient, the method comprising analysing a sample from the patient to determine whether the patient has a D-amino acid oxidase (DAO) abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased risk of the late-onset neurodegenerative disease.

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DISEASE-ASSOCIATED GENE

This invention relates to disease-associated genes, and in particular to genes associated with late-onset neurodegenerative disease.

Amyotrophic lateral sclerosis (ALS), also known as motor neurone disease, is a chronic late-onset neurodegenerative condition which targets neurones of the motor system in the spinal cord, brain stem and cerebral cortex causing progressive muscle weakness, atrophy, paralysis and respiratory failure. Onset is usually in the 5th to 6th decade and may affect limb or bulbar function initially. The prevalence of the condition world-wide is 4-8 per 100,000 with an annual incidence ranging from 0.5-3 per 100,000 (Haverkamp *et al* 1995). At present the aetiology of the disease is unknown and treatment is largely palliative. A small proportion of cases (5-10%) show autosomal dominant inheritance and these cases are indistinguishable from the more common sporadic form of the disease, sporadic amyotrophic lateral sclerosis (SALS). Familial amyotrophic lateral sclerosis (FALS) has provided a tangible focus for the identification of disease-associated genes and hence a greater understanding of the pathogenesis of the disease on which to develop new treatments.

The first FALS gene identified (ALS1 MIM 105400) was copper/zinc-dependent superoxide dismutase (SOD1) (Rosen *et al* 1993). Over 90 mutations in SOD1 have been detected in a proportion of FALS cases (~20%) and current evidence points to a gain of function property of the mutation being responsible for the disease which alters enzyme function, potentially increasing the generation of toxic free radicals and promoting cell death through both apoptotic and non-apoptotic mechanisms (see reviews by Cleveland and Rothstein 2001 and Beckman *et al* 2001). However the disease mechanism in the majority of cases (~80%) is as yet unknown.

A further locus has been reported for autosomal dominant adult-onset FALS on 18q (Hand *et al* 2001). Other rarer forms of ALS have also been reported, which include autosomal dominant juvenile-onset ALS (ALS4 MIM 602433) which maps to chromosome 9q34 (Chance *et al* 1998, Blair *et al* 2000) and three autosomal recessive forms of juvenile-onset ALS. The most prevalent form (ALS5 MIM 602099) has onset from the first/second decade, has little effect on life expectancy and maps to 15q15.1-q21.1 (Hentati *et al* 1998). A second juvenile-onset autosomal recessive locus has been mapped to 2q33 (ALS2 MIM 205100) and recently the causative gene identified as a GTPase-like protein (Hadano *et al* 2001).

Alzheimer's disease (AD) is a late-onset neurodegenerative disease characterised by a slowly progressive form of dementia, *ie* a progressive, acquired impairment of intellectual functions. Memory impairment is a necessary feature for the diagnosis, and change in one of the following areas must also be present: language, decision-making ability, judgement, attention, and other related areas of cognitive function and personality. The most important risk factors for dementia are old age and a family history of dementia. About 10 percent of all people over 70 have significant memory problems and about half of those are due to AD. The number of people with AD doubles each decade past age 70, and as women usually live longer than men, they are more likely to develop AD.

AD can be classified into two types - early onset and late onset. In early onset AD symptoms first appear before age 60, whilst late onset AD, which is the most common form of the disease, develops in people 60 and older and is thought to be less likely to occur in families.

AD can be confirmed by microscopic examination of a sample of brain tissue after death. The brain tissue shows "neurofibrillary tangles" (twisted fragments of protein within nerve cells that clog up the cell), "neuritic

plaques" (abnormal clusters of dead and dying nerve cells, other brain cells, and protein), and "senile plaques" (areas where products of dying nerve cells have accumulated around protein). Although these changes occur to some extent in all brains with age, there are many more of them in the brains of people with AD.

The formation of lesions made of fragmented brain cells surrounded by amyloid-family proteins are characteristic of the disease. Interestingly, these lesions and their associated proteins are closely related to similar structures found in Down's Syndrome. Tangles of filaments largely made up of a protein associated with the cytoskeleton have also been observed in samples taken from AD brain tissue.

The destruction of neurones in AD leads to a decrease in neurotransmitters, particularly acetylcholine, serotonin, and norepinephrine, with acetylcholine being the most affected. By causing both structural and chemical problems in the brain, AD appears to disconnect areas of the brain that normally work together.

Currently, mutations in four genes, situated on chromosomes 1, 14, 19, and 21, are believed to play a role in the disease. The best-characterised of these are PS1 (or AD3) on chromosome 14, PS2 (or AD4) on chromosome 1 and amyloid precursor protein (APP) on chromosome 21. The apolipoprotein E E4 allele is thought to represent a significant risk factor for both familial and sporadic forms of AD.

Parkinson's disease (PD) is a late-onset neurodegenerative disease that affects approximately 2 out of 1,000 people, and most often develops after age 50. It does occasionally occur in younger adults, and rarely in children. It affects both men and women and is one of the most common neurological disorders of the elderly. In some cases the disease occurs within families, especially

when it affects young people. PD is a neurodegenerative disease that manifests as a tremor, muscular stiffness and difficulty with balance and walking. A classic pathological feature of the disease is the presence of an inclusion body, called the Lewy body, in many regions of the brain.

Parkinson's disease is caused by progressive deterioration of the nerve cells of the substantia nigra, a part of the brain that controls muscle movement (the basal ganglia and the extrapyramidal area). Dopamine, which is one of the substances used by cells to transmit impulses (transmitters), is normally produced in this area. Deterioration of this area of the brain reduces the amount of dopamine available to the brain. Insufficient dopamine disturbs the balance between dopamine and other transmitters, such as acetylcholine. Without dopamine, the nerve cells cannot properly transmit messages, and this results in impaired control of the execution of voluntary movement causing akinesia and tremor. The exact reason that the cells of the brain deteriorate is unknown. The disorder may affect one or both sides of the body, with varying degrees of loss of function.

Until relatively recently, Parkinson's disease was not though to be heritable, and research was primarily focused on environmental risk factors such as viral infection or neurotoxins. However, a positive family history was gradually perceived to be a risk factor, a view that was confirmed when a candidate gene for some cases of PD was mapped to chromosome 4. Mutations in this gene have now been linked to several Parkinson disease families. The product of this gene, a protein called alpha-synuclein, is a familiar culprit: a fragment of it is a known constituent of AD plaques.

Since alpha-synuclein fragments are implicated in both PD and AD, there may be shared pathogenic mechanisms between the two, therefore research into one disease may aid understanding of the other.

There is no known cure for Parkinson's disease. Treatment is aimed at controlling the symptoms. Medications control symptoms primarily by controlling the imbalance between the transmitters.

Despite the advances in our understanding of the late-onset neurodegenerative diseases, the pathological basis of these diseases has not been fully elucidated. Identification of genetic factors involved in ALS, AD and PD would be expected to lead to improvements in the treatment of these diseases.

We have now identified a further FALS locus on chromosome 12q, and have localised the area of linkage to a 20cM interval between the markers D12S1706 and D12S354.

D-amino acid oxidase (DAO), a candidate gene in this region, was sequenced in at least one affected individual from each family showing potential linkage to chromosome 12. A disease-associated mutation, transforming a C to T in codon 199, was detected in exon 6 of the DAO gene. This results in a coding change Arg to Trp at position 199 (R199W) which was shown to be significantly associated with disease in this family and absent from unaffected individuals in this family.

DAO, or DAO abnormalities, have not been previously implicated in late-onset neurodegenerative diseases such as ALS, PD or AD. The properties and distribution of DAO are consistent with it playing an important role in the brain stem and spinal cord and hence a mutation at the active site of DAO is likely to lead to pathological consequences which could underlie the pathogenesis of late-onset neurodegenerative disease. Without being bound by theory, it is believed that a build up of D-amino acids in the brain occurs with age leading to neurotoxicity and late-onset neurodegenerative disease, which is exacerbated by the presence of a DAO abnormality.

DAO (EC. 1.4.3.3) is a flavin adenine dinucleotide (FAD) dependent oxidase which catalyses the oxidative deamination of D-amino acids which are present in most organisms and mammalian tissues. DAO is one of the principal flavoproteins of peroxisomes found in mammalian kidney, liver and brain. The enzyme functions in the catabolism of small neutral and basic amino acids which may arise from the racemisation of L-amino acids or from fungi, bacteria and insects. Although D-amino acids are normally associated with a bacterial origin, substantial amounts of D-amino acids have been demonstrated in mammalian tissue. Recently a serine racemase has been identified in rat brain (Wolosker *et al* 1999) which indicates a potential physiological role for D-amino acids in brain.

In the mammalian central nervous system (CNS), DAO is confined mainly to brain stem, spinal cord and cerebellum, and is scarce in the mammalian forebrain (Horiike *et al* 2001, Horiike *et al* 1994). The enzyme is localised to astrocytes and glial cells including Bergmann glial cells, and is absent from neurones, endothelial cells, oligodendrocytes or ependymal cells. This distribution of enzyme activity is likely to be responsible for the low concentrations of D-serine found in the brain stem and spinal cord where DAO enzyme activity is high compared to the forebrain (0.4 μmol/g wet weight) where enzyme activity is low and little metabolism of D-serine would occur.

Without being limited by theory, it is believed that the DAO abundantly expressed in the spinal cord is in sufficient proximity to the neurones to affect D-amino acid levels in and around the neurones. A DAO abnormality, such as a DAO mutation or deficiency, could have a direct effect on neurones leading to a build-up of D-amino acids in the neurones with toxic effects.

The DAO gene is present as a single copy on chromosome 12. The gene has 11 exons and spans 20kb (Fukui and Miyake 1992). The protein consists of 347 amino acids and is 39.4 kDa in size. The enzyme exists as a homodimer

with one molecule of noncovalently bound FAD as coenzyme and catalyses the oxidation of neutral and basic D-amino acids to their corresponding ketoacids. The first stage in catalysis is the oxidation of the D-amino acids to the corresponding 2-imino acid forming a reduced enzyme-imino complex. This is followed by electron transfer from the complex of O_2. With non-cyclic amino acids the 2-imino acid is then hydrolysed to the corresponding 2-oxo acid ammonia. The mechanism of the enzyme activity involves hydride transfer without acid base catalysis (Todone *et al* 1997) and the reduced $FADH_2$ formed rapidly reacts with O_2 to generate the oxidised cofactor and release H_2O_2. The H_2O_2 is then immediately removed by the activity of other enzymes such as catalase.

The substrate binding site is located in a hydrophobic cavity which excludes solvents and is covered by a "lid" type structure which is a loop comprising residues 216-228. This region is in close proximity to the site of the mutation that we have identified (codon 199). Arg at the equivalent position to codon 199 of human DAO is highly conserved (see Figure 5, and Figure 1 of Pilone (2000)). Arg to Trp substitution at position 199 is likely to affect the catalytic activity of the enzyme through conformational effects produced by replacing the charged Arg by an aromatic indole nucleus.

The R199W mutation in DAO appears to be transmitted in an autosomal dominant fashion and causes ALS when present in the heterozygous form.

A first aspect of the invention provides a method of determining an increased risk of a late-onset neurodegenerative disease to a patient, the method comprising analysing a sample from the patient to determine whether the patient has a D-amino acid oxidase (DAO) abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased risk of the late-onset neurodegenerative disease.

By the term "determining an increased risk of a late-onset neurodegenerative disease to a patient", we include the meaning of determining an increased likelihood of the patient developing a late-onset neurodegenerative disease, or determining an increased likelihood of the patient having an increased rate of progression of the late-onset neurodegenerative disease, or determining an increased likelihood of the late-onset neurodegenerative disease having an increased severity in the patient.

More preferably, determining an increased risk of a late-onset neurodegenerative to a patient comprises determining an increased likelihood of any two of developing a late-onset neurodegenerative disease, having an increased rate of progression of the late-onset neurodegenerative disease, or of the late-onset neurodegenerative disease having an increased severity in the patient.

Determining an increased risk of a late-onset neurodegenerative disease to a patient can, in another embodiment, comprise all three of determining an increased likelihood of the patient developing the disease, determining an increased likelihood of the patient having an increased rate of progression of the disease, and determining an increased likelihood of the disease having an increased severity in the patient.

The invention includes a method of determining an increased likelihood of a patient *i)* developing a late-onset neurodegenerative disease, *ii)* having an increased rate of progression of a late-onset neurodegenerative disease, or *iii)* having an increased severity of a late-onset neurodegenerative disease, the method comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased likelihood of *i)* developing a late-onset neurodegenerative disease, *ii)* having an increased rate of progression of a late-onset neurodegenerative disease, or *iii)* having an

increased severity of a late-onset neurodegenerative disease.

The invention includes a method of determining an increased likelihood of a patient developing ALS, comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased likelihood of developing ALS.

The invention also includes a method of determining an increased likelihood of an increased rate of progression of ALS in a patient, comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased likelihood of an increased rate of progression of ALS.

The invention further includes a method of determining an increased likelihood of an increased severity of ALS in a patient, comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the ALS has an increased likelihood of an increased severity in the patient.

The invention also includes a method of determining an increased likelihood of a patient developing AD comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased likelihood of developing AD.

The invention also includes a method of determining an increased likelihood of an increased rate of progression of AD in a patient, comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that

the patient has an increased likelihood of an increased rate of progression of AD.

The invention further includes a method of determining an increased likelihood of an increased severity of AD in a patient, comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the AD has an increased likelihood of an increased severity in the patient.

The invention also includes a method of diagnosing an increased likelihood of a patient developing PD comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased likelihood of developing PD.

The invention also includes a method of determining an increased likelihood of an increased rate of progression of PD in a patient, comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased likelihood of an increased rate of progression of PD.

The invention further includes a method of determining an increased likelihood of an increased severity of PD in a patient, comprising analysing a sample from the patient to determine whether the patient has a DAO abnormality, wherein the presence of a DAO abnormality is an indication that the PD has an increased likelihood of an increased severity in the patient.

In this first aspect of the invention, by "D-amino acid oxidase" (DAO) we mean the flavin adenine dinucleotide (FAD) dependent oxidase, from the same species as the patient from which the sample is taken, which catalyses the

oxidative deamination of D-amino acids (EC. 1.4.3.3).

Preferably, the patient is a human patient, and thus DAO is human DAO. Human DAO has the amino acid sequence listed in Genbank Accession No. NP_001908 and in Figure 5 (SEQ ID No. 1). The cDNA sequence of human DAO is listed in Genbank Accession No. NM_001917.

It is appreciated that by "DAO" we do not necessarily mean human DAO. Unless otherwise specified, by "DAO" we include DAO from non-human species when from the context it is clear that the invention does not specifically require human DAO.

Preferably, the sample to be analysed is a blood, plasma, serum, urine, saliva, skin, cerebrospinal fluid or leukocyte, preferably polymorphonuclear leukocyte (Robinson *et al*, *J Cell Biol*, vol. 77 (1), pp 59-71, 1978), sample from the patient.

By "DAO abnormality" we include the meaning that the patient does not have normal DAO activity. We include the meaning that the DAO gene is not expressed or has a reduced level of expression (whether affected at the level of transcription, mRNA stability or translation); or that the DAO activity is reduced or absent or outside of the normal range; or that the DAO has an altered substrate specificity; or that the DAO has altered cellular localisation or altered tissue or cell-type expression pattern; or the DAO has gained a function. DAO abnormalities may be caused by DNA mutations within the DAO gene and promoter/ enhancer regions.

When the enzyme DAO is functioning, it catabolises D-amino acids with the exception of the acidic amino acids D-glu and D-asp; hence the D-amino acid levels in the sample are reduced. Conversely, when there is a DAO abnormality and DAO enzyme activity is reduced or absent, D-amino acids

accumulate and the D-amino acid levels in the sample are higher.

Thus in a preferred embodiment of the invention, the method for analysing a sample from a patient to determine whether the patient has a DAO abnormality comprises determining the D-amino acid level in the sample. The method may further comprise the step of comparing the D-amino acid level with the level expected for an individual not considered to have a DAO abnormality, or with the level determined from an individual or group of individuals not considered to have a DAO abnormality, for example non-affected family members. A D-amino acid level in the sample greater than about 100 nmol/g, or about 200 nmol/g, or about 300 nmol/g, or about 400 nmol/g, or about 500 nmol/g, or about 600 nmol/g, or about 700 nmol/g, or about 800 nmol/g, or about 900 nmol/g, or about 1000 nmol/g, or greater than about 2000 nmol/g wet weight may indicate that the patient has a DAO abnormality.

The method can comprise measuring total D-amino acid levels in the sample. However, as DAO does not catabolise D-glu and D-asp, the method preferably involves measuring the levels of a specific D-amino acid or more than one specific D-amino acid with the exception of D-glu and D-asp.

As the aliphatic and aromatic D-amino acids are more efficient substrates for DAO than the polar and basic D-amino acids (Pilone 2000), the method preferably involves measuring the levels of a specific aliphatic or aromatic D-amino acid or more than one aliphatic and aromatic D-amino acid.

Levels of D-ser, D-leu and D-pro have been found to be raised in the serum of mice lacking DAO activity (Hashimoto *et al* 1993, and Hamase *et al, Anal Biochem*, vol. 298 (2) pp 253-258, 2001, both of which are incorporated herein by reference). Thus, most preferably, D-ser, D-leu or D-pro levels are measured.

Bruckner & Hausch (*J Chromatography*, vol. 614 (1) pp 7-17, 1993) used a capillary gas chromatographic method to determine the presence of D-amino acids (D-ala, D-asp, D-asn, S-ser, D-pro, D-leu, D-glu, D-gln) in the serum of patients with renal failure, which may include reduced renal DAO activity, and in normal patients.

Hence, in one preferred embodiment, the method includes determining the levels of D-ala, D-asn, S-ser, D-pro, D-leu, or D-gln, particularly levels of D-ser, D-pro or D-leu, in serum from the patient.

When the enzyme DAO is functioning, it catabolises D-amino acids and hence the D/L ratio in the sample is reduced. Conversely, when there is a DAO abnormality and the enzyme is not functioning, D-amino acids accumulate and the D/L ratio in the sample is higher.

Thus in another preferred embodiment of the invention, the method for analysing a sample from a patient to determine whether the patient has a DAO abnormality comprises determining the D- to L-amino acid ratio (D/L ratio) in the sample. The ratio may be compared with the ratio expected for an individual not considered to have a DAO abnormality, or with the level determined from an individual or group of individuals not considered to have a DAO abnormality, for example non-affected family members. Typically, a D/L ratio in the sample greater than about 0.1 may indicate that patient has a DAO abnormality. Preferably, a D/L ratio in the sample greater than about 0.15 indicates that patient has a DAO abnormality. More preferably, a D/L ratio in the sample greater than about 0.2, or greater than about 0.25, or greater than about 0.3, or greater than about 0.35, or greater than about 0.4, or greater than about 0.45, or greater than about 0.5, or greater than about 1.0 indicates that patient has a DAO abnormality.

The method can comprise measuring total total D/L ratios in the sample.

However, as DAO does not catabolise D-glu and D-asp, the method preferably involves measuring the D/L ratio of a specific D-amino acid or more than one specific D-amino acid, with the exception of D-glu and D-asp. Most preferably, the D/L ratio of D-ser is measured.

As the aliphatic and aromatic D-amino acids are more efficient substrates for DAO than the polar and basic D-amino acids (Pilone 2000), the method preferably involves measuring the D/L ratio of a specific aliphatic or aromatic D-amino acid or more than one aliphatic and aromatic D-amino acid.

It is appreciated that analysis of total levels and D/L ratios of individual D-amino acids may determine whether the patient has a DAO abnormality manifesting as an altered substrate specificity

Suitable methods for measuring D-amino acid levels are described in Zhujun *et al* (*Microchemical Journal*, vol. 52, pp. 131-138, 1995); Hamase *et al* 2001; Gutheil *et al* (*Anal Biochem*, vol. 287 (2), pp. 196-202, 2000);Bruckner & Hausch 1993; and D'Aniello *et al* (*J. Biol. Chem.*, vol. 268 (36), pp. 26941-9, 1993). The disclosure of each of these references relating to the determination of D-amino acid levels is incorporated herein by reference.

Some DAO abnormalities, such as a coding region mutation which leads to an amino acid substitution in the active site of the enzyme, may cause the enzyme to have reduced or absent DAO activity, even though DAO protein levels remain unaffected.

Thus, in another preferred embodiment of the invention, the method for analysing a sample from a patient to determine whether the patient has a DAO abnormality comprises determining the activity of DAO in the sample. Typically, a DAO activity in the sample below a predetermined normal range or level indicates that the patient has a DAO abnormality.

As is discussed in Example 1, the level of DAO activity from the spinal cord of control individuals without a DAO abnormality was $1.5 \pm 0.2 \times 10^{-3}$ Units/mg protein (mean ± SEM). In contrast, the level of DAO activity from the spinal cord of FALS patient 3.7 with the heterozygous R199W DAO abnormality was 0.065×10^{-3} Units/mg protein.

Thus, in an embodiment, a DAO activity in a sample below about 1.3×10^{-3} Units/mg protein indicates that the patient has a DAO abnormality. More preferably, a DAO activity below about 1.0, or 0.9, or 0.8, or 0.7, or 0.6, or 0.5, or 0.4, or 0.3, or 0.2, or 0.1×10^{-3} Units/mg protein indicates that the patient has a DAO abnormality. Still more preferably, a DAO activity below about 0.09, or 0.08, or 0.07, or 0.06, or 0.05, or 0.04, or 0.03, or 0.02, or 0.01 $\times 10^{-3}$ Units/mg protein indicates that the patient has a DAO abnormality.

Some DAO abnormalities may cause the enzyme to have an altered, ie reduced, increased, absent or new, susceptibility to inhibitors. For example, DAO has been shown to be inhibited by creatinine (Nohara *et al*, *Nephron*, vol. 91 (2), pp 281-285, 2002). Thus, in another preferred embodiment of the invention, the method for analysing a sample from a patient to determine whether the patient has a DAO abnormality comprises determining the effect of DAO inhibitors on DAO in the sample. Typically, a reduced, increased, absent or new susceptibility to a DAO inhibitor indicates that the patient has a DAO abnormality.

Some DAO abnormalities, such as promoter region mutations or nonsense mutations may result in a reduced amount of DAO in a sample compared to the levels that should normally be present, or may result in the absence of DAO from a sample where it should normally be present. Alternatively, a promoter or enhancer region mutation may result in an increased amount of DAO in a sample compared to the levels that should normally be present, or may result in the presence of DAO in a sample where it should normally be

absent.

Thus, in yet another preferred embodiment of the invention, the method for analysing a sample from a patient to determine whether the patient has a DAO abnormality comprises determining the amount of DAO in the sample, wherein an amount of DAO outside a predetermined normal range for the sample is an indication that the patient has a DAO abnormality.

The sample containing DAO protein derived from the patient is conveniently a sample of the tissue in which DAO is usually present. Preferably, the sample is a liquid sample such as blood, plasma, serum, saliva, urine or cerebrospinal fluid. The sample may also be a leukocyte sample or a skin sample. Alternatively, and less preferred, the sample may be a tissue sample such a liver biopsy.

By "the amount of DAO protein" is meant the amount of DAO protein per unit mass of sample tissue or per unit volume of a liquid sample or per number of sample cells. To determine whether the amount of DAO indicates the presence of a DAO abnormality, the measured DAO level is compared to the amount of DAO protein per unit mass of known normal tissue, or per unit volume of a known normal liquid sample, or per unit number of normal cells.

The amount or level of DAO may be determined in a sample in any suitable way. Assaying DAO protein levels in a biological sample can occur using any method known in the art. Preferred for assaying DAO protein levels in a biological sample are antibody-based techniques. For example, DAO protein expression in tissues can be studied with classical immunohistological methods. In these, specific recognition is provided by the primary antibody (polyclonal or monoclonal) but the secondary detection system can utilise fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunohistological staining of tissue section for pathological examination is

obtained. Tissues can also be extracted, *eg* with urea and neutral detergent, for the liberation of DAO protein for Western blot or dot/slot assay (Jalkanen, M, *et al*, J. Cell. Biol. 101:976-985 (1985); Jalkanen, M, *et al*, J. Cell. Biol. 105:3087-3096 (1987)). In this technique, which is based on the use of cationic solid phases, quantitation of DAO protein can be accomplished using isolated DAO protein as a standard. This technique can also be applied to body fluids. With these samples, a molar concentration of DAO protein will aid to set standard values of DAO protein content for different body fluids, like serum, plasma, urine, spinal fluid, etc. The normal appearance of DAO protein amounts can then be set using values from healthy individuals, which can be compared to those obtained from a test subject.

Other antibody-based methods useful for detecting DAO protein include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, a DAO protein-specific monoclonal antibody can be used both as an immunoadsorbent and as an enzyme-labelled probe to detect and quantify the DAO protein. The amount of DAO protein present in the sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm. Such an ELISA for detecting a tumour antigen is described in Iacobelli *et al*, Breast Cancer Research and Treatment 11: 19-30 (1988). In another ELISA assay, two distinct specific monoclonal antibodies can be used to detect DAO protein in a body fluid. In this assay, one of the antibodies is used as the immunoadsorbent and the other as the enzyme-labelled probe.

The above techniques may be conducted essentially as a "one-step" or a "two-step" assay. The "one-step" assay involves contacting DAO protein with immobilised antibody and, without washing, contacting the mixture with the labelled antibody. The "two-step" assay involves washing before contacting the mixture with the labelled antibody. Other conventional methods may also

be employed as suitable. It is usually desirable to immobilise one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed from the sample.

Suitable labels include radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99}mTc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Further suitable labels for the DAO protein-specific antibodies of the present invention include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholine esterase.

Examples of suitable radioisotopic labels include ^{3}H, ^{111}In, ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{57}To, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{90}Y, ^{67}Cu, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd and ^{111}In. Examples of suitable non-radioactive isotopic labels include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Tr, and ^{56}Fe.

Examples of suitable fluorescent labels include an ^{152}Eu label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an o-phthaldehyde label, and a fluorescamine label.

Examples of suitable toxin labels include diphtheria toxin, ricin, and cholera toxin.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt

label, an oxalate ester label, a luciferin label, a luciferase label, and an aequorin label.

Typical techniques for binding the above-described labels to antibodies are provided by Kennedy *et al*, Clin. Chim. Acta 70:1-31 (1976), and Schurs *et al*, Clin. Chim. Acta 81:1-40 (1977). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleintide method, the m-maleimidobenzyl-N-hydroxy-succinimide ester method, all of which methods are incorporated by reference herein.

The antibodies may be monoclonal or polyclonal. Suitable monoclonal antibodies may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and applications", J G R Hurrell (CRC Press, 1982), both of which are incorporated herein by reference.

DAO-protein specific antibodies for use in the present invention can be raised against the intact DAO protein or an antigenic polypeptide fragment thereof, which may be presented together with a carrier protein, such as an albumin, to an animal system (such as rabbit or mouse) or, if it is long enough (at least about 25 amino acids), without a carrier.

It is appreciated that antibodies can be raised against DAO from a range of species, including humans and other mammals, as well as DAO from non-mammalian species.

It is further appreciated that as DAO is a conserved protein, an antibody raised against intact DAO protein from one species may be reactive against DAO from another species, although typically at a lower affinity. It is also appreciated that an antibody raised against a conserved antigenic polypeptide

fragment of DAO, such as at the active site, is more likely to be reactive against DAO from multiple species with high affinity.

In one preferred embodiment, the antibodies are reactive with DAO mutated at position 199 (DAO^{199mut}), *ie* with DAO that does not have Arg at position 199, as described in more detail with respect to the ninth aspect of the invention. Preferably, DAO^{199mut} contains Trp at amino acid position 199 (DAO 199W). In this embodiment, the antibodies preferably have a greater affinity for DAO^{199mut} than for wild-type DAO. More preferably, the antibodies have at least 1.5, or at least 2, or at least 5, or at least 10, or at least 50, or at least 100, or at least 500, or at least 1,000, or at least 5,000 times greater affinity for DAO^{199mut} than for wild-type DAO. Most preferably, the antibodies have at least 10,000 times greater affinity for DAO^{199mut} than for wild-type DAO.

As used herein, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')2 fragments) which are capable of specifically binding to DAO protein. Fab and F(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al*, J. Nucl. Med. 24:316-325 (1983)). Thus, these fragments are preferred. Alternatively, DAO protein-binding fragments can be produced through the application of recombinant DNA technology or through synthetic chemistry. It is further appreciated that other antibody-like molecules may be used in the method of the inventions including, for example, antibody derivatives which retain their antigen-binding sites, synthetic antibody-like molecules such as single-chain Fv fragments (ScFv) and domain antibodies (dAbs), and other molecules with antibody-like antigen binding motifs.

The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing the DAO protein or an antigenic

fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of DAO protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In the most preferred method, the antibodies of the present invention are monoclonal antibodies (or DAO protein binding fragments thereof). Such monoclonal antibodies can be prepared using hybridoma technology (Kohler *et al*, Nature 256:495 (1975); Kohler *et al*, Eur. J. Immunol 6:511 (1976); Kohler *et al*, Eur. J. Immunol. 6:292 (1976); Hammerling *et al*, in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., (1981) pp. 563-681). In general, such procedures involve immunising an animal (preferably a mouse) with a DAO protein antigen or, more preferably, with a DAO protein-expressing cell. Suitable cells can be recognised by their capacity to bind anti-DAO protein antibody. Such cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% foetal bovine serum (inactivated at about 56°C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 g/ml of streptomycin. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP20), available from the American Type Culture Collection, Rockville, Maryland. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands *et al* (Gastroenterology 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the DAO protein antigen.

Alternatively, additional antibodies capable of binding to the DAO protein antigen may be produced in a two-step procedure through the use of anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and that, therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, DAO-protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the DAO protein-specific antibody can be blocked by the DAO protein antigen. Such antibodies comprise anti-idiotypic antibodies to the DAO protein-specific antibody and can be used to immunize an animal to induce formation of further DAO protein-specific antibodies.

In a preferred embodiment of the invention, antibodies will immunoprecipitate DAO proteins from solution as well as react with DAO protein on western or immunoblots of polyacrylamide gels. In another preferred embodiment, antibodies will detect DAO proteins in paraffin or frozen tissue sections using immunocytochemical techniques. For example, a method involving FITC immunofluorescence may conveniently be used.

Preferred embodiments relating to methods for detecting DAO protein include enzyme linked immunosorbent assays (ELISA), radioimmunoassay (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays using monoclonal and/or polyclonal antibodies. Exemplary sandwich assays are described by David *et al* in US Patent Nos. 4,376,110 and 4,486,530, hereby incorporated by reference. Antibody staining of cells on slides may be used, using antibodies to DAO in methods well known in cytology laboratory diagnostic tests, as well known to those skilled in the art.

In addition to assaying DAO protein levels in a biological sample obtained from an individual, DAO protein can also be detected *in vivo* by imaging.

Thus in another preferred embodiment, the invention provides a method for analysing a patient to determine whether the patient has a DAO abnormality, comprising determining the amount of DAO in a tissue in the patient by *in vivo* imaging, wherein an amount of DAO outside a predetermined normal range for the sample, typically below the normal range, is an indication that the patient has a DAO abnormality. Antibody labels or markers for *in vivo* imaging of DAO protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or caesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labelling of nutrients for the relevant hybridoma.

A DAO protein-specific antibody or antibody fragment which has been labelled with an appropriate detectable imaging moiety, such as a radioisotope (for example, ^{131}I, ^{112}In, ^{99}mTc), a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously or intraperitoneally) into the patient to be examined for late-onset neurodegenerative disease.

It is appreciated that the size of the patient and the sensitivity of the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of 99mTc. The labelled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain DAO protein. A method for *in vivo* imaging of tumours is described by S.W. Burchiel *et al*, "Immunopharmaco-kinetics of Radiolabeled Antibodies and Their Fragments"

(Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982)).

Where *in vivo* imaging is used to detect enhanced levels of DAO protein for diagnosis in humans, it may be preferable to use "humanized" chimeric monoclonal antibodies. Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric antibodies are known in the art. See, for a review, Morrison, Science 229:1202 (1985); Oi *et al*, BioTechniques 4:214 (1986); Cabilly *et al*, U.S. Patent No. 4,816,567; Taniguchi *et al*, EP 171496; Morrison *et al*, EP 173494; Neuberger *et al*, WO 8601533; Robinson *et al*, WO 870267 1; Boulianne *et al*, Nature 312:643 (1984); Neuberger *et al*, Nature 314:268 (1985).

^{111}In is a preferred isotope where *in vivo* imaging is used since it avoids the problem of dehalogenation of the ^{121}I or ^{131}I-labeled monoclonal antibody by the liver. In addition, this radionucleotide has a more favourable gamma emission energy for imaging (Perkins *et al*, Eur. J. Nucl. Med. 10:296-301 (1985); Carasquillo *et al*, J. Nucl. Med. 28:281-287 (1987)). For example, ^{111}In coupled to monoclonal antibodies with 1-(P-isothiocyanatobenzyl)-DPTA has shown little uptake in non-tumorous tissues, particularly the liver, and therefore enhances specificity of localisation (Esteban *et al*, J. Nucl. Med. 28:861-870 (1987)).

Examples of nuclear magnetic resonance contrasting agents include heavy metal nuclei such as Gd, Mn and Fe.

In yet another preferred embodiment of the invention, the method for analysing a sample from a patient to determine whether the patient has a DAO abnormality comprises determining whether nucleic acid in the sample contains a mutation in a DAO gene, wherein the presence of a DAO mutation

is an indication that the patient has a DAO abnormality.

It is appreciated that a mutation in the transcribed regions of the DAO gene may be detected by analysing either RNA, cDNA or genomic DNA. However, a DAO mutation in a non-coding region, such as an intronic splice-site mutation or a promoter or enhancer mutation, is usually detected by analysing genomic DNA.

Typically, analysing the sample comprises amplifying DAO-encoding nucleic acid, and analysing the amplified nucleic acid. If the nucleic acid in the sample from the patient is RNA, the method may further comprise the prior step of reverse transcribing RNA in the sample.

A range of methods are known for analysing a nucleic acid for a mutation. Suitable techniques for isolation, manipulation, modification, amplification and analysis of nucleic acids are well known in the art and are described herein and, for example, in Sambrook *et al* (2001) "*Molecular Cloning, a Laboratory Manual*", 3rd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

A DAO abnormality can be detected by identifying loss of the gene encoding the protein, or mutations in the protein coding sequence or in associated DNA regions which govern the expression or processing of the coding region, leading to reduced or nil expression of the protein or expression of functionally inactive or functionally altered versions of the protein. Such genetic assay methods include the standard techniques of restriction fragment length polymorphism assays and PCR-based assays.

The assay may involve any suitable method for identifying such polymorphisms, such as: sequencing of the DNA at one or more of the relevant positions;

differential hybridisation of an oligonucleotide probe designed to hybridise at the relevant positions of either the wild-type or mutant sequence; denaturing gel electrophoresis following digestion with an appropriate restriction enzyme, preferably following amplification of the relevant DNA regions; S1 nuclease sequence analysis; non-denaturing gel electrophoresis, preferably following amplification of the relevant DNA regions; conventional RFLP (restriction fragment length polymorphism) assays; selective DNA amplification using oligonucleotides which are matched for the wild-type sequence and unmatched for the mutant sequence or *vice versa*; or the selective introduction of a restriction site using a PCR (or similar) primer matched for the wild-type or mutant genotype, followed by a restriction digest. The assay may be indirect, *ie* capable of detecting a mutation at another position or gene which is known to be linked to one or more of the mutant positions. The probes and primers may be fragments of DNA isolated from nature or may be synthetic.

A non-denaturing gel may be used to detect differing lengths of fragments resulting from digestion with an appropriate restriction enzyme. The DNA is usually amplified before digestion, for example using the polymerase chain reaction (PCR) method and modifications thereof. Otherwise 10-100 times more DNA would need to be obtained in the first place, and even then the assay would work only if the restriction enzyme cuts DNA infrequently.

Amplification of DNA may be achieved by the established PCR method as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491 or by developments thereof or alternatives such as the ligase chain reaction, QB replicase and nucleic acid sequence-based amplification.

An "appropriate restriction enzyme" is one which will recognise and cut the wild-type sequence and not the mutated sequence or *vice versa*. The sequence which is recognised and cut by the restriction enzyme (or not, as the case may be) can be present as a consequence of the mutation or it can be introduced into

the normal or mutant allele using mismatched oligonucleotides in the PCR reaction. It is convenient if the enzyme cuts DNA only infrequently, in other words if it recognises a sequence which occurs only rarely.

In another method, a pair of PCR primers are used which match (*ie* hybridise to) either the wild-type DAO gene or to a mutated DAO but not to both. Whether amplified DNA is produced will then indicate the wild-type or mutated DAO genotype (and hence phenotype). However, this method relies partly on a negative result (*ie* the absence of amplified DNA) which could be due to a technical failure. It is therefore less reliable and/or requires additional control experiments.

A preferable method employs similar PCR primers but, as well as hybridising to only one of the wild-type or mutant sequences, they introduce a restriction site which is not otherwise there in either the wild-type or mutant sequences.

Kits and assay components comprising PCR primers and oligonucleotides for hybridisation as described above form further embodiments of the invention. Suitable DAO oligonucleotides include those listed in Table 2.

The primer kit of the present invention is useful for determination of the nucleotide sequence of the DAO gene using the polymerase chain reaction. The kit comprises a set of pairs of single stranded DNA primers which can be annealed to sequences within or surrounding the DAO gene in order to prime amplifying DNA synthesis of the gene itself. The complete set allows synthesis of all of the nucleotides of the DAO gene coding sequences, *ie* the exons. The set of primers preferably allows synthesis of both intron and exon sequences, as gene mutations may be found in introns. The kit can also contain DNA polymerase, preferably a thermophilic DNA polymerase, more preferably *Taq* polymerase, and suitable reaction buffers. Such components are known in the art.

In order to facilitate subsequent cloning of amplified sequences, primers may have restriction enzyme sites appended to their 5' ends. Thus, all nucleotides of the primers are derived from DAO gene sequences or sequences adjacent to that gene except the few nucleotides necessary to form a restriction enzyme site. Such enzymes and sites are well known in the art. The primers themselves can be synthesised using techniques which are well known in the art. Generally, the primers can be made using synthesising machines which are commercially available. Given the sequence of the DAO gene (cDNA sequence: Genbank Accession No. NM_001917; genomic sequence: Genbank Accession No. NT_009660), design of particular primers is well within the ability of a person of skill in the art.

The nucleic acid probes provided by the present invention are useful for a number of purposes. They can be used in Southern hybridization to genomic DNA and in the RNase protection method for detecting point mutations already discussed above. The probes can be used to detect PCR amplification products. They may also be used to detect mismatches with the DAO gene or mRNA using other techniques. Mismatches can be detected using either enzymes (*eg* S1 nuclease), chemicals (*eg* hydroxylamine or osmium tetroxide and piperidine), or changes in electrophoretic mobility of mismatched hybrids as compared to totally matched hybrids or by single-stranded conformational polymorphism (SSCP). These techniques are known in the art. Generally, the probes are complementary to DAO gene coding sequences, although probes to certain introns are also contemplated. An entire battery of nucleic acid probes may be used to compose a kit for detecting loss of wild-type DAO genes. The kit allows for hybridisation to the entire DAO gene. The probes may overlap with each other or be contiguous.

If a riboprobe is used to detect mismatches with mRNA, it is complementary to the mRNA of the human wild-type DAO gene. The riboprobe thus is an anti-

sense probe in that it does not code for the DAO protein because it is of the opposite polarity to the sense strand. The riboprobe generally will be radioactively labelled which can be accomplished by any means known in the art. If the riboprobe is used to detect mismatches with DNA it can be of either polarity, sense or anti-sense. Similarly, DNA probes also may be used to detect mismatches.

Nucleic acid probes may also be complementary to mutant alleles of the DAO gene. These are useful to detect similar mutations in other patients on the basis of hybridisation rather than mismatches. These are discussed above and referred to as allele-specific probes. As mentioned above, the DAO gene probes can also be used in Southern hybridisation to genomic DNA to detect gross chromosomal changes such as deletions and insertions. In addition, the probes can be used to detect DAO gene mRNA in tissue to determine if expression is diminished as a result of loss of wild-type DAO genes. Provided with the DAO gene coding sequence (Genbank Accession No. NM_001917) design of particular probes is well within the skill of the ordinary artisan.

Preferably, levels of mRNA encoding the DAO protein are assayed using the RT-PCR method described in Makino *et al*, Technique 2:295-301 (1990).

Briefly, this method involves adding total RNA isolated from a biological sample in a reaction mixture containing a reverse transcriptase (RT) primer and appropriate buffer. After incubating for primer annealing, the mixture can be supplemented with a RT buffer, dNTPs, dithiothreitol (DTT), RNase inhibitor and reverse transcriptase. After incubation to achieve reverse transcription of the RNA, the RT products are then subject to PCR using labelled primers. Alternatively, rather than labelling the primers, a labelled dNTP can be included in the PCR reaction mixture. PCR amplification can be performed in a DNA thermal cycler according to conventional techniques. After a suitable number of rounds to achieve amplification, the PCR reaction

mixture is electrophoresed on a polyacrylamide gel. After drying the gel, the radioactivity of the appropriate bands is quantified using an imaging analyser. By this method, the radioactivity of the "amplicons" in the polyacrylamide gel bands are linearly related to the initial concentration of the target mRNA.

RT and PCR reaction ingredients and conditions, reagent and gel concentrations, and labelling methods are well known in the art. Variations on the RT-PCR method will be apparent to the skilled artisan. Any set of oligonucleotide primers which will amplify reverse transcribed target mRNA can be used and can be designed based upon the sequence of the DAO gene.

In one preferred embodiment, the patient from whom the sample is taken is a foetus.

Typically, DAO nucleic acid from the patient is compared to DAO nucleic acid of a relative of the patient, more preferably to DAO nucleic acid of more than one relative of the patient. Most preferably, the nucleic acid from the patient is compared with the nucleic acid of at least one relative of the patient who has the late-onset neurodegenerative disease, and with the nucleic acid of at least one relative of the patient who does not have the late-onset neurodegenerative disease. Preferably, the relative who does not have the late-onset neurodegenerative disease is of an age to have shown symptoms of the disease if he or she were likely to do so.

The presence of a mutation in the DAO gene of the patient, that is also present in relatives of the patient with the disease and not found in relatives without the disease, is an indication that the patient has a DAO abnormality.

Thus the presence of a mutation in the DAO gene of the patient, that is present in relatives of the patient with the disease and not found in relatives without the disease, is an indication that the patient has an increased likelihood of

developing the late-onset neurodegenerative disease.

In a preferred embodiment, the DAO mutation causes an Arg to Trp change at amino acid 199 of DAO (DAO R199W). This mutation severely reduces the activity of the DAO enzyme, see Example 1.

A second aspect of the invention provides a pharmaceutical composition comprising DAO and a pharmaceutically acceptable carrier.

It is appreciated that non-human DAO may be suitable for use in the composition of the second aspect of the invention. Preferably, the DAO is from the species to which the pharmaceutical composition is to be administered, or has a specificity and activity similar to that of the DAO from the species to which the composition is to be administered. Thus for veterinary administration, DAO from the non-human animal species to be treated is preferred, and for human administration, human DAO is preferred.

The invention contemplates the use of fragments and modifications of wild type DAO as long as the DAO maintains its FAD-dependent ability to catalyse the oxidative deamination of D-amino acids. For example, non-human DAO can be humanised to reduce the risk of an unwanted immune-response against the protein by a human patient.

The pharmaceutically acceptable carrier(s) must be "acceptable" in the sense of being compatible with the DAO protein and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free; however, other acceptable carriers may be used.

The pharmaceutical compositions or formulations of the invention may be for topical administration or for parenteral or intravenous administration.

The pharmaceutical compositions or formulations may conveniently be

presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the DAO, *ie* the active ingredient, with the pharmaceutically acceptable carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (*eg* povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (*eg* sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges

comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

In a third aspect, the invention provides a pharmaceutical composition comprising a nucleic acid molecule that encodes DAO and a pharmaceutically acceptable carrier.

It is appreciated that nucleic acid encoding non-human DAO may be suitable for use in the composition of the third aspect of the invention. Preferably, the

nucleic acid encodes DAO from the species to which the pharmaceutical composition is to be administered, or encodes DAO which has a specificity and activity similar to that of the DAO from the species to which the composition is to be administered. Thus for veterinary administration, DNA nucleic acid encoding DAO from the species to be treated is preferred, and for human administration, nucleic acid encoding human DAO is preferred.

The invention contemplates the use of nucleic acid encoding fragments and modifications of wild type DAO as long as the DAO maintains its FAD-dependent ability to catalyse the oxidative deamination of D-amino acids. For example, nucleic acid encoding non-human DAO can be humanised to reduce the risk of an unwanted immune-response in humans against the protein.

It is appreciated that the nucleic acid encoding DAO may be RNA or DNA, and the DNA may be single or double stranded, and may be cDNA or genomic DNA

Preferably, the nucleic acid encoding human DAO consists of or comprises the human DAO cDNA sequence listed in Genbank Accession No. NM_001917, or encodes DAO having the amino acid sequence listed in Genbank Accession No. NP_001908 and in Figure 5 (SEQ ID No. 1), or the complement thereof.

The recombinant polynucleotide encoding DAO may be expressed from any suitable genetic construct as is described below and delivered to the patient. Typically, the genetic construct which expresses the DAO comprises the DAO coding sequence operatively linked to a promoter which can express the transcribed polynucleotide (eg mRNA) molecule in a cell to which it has been delivered, and which may be translated to synthesise the DAO polypeptide. Suitable promoters will be known to those skilled in the art, and may include promoters for ubiquitously expressed, for example housekeeping genes or for

tissue-specific genes, depending upon where it is desired to express the DAO polypeptide, as discussed further below.

Preferably, the genetic construct is adapted for delivery to a human cell, more preferably, to a human neuronal cell.

In a fourth aspect the invention provides a method for treating a patient with a late-onset neurodegenerative disease comprising the step of providing to the patient an effective amount of DAO, or a pharmaceutical composition as described in the second or third aspect of the invention.

The invention includes a method for treating a patient with ALS comprising the step of providing to the patient an effective amount of DAO, or a pharmaceutical composition as described in the second or third aspect of the invention.

The invention also includes a method for treating a patient with AD comprising the step of providing to the patient an effective amount of DAO, or a pharmaceutical composition as described in the second or third aspect of the invention.

The invention includes a method for treating a patient with PD comprising the step of providing to the patient an effective amount of DAO, or a pharmaceutical composition as described in the second or third aspect of the invention.

In one embodiment, providing DAO to the patient comprises administering DAO protein or a pharmaceutical composition containing DAO protein, such as a composition described above with reference to the second aspect of the invention, to the patient.

Preferably, if the late-onset neurodegenerative disease is ALS, the method

further comprises administering to the patient an agent selected from *i*) methylcobalamin, *ii*) 1-(2-naphth-2-ylethyl)-4-(3-trifluoromethylphenyl)-1,2,3,6- tetrahydropyridine, *iii*) a non-cysteine glutathione precursor, *iv*) a glutathione derivative, *v*) topiramate, or v*i*) 2-amino-6-(trifluoromethoxy)benzothiazole, or a pharmaceutically acceptable salt thereof.

Preferably, if the late-onset neurodegenerative disease is PD, the method further comprises administering either or both of L-DOPA and a DOPA decarboxylase inhibitor to the patient. Additionally or alternatively, the method also comprises stereotaxic stimulation of the patient.

Preferably, if the late-onset neurodegenerative disease is AD, the method further comprises administering to the patient an acetylcholine esterase inhibitor.

In another embodiment, providing DAO to the patient comprises administering a nucleic acid that encodes DAO or a pharmaceutical composition containing a nucleic acid that encodes DAO, such as a composition described above with reference to the third aspect of the invention, to the patient.

Means and methods of introducing a genetic construct into a cell in an animal body are known in the art. For example, the constructs of the invention may be introduced into the cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the (dividing) cell. Targeted retroviruses are available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into pre-existing viral *env* genes (see Miller & Vile (1995) *Faseb J.* 9, 190-199 for a review of this and other targeted vectors for gene therapy).

It will be appreciated that retroviral methods, such as those described below, may only be suitable when the cell is a dividing cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviral DNA constructs which encode DAO may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a neo^R gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at -70°C. For the introduction of the retrovirus into the target cells, it is convenient to inject directly retroviral supernatant to which 10 μg/ml Polybrene has been added. The injection may be made into the area in which the target cells are present. It will thus be appreciated that retroviral delivery may be a less preferred delivery means in relation to the present invention.

Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes liposomes (Nässander *et al* (1992) *Cancer Res.* **52**, 646-653).

It will be appreciated that "naked DNA" and DNA complexed with cationic and neutral lipids may also be useful in introducing the DNA of the invention into cells of the patient to be treated. Non-viral approaches to gene therapy are described in Ledley (1995) *Human Gene Therapy* **6**, 1129-1144. Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al*

(1995) *Gene Therapy* **2**, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* **274**, 373-376 are also useful for delivering the genetic construct of the invention to a cell. Thus, it will be appreciated that a further aspect of the invention provides a virus or virus-like particle comprising a DAO-encoding genetic construct. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia and parvovirus.

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cell types which over-express a cell surface protein for which antibodies are available. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* **257**, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct of the invention for delivery to the target cells, for example, by forming the liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80,000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end-over-end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80,000 x g for 45 min. Immunoliposomes may be injected, for example intraperitoneally or directly into a site where the target cells are present.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct or other genetic construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA *via* electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and penton proteins, is internalised into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle, for example, as described below.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulphide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs or other genetic constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs of the invention are supplied to the target cells, a high level of expression from

the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other genetic constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example transferrin linked to the DNA construct or other genetic construct of the invention, the construct is taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex retroviral constructs; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

It may be desirable to locally perfuse an area comprising target cells with the suitable delivery vehicle comprising the genetic construct for a period of time; additionally or alternatively the delivery vehicle or genetic construct can be injected directly into accessible areas comprising target cells. It will be appreciated that in treating a case of late-onset neurodegenerative disease, it may be beneficial to deliver the delivery vehicle or genetic construct systemically; however, it may also or alternatively be beneficial to deliver the delivery vehicle or genetic construct to the nervous system, and especially to particular neurones therein, as a priority, for example by injection to motor neurons in spinal cord.

The genetic constructs of the invention can be prepared using methods well known in the art.

It will be appreciated that it may be desirable to be able to regulate temporally expression of the DAO in the cell. Thus, it may be desirable that expression of the DAO is directly or indirectly (see below) under the control of a promoter that may be regulated, for example by the concentration of a small molecule that may be administered to the patient when it is desired to activate or repress (depending upon whether the small molecule effects activation or repression of the promoter) expression of the said DAO. It will be appreciated that this may be of particular benefit if the expression construct is stable *ie* capable of expressing the DAO (in the presence of any necessary regulatory molecules) in the cell for a period of at least one week, one, two, three, four, five, six, eight months or one or more years.

A preferred construct of the invention may comprise a regulatable promoter. Examples of regulatable promoters include those referred to in the following papers: Rivera *et al* (1999) *Proc Natl Acad Sci USA* **96(15)**, 8657-62 (control by rapamycin, an orally bioavailable drug, using two separate adenovirus or adeno-associated virus (AAV) vectors, one encoding an inducible human growth hormone (hGH) target gene, and the other a bipartite rapamycin-regulated transcription factor); Magari *et al* (1997) *J Clin Invest* **100(11)**, 2865-72 (control by rapamycin); Bueler (1999) *Biol Chem* **380(6)**, 613-22 (review of adeno-associated viral vectors); Bohl *et al* (1998) *Blood* **92(5)**, 1512-7 (control by doxycycline in adeno-associated vector); Abruzzese *et al* (1996) *J Mol Med* **74(7)**, 379-92 (reviews induction factors e.g., hormones, growth factors, cytokines, cytostatics, irradiation, heat shock and associated responsive elements). Tetracycline-inducible vectors may also be used. These are activated by a relatively non toxic antibiotic that has been shown to be useful for regulating expression in mammalian cell cultures. Also, steroid-based inducers may be useful especially since the steroid receptor complex enters the nucleus where the DNA vector must be segregated prior to transcription.

This system may be further improved by regulating the expression at two levels, for example by using a tissue-specific promoter and a promoter controlled by an exogenous inducer/repressor, for example a small molecule inducer, as discussed above and known to those skilled in the art. Thus, one level of regulation may involve linking the DAO gene to an inducible promoter whilst a further level of regulation entails using a tissue-specific promoter to drive the gene encoding the requisite inducible transcription factor (which controls expression of the DAO gene from the inducible promoter). Control may further be improved by cell-type-specific targeting of the genetic construct.

Techniques and promoters described in WO99/55359 (concerning nerve sprouting/nerve regeneration) may be useful. For example, WO99/55359 describes a promoter specific for peptidergic nerves. Expression in other neurone types may also be achieved using a promoter specific for that neurone type, as known to those skilled in the art. The cell-type specific promoter may be used directly to control the DAO gene, or to control the gene encoding the inducible transcription factor. Further levels of control may also be used, as will be apparent to those skilled in the art.

In a particularly preferred embodiment of the invention, eukaryotic expression vectors encoding DAO and targeted to mammalian motor nerve endings may be employed in the treatment of a patient suffering from a late-onset neurodegenerative disease such as ALS, AD or PD. Alternatively, the DAO could be linked to the surface of liposomal or viral delivery vehicle to give cholinergic specificity. Such a targeted viral-based approach may be beneficial as many non-virulent systems are commercially available, for example as discussed above, especially those that include membrane fusion elements and allow intracellular delivery of genes.

Alternatively, or in addition, neuron-specificity may be achieved by placing

the nucleic acid encoding DAO under the control of a cholinergic specific promoter (see, for example, Naciff *et al* (1999) *J. Neurochem* **72**, 17-28, which describes the identification of a 6.4-kb DNA fragment from the mouse vesicular acetylcholine transporter (VAChT) gene, encompassing 633 bp of the 5'-flanking region of the mouse vesicular acetylcholine transporter, the entire open reading frame of the VAChT gene, contained within the first intron of the ChAT gene, and sequences upstream of the start coding sequences of the ChAT gene, which is capable of directing cholinergic neuron-specific expression). This system may be further improved by regulating the expression at two levels, for example by using an exogenous inducer, for example a small molecule inducer, as known to those skilled in the art. Only upon addition of the low molecular weight inducer would expression of DAO occur; in this way, the time and extent of the protein's production is carefully regulated. One level of regulation may involve linking the DAO gene to an inducible promoter whilst a further level of regulation entails using the cholinergic promoter to drive the gene encoding the requisite inducible transcription factor (which controls expression of the DAO gene from the inducible promoter).

It will be appreciated that the expressed DAO protein must also be produced at an appropriate level relative to other synaptic proteins for optimal functioning.

Thus, a further aspect of the invention provides a gene therapy delivery system comprising an inactive neurotoxin having specificity for a target nerve cell and a polynucleotide that encodes DAO and comprises a target nerve cell-specific promoter. The inactive neurotoxin may have specificity for a cholinergic neuron; it may be an inactive botulinum neurotoxin, for example an inactive botulinum toxin A, botulinum toxin B, botulinum toxin C, botulinum toxin D, botulinum toxin E, botulinum toxin F or botulinum toxin

G neurotoxin. The target nerve cell-specific promoter may be specific for cholinergic neurons; it may be a promoter for vesicular acetylcholine transporter (VAChT), for example a promoter from mouse VAChT as described in Naciff *et al* (1999) *J Neurochem* **72(1)**, 17-28.

It will be appreciated that the methods or constructs of the invention may be evaluated in, for example, dissociated primary neuronal cell cultures and/or nerve-muscle co-cultures, as known to those skilled in the art, before evaluation in whole animals. The methods described in de Pavia *et al* (1999) *Proc Natl Acad Sci USA* **96**, 3200-3205 may also be used in the evaluation of the methods or constructs of the invention.

The aforementioned recombinant polynucleotides encoding DAO or a formulation thereof may be administered by any conventional method including oral and parenteral (eg subcutaneous or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time. It is preferred that the DAO, construct or formulation is administered by injection, preferably intramuscular injection. It will be appreciated that an inducer, for example small molecule inducer as discussed above may preferably be administered orally.

Further delivery or targeting strategies may include the following. Ballistic compressed air driven DNA/protein coated nanoparticle penetration (*ie* BioRad device) of cells in culture or *in vivo* may be used. Plasmids for delivery should have cell-type specific promoters.

It will be appreciated that the DAO, polynucleotide or construct of the invention can be delivered to a locus by any means appropriate for localised administration of a drug. For example, a solution of the construct can be injected directly to a site or can be delivered by infusion using an infusion pump. The construct, for example, also can be incorporated into an

implantable device which when placed at the desired site, permits the construct to be released into the surrounding locus.

The nucleic acid construct encoding DAO, for example, may be administered via a hydrogel material. The hydrogel is non-inflammatory and biodegradable. Many such materials now are known, including those made from natural and synthetic polymers. In a preferred embodiment, the method exploits a hydrogel which is liquid below body temperature but gels to form a shape-retaining semisolid hydrogel at or near body temperature. Preferred hydrogel are polymers of ethylene oxide-propylene oxide repeating units. The properties of the polymer are dependent on the molecular weight of the polymer and the relative percentage of polyethylene oxide and polypropylene oxide in the polymer. Preferred hydrogels contain from about 10% to about 80% by weight ethylene oxide and from about 20% to about 90% by weight propylene oxide. A particularly preferred hydrogel contains about 70% polyethylene oxide and 30% polypropylene oxide. Hydrogels which can be used are available, for example, from BASF Corp., Parsippany, NJ, under the tradename Pluronic^{R}.

In this embodiment, the hydrogel is cooled to a liquid state and the construct, for example, is admixed into the liquid to a concentration of about 1 mg nucleic acid per gram of hydrogel. The resulting mixture then is applied onto the surface to be treated, for example by spraying or painting during surgery or using a catheter or endoscopic procedures. As the polymer warms, it solidifies to form a gel, and the construct diffuses out of the gel into the surrounding cells over a period of time defined by the exact composition of the gel.

The construct, for example, can be administered by means of other implants that are commercially available or described in the scientific literature, including liposomes, microcapsules and implantable devices. For example,

implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as ethylenevinyl acetate (EVAc), polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof can be used to locally deliver the construct. The construct can be incorporated into the material as it is polymerised or solidified, using melt or solvent evaporation techniques, or mechanically mixed with the material. In one embodiment, the oligonucleotides are mixed into or applied onto coatings for implantable devices such as dextran coated silica beads, stents, or catheters.

The construct, for example, may be administered to the patient systemically for both therapeutic and prophylactic purposes. The construct, for example may be administered by any effective method, as described above, for example, parenterally (eg intravenously, subcutaneously, intramuscularly) or by oral, nasal or other means which permit the construct, for example, to access and circulate in the patient's bloodstream. Construct administered systemically preferably are given in addition to locally administered construct, but also have utility in the absence of local administration. A dosage in the range of from about 0.1 to about 10 grams per administration to an adult human generally will be effective.

Vectors, constructs, tissue-specific promoters and routes of administration for gene therapy aspects of this invention are known to a person of skill in the art, and are described for example in WO 01/18038, which is incorporated herein by reference.

When the late-onset neurodegenerative disease is ALS, the nucleic acid is preferably targeted to motor neurones. One method for targeting DAO nucleic acid to motor neurones includes using a viral vector such as an adenovirus construct. The viral vector containing the DAO construct is injected into

muscle, and retrograde transport uptakes and transfers the vector into the nerve terminals in the muscle, along the axons, to the motor neurone cell body which lies in the spinal cord grey matter.

When the late-onset neurodegenerative disease is PD, the nucleic acid is preferably administered to the substantia nigra. One method for targeting DAO nucleic acid to the substantia nigra includes direct stereotaxic intracerebral injection into the substantia nigra, as is used, for example, for foetal tissue grafts.

When the late-onset neurodegenerative disease is AD, the nucleic acid is preferably administered to the basal forebrain. One method for targeting DAO nucleic acid to the basal forebrain includes injection to the basal forebrain, as is known in the art targeting neurotrophic factors to the basal forebrain in animals. Alternatively, intraventricular injection can be used to target a wider range of cortical regions.

In a fifth aspect, the invention provides DAO for use in medicine. Preferably, in this aspect, the DAO is in a pharmaceutical composition as defined above in the second aspect of the invention.

In a sixth aspect, the invention provides a nucleic acid molecule that encodes DAO for use in medicine. Preferably, in this aspect, the nucleic acid molecule that encodes DAO is in pharmaceutical composition as defined above in the third aspect of the invention.

The invention includes a nucleic acid molecule that encodes DAO in a disabled herpes simplex viral vector for use in medicine.

A seventh aspect of the invention provides the use of DAO in the preparation of a medicament for treating a late-onset neurodegenerative disease. Preferably, in this aspect, the DAO is as defined above in the second aspect of

the invention.

The invention includes the use of DAO in the preparation of a medicament for treating a patient with ALS.

The invention includes the use of DAO in the preparation of a medicament for treating a patient with AD.

The invention includes the use of DAO in the preparation of a medicament for treating a patient with PD.

An eighth aspect of the invention provides the use of a nucleic acid molecule that encodes DAO in the preparation of a medicament for treating a late-onset neurodegenerative disease. Preferably, in this aspect, the nucleic acid molecule that encodes DAO may be as defined above in the third aspect of the invention. Further preferably, in this aspect, the medicament that comprises a nucleic acid molecule that encodes DAO may be administered as defined above in the fourth aspect of the invention.

The invention includes the use of a nucleic acid molecule that encodes DAO in the preparation of a medicament for treating a patient with ALS.

The invention includes the use of a nucleic acid molecule that encodes DAO in the preparation of a medicament for treating a patient with AD.

The invention includes the use of a nucleic acid molecule that encodes DAO in the preparation of a medicament for treating a patient with PD.

A ninth aspect of the invention provides DAO mutated at position 199 (DAO^{199mut}), *ie* that does not have Arg at position 199. As can be seen from the sequence alignment in Figure 5, and in Figure 1 of Pilone (2000), DAO is highly conserved, and species ranging from mammals to yeast and bacteria all

have Arg at the amino acid equivalent to position 199 of human DAO. This conserved Arg is at position 199 in pig, cow and rabbit; position 198 in rat, mouse and *R. gracilis*; position 193 in *S. coelicolor*; position 207 in *S. pombe;* position 214 in *F. solani;* and position 213 in *T. variabilis*. The invention includes DAO from any species in which the amino acid at the position equivalent to position 199 in human DAO is mutated. For convenience, all such DAOs will be collectively known as DAO^{199mut}.

Least preferably, DAO^{199mut} has an amino acid with a basic side chain (Lys, His) at position 199. More preferably, DAO^{199mut} has an amino acid with an aliphatic side chain (Gly, Ala, Val, Leu, Ile, Pro); an aliphatic hydroxyl side chain (Ser, Thr); a sulphur containing side chain (Cys, Met); or an acidic or amide side chain (Asp, Glu, Asn Gln) at position 199. Yet more preferably, DAO^{199mut} has an amino acid with an aromatic side chain (Phe, Tyr, Trp) at position 199. Most preferably, DAO^{199mut} contains Trp at amino acid position 199 (DAO 199W) in place of Arg.

Preferably, the DAO^{199mut} is mammalian DAO^{199mut}, more preferably human DAO^{199mut}.

A tenth aspect of the invention provides a nucleic acid encoding DAO^{199mut} as defined in the ninth aspect of the invention, or the complement thereof. Preferably, the nucleic acid encodes DAO 199W.

An eleventh aspect of the invention provides a vector comprising the nucleic acid as defined in the tenth aspect of the invention.

Typical prokaryotic vector plasmids are: pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories (Richmond, CA, USA); p*Trc*99A, pKK223-3, pKK233-3, pDR540 and pRIT5 available from Pharmacia (Piscataway, NJ, USA); pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A,

pNH18A, pNH46A available from Stratagene Cloning Systems (La Jolla, CA 92037, USA).

A typical mammalian cell vector plasmid is pSVL available from Pharmacia (Piscataway, NJ, USA). This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells. An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia (Piscataway, NJ, USA). This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems (La Jolla, CA 92037, USA). Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

Generally, the nucleic acid encoding DAO^{199mut} is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. It may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host prior to insertion into the vector, although such controls are generally available in the expression vector. Thus, the insert nucleic acid encoding DAO^{199mut} may be operatively linked to an appropriate promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters and the promoters of retroviral LTRs. Other suitable promoters will be known to the skilled artisan. The expression constructs desirably also contain sites for transcription initiation and termination, and in the transcribed region, a ribosome binding site for translation. (Hastings *et al*, International Patent No. WO 98/16643, published 23 April 1998)

Methods well known to those skilled in the art can be used to construct expression vectors containing the coding sequence and, for example appropriate transcriptional or translational controls. One such method involves ligation via homopolymer tails. Homopolymer polydA (or polydC) tails are added to exposed 3' OH groups on the DNA fragment to be cloned by terminal deoxynucleotidyl transferases. The fragment is then capable of annealing to the polydT (or polydG) tails added to the ends of a linearised plasmid vector. Gaps left following annealing can be filled by DNA polymerase and the free ends joined by DNA ligase.

Another method involves ligation via cohesive ends. Compatible cohesive ends can be generated on the DNA fragment and vector by the action of suitable restriction enzymes. These ends will rapidly anneal through complementary base pairing and remaining nicks can be closed by the action of DNA ligase.

A further method uses synthetic molecules called linkers and adaptors. DNA fragments with blunt ends are generated by bacteriophage T4 DNA polymerase or *E.coli* DNA polymerase I which remove protruding 3' termini and fill in recessed 3' ends. Synthetic linkers, pieces of blunt-ended double-stranded DNA which contain recognition sequences for defined restriction enzymes, can be ligated to blunt-ended DNA fragments by T4 DNA ligase. They are subsequently digested with appropriate restriction enzymes to create cohesive ends and ligated to an expression vector with compatible termini. Adaptors are also chemically synthesised DNA fragments which contain one blunt end used for ligation but which also possess one preformed cohesive end.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the polynucleotide of the invention is to use the

polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

A twelfth aspect of the invention provides a host cell comprising a nucleic acid as defined in the tenth aspect of the invention or a vector as defined in the eleventh aspect of the invention.

The host cell can be either prokaryotic or eukaryotic. If the DAO^{199mut} polynucleotide, in the vector, is to be expressed as a glycoprotein, the host cell is a eukaryotic host cell, and preferably a mammalian host cell.

Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

In a preferred embodiment, the host cell is a mammalian neuronal cell, such as

ND7.

Preferably, the host cell is a human neuronal cell.

Transformation of appropriate cell hosts with a vector is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (2001) *Molecular Cloning, A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cell, bacterial cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* **2**, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5x PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Physical methods may be used for introducing DNA into animal and plant cells. For example, microinjection uses a very fine pipette to inject DNA molecules directly into the nucleus of the cells to be transformed. Another

example involves bombardment of the cells with high-velocity microprojectiles, usually particles of gold or tungsten that have been coated with DNA.

Successfully transformed cells, *ie* cells that contain nucleic acid encoding DAO^{199mut} can be identified by well known techniques. For example, one selection technique involves incorporating into the expression vector a DNA sequence (marker) that codes for a selectable trait in the transformed cell. These markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture, and tetracyclin, kanamycin or ampicillin resistance genes for culturing in *E.coli* and other bacteria. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

The marker gene can be used to identify transformants but it is desirable to determine which of the cells contain recombinant DNA molecules and which contain self-ligated vector molecules. This can be achieved by using a cloning vector where insertion of a DNA fragment destroys the integrity of one of the genes present on the molecule. Recombinants can therefore be identified because of loss of function of that gene.

Another method of identifying successfully transformed cells involves growing the cells resulting from the introduction of an expression construct of the present invention to produce the DAO^{199mut} protein. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when

the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

Host cells that have been transformed by the recombinant DAO^{199mut} nucleic acid, typically in a vector as described above, are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the DAO^{199mut}, which can then be recovered.

The DAO^{199mut} protein can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

For example, for expression in a baculovirus system, recombinant DNA encoding the DAO^{199mut} nucleic acid may be cloned into a suitable transfer vector such as pMelBac (Invitrogen). Co-transfection with baculovirus DNA (eg Bac-N-Blue/Invitrogen) results in a recombinant baculovirus encoding the spike gene. Infection of a suitable insect cell line (e.g. Sf9, Sf21, High Five/Invitrogen) at an appropriate multiplicity of infection leads to expression of the recombinant spike protein. Protein expression is confirmed by western

blotting or ELISA using appropriate reagents.

The invention thus includes a method of obtaining DAO^{199mut}. The method comprises culturing a host cell comprising a DAO^{199mut} nucleic acid, typically in a vector; expressing the protein in the host cell, and purifying the protein. The invention further includes the protein obtainable by this method.

In a thirteenth aspect, the invention includes a cell culture comprising the host cell as defined in twelfth aspect of the invention. Preferably, the cell culture is a mammalian or human neuronal cell culture comprising mammalian or human neuronal cells as defined in twelfth aspect of the invention.

Preferably, the cell culture expresses DAO^{199mut}, more preferably DAO 199W, and most preferably human DAO 199W.

It is appreciated that a cell culture expressing a particular gene, or having a particular activity does not necessitate every cell within that culture expressing the particular gene or having the particular activity. Rather, we include the meaning that a sufficient proportion of the cells within the culture express the particular gene or have the particular activity so that the culture, when considered as a whole, expresses the gene or has the activity.

In a preferred embodiment, the cell culture may have reduced or absent endogenous DAO activity. If the host cells in the culture normally express DAO, then a reduced or absent activity may be achieved by "knocking-out" the functional, endogenous copies of the DAO gene within the cell or cells used to found the culture. Suitable methods for preventing expression of a specific gene in a cell, such as by "knocking-out" functional, endogenous copies of the gene within the cell, are well known in the art and are described, for example, in Sambrook *et al* (2001) "*Molecular Cloning, a Laboratory Manual*", 3rd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory

Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

Preferably, the expression of DAO^{199mut} in the cell culture is inducible. Inducible expression is typically controlled via an inducible promoter. Suitable inducible promoters are known in the art and include the β-galactosidase promoter that is induced by IPTG; and the metallothionein promoters that are induced by the addition of heavy metals.

Additionally or alternatively, the expression of DAO^{199mut} in the cell culture is transient.

Suitable methods and promoters for inducible and transient expression of a nucleic acid, such as that encoding DAO^{199mut}, are well known in the art and are described, for example, in Sambrook *et al* (2001) "*Molecular Cloning, a Laboratory Manual*", 3rd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

In a preferred embodiment, the host cell as defined in twelfth aspect of the invention or the cell culture as defined above with respect to the thirteenth aspect of the invention, has reduced or absent endogenous SOD1 activity.

Additionally or alternatively, the host cell as defined in twelfth aspect of the invention or the cell culture as defined above with respect to the thirteenth aspect of the invention, has reduced or absent endogenous HSP27 activity.

Thus the invention includes cells, and a culture comprising the cells, that

(1) contain nucleic encoding DAO^{199mut}, and preferably express DAO^{199mut}.

(2) contain nucleic encoding DAO^{199mut}, and preferably express

DAO^{199mut}, and have reduced or no endogenous DAO activity.

(3) contain nucleic encoding DAO^{199mut}, and preferably express DAO^{199mut}, and have reduced or no endogenous SOD1 activity.

(4) contain nucleic encoding DAO^{199mut}, and preferably express DAO^{199mut}, and have reduced or no endogenous HSP27 activity.

(5) contain nucleic encoding DAO 199W, and preferably express DAO^{199mut}, and have reduced or no endogenous DAO activity, and have reduced or no endogenous SOD1 activity.

(6) contain nucleic encoding DAO^{199mut}, and preferably express DAO^{199mut}, and have reduced or no endogenous DAO activity, and have reduced or no endogenous HSP27 activity.

(7) contain nucleic encoding DAO^{199mut}, and preferably express DAO^{199mut}, and have reduced or no endogenous SOD1 activity, and have reduced or no endogenous HSP27 activity.

(8) contain nucleic encoding DAO^{199mut}, and preferably express DAO^{199mut}, and have reduced or no endogenous DAO activity, and have reduced or no endogenous SOD1 activity, and have reduced or no endogenous HSP27 activity.

In a fourteenth aspect, the invention provides a method of screening for a protective agent, comprising

(a) providing a cell culture as defined in the thirteenth aspect of the invention;

(b) expressing DAO^{199mut};

(c) providing a test agent; and

(d) assessing at least one characteristic of the cells in the culture,

wherein an improvement in the at least one characteristic of the cells in the presence of the test agent indicates that the test agent is a protective agent.

Preferably, the cells in the culture are mammalian cells, more preferably mammalian neuronal cells, most preferably, human neuronal cells.

Preferably, DAO^{199mut} is DAO 199W. More preferably DAO^{199mut} is human DAO 199W.

Hence, the protective agent identified by the method is a preferably a mammalian protective agent, more preferably a mammalian neuronal protective agent, and most preferably, a human neuronal protective agent.

It is appreciated that typically step (a) is performed first, and step (d) is performed last. Steps (b) and (c) can be performed in either order or substantially simultaneously.

It is further appreciated that step (b) can be performed substantially simultaneously with step (a), *ie* in a combined step of providing a neuronal cell culture that expresses DAO^{199mut}.

It is further appreciated that step (d), assessing at least one characteristic of the neuronal cells in the culture, may be an ongoing analysis, and, in addition to being performed after steps (a), (b) and (c), may also comprise initially assessing the at least one characteristic of the neuronal cells in the culture prior to step (b) or prior to step (c), or both of steps (b) and (c).

Typically, the protective agent identified by this method protects the cells against toxic effects of DAO^{199mut}.

In a preferred embodiment the method of screening for a protective agent further comprises (e) providing D-amino acids to the culture. Step (e) is typically performed before step (b).

Typically, the protective agent identified by this method protects cells against DAO abnormality, or protects cells against the accumulation of D-amino acids, or protects against late-onset neurodegenerative disease. Preferably, the protective agent identified by this method protects neuronal cells against DAO abnormality, or protects neuronal cells against the accumulation of D-amino acids, or protects humans against late-onset neurodegenerative disease

Thus the invention includes a method of screening for an agent that protects against ALS.

The invention also includes a method of screening for an agent that protects against AD.

The invention further includes a method of screening for an agent that protects against PD.

Typically, the at least one characteristic of the neuronal cells is selected from cell viability, cell survival, cell growth, apoptotic and non-apoptotic cell-death, and enzyme activities.

Suitable assays and methods for performing this aspect of the invention are known in the art, and are described herein, for example, in Example 3 and in Patel, Y. *et al* (2002) "Neuroprotective effects of copper/zinc-dependent superoxide dismutase against a wide range of death-inducing stimuli and proapoptotic effect of familial amyotrophic lateral sclerosis mutations" *Molecular Brain Research* **109**: 189-197.

The invention includes a protective agent identified by the methods of the

fourteenth aspect of the invention.

A fifteenth aspect of the invention provides an experimental model for screening for a neuronal protective agent, the model comprising non-human neuronal tissue with reduced or absent endogenous DAO activity.

The non-human neuronal tissue may be derived from a non-human animal with reduced or absent endogenous DAO activity. Additionally or alternatively, the endogenous DAO activity can be inhibited by use of DAO inhibiting compounds such as benzoate (Moses *et al* (1996)).

Preferably, the experimental model expresses DAO^{199mut}, more preferably DAO 199W, and most preferably human DAO 199W.

Preferably, the expression of DAO^{199mut} in the experimental model is inducible.

Additionally or alternatively, the expression of DAO^{199mut} may be transient.

In a preferred embodiment, the experimental model comprises a cerebral artery occlusion, neonatal axotomy or lesions of the substantia nigra. Suitable experimental models include those described in Example 4, and in Akbar, M.T. *et al* (2003) "The neuroprotective effects of heat shock protein 27 overexpression in transgenic animals against kainate-induced seizures and hippocampal cell death" *Journal of Biological Chemistry* **278:** 19956 - 19965.

A sixteenth aspect of the invention provides a method of screening for a protective agent, the method comprising:

(a) providing an experimental model according to the fifteenth aspect of the invention;

(b) expressing DAO^{199mut};

(c) providing a test agent; and

(d) assessing at least one characteristic of the tissue in the model,

wherein an improvement in the at least one characteristic of the tissue in the model in the presence of the test agent indicates that the test agent is a protective agent.

Preferably, the tissue in the culture is mammalian tissue, more preferably mammalian neuronal tissue.

Hence, the protective agent identified by the method is a preferably a mammalian protective agent, more preferably a mammalian neuronal protective agent.

It is appreciated that typically step (a) is performed first, and step (d) is performed last. Steps (b) and (c) can be performed in either order or substantially simultaneously.

It is further appreciated that step (b) can be performed substantially simultaneously with step (a), *ie* in a combined step of providing an experimental model that expresses DAO^{199mut}.

It is further appreciated that step (d), assessing at least one characteristic of the tissue in the model, may be an ongoing analysis, and, in addition to being performed after steps (a), (b) and (c), may also comprise initially assessing the at least one characteristic of the tissue in the model prior to step (b) or prior to step (c), or both of steps (b) and (c).

Typically, the protective agent identified by this method protects mammalian tissue, and preferably mammalian neuronal tissue, against the toxic effects of DAO^{199mut}.

In a preferred embodiment the method of screening for a protective agent further comprises (e) providing D-amino acids to the model. Step (e) is typically performed before step (b).

Typically, the protective agent identified by this method protects tissue against DAO abnormality, or protects tissue against the accumulation of D-amino acids, or protects against late-onset neurodegenerative disease. Preferably, the protective agent identified by this method protects mammalian tissue, and preferably mammalian neuronal tissue, against DAO abnormality or the accumulation of D-amino acids. Further preferably, the protective agent protects humans, against late-onset neurodegenerative disease.

Thus the invention includes a method of screening for an agent that protects against ALS.

The invention also includes a method of screening for an agent that protects against AD.

The invention further includes a method of screening for an agent that protects against PD.

For example, the invention includes screening for protection against loss of *substantia nigra* (SN) cells following 6-hydroxydopamine or MPTP or rotenone lesion of the SN. Cell loss or cell survival is assessed histologically, cytochemically by loss of tyrosine hydroxylase positive cells, or behaviourally by protection against response to dopaminergic agents such as apomorphine and amphetamine.

Typically, the at least one characteristic of the neuronal tissue is selected from tissue survival, apoptotic and non-apoptotic cell-death, and enzyme activities. Suitable assays and methods for performing this aspect of the invention are known in the art, and are described herein, for example, in Example 4.

The invention includes a protective agent identified by the methods of the sixteenth aspect of the invention.

A seventeenth aspect of the invention provides a transgenic non-human mammal having one or both endogenous copies of the DAO gene knocked-out.

Preferably, the non-human mammal with one or both endogenous copies of the DAO gene knocked-out expresses either human DAO or DAO^{199mut} or both.

An eighteenth aspect of the invention provides a non-human mammal that expresses either human DAO or DAO^{199mut}. Preferably, the non-human mammal has reduced or no endogenous DAO activity.

Preferably, the DAO^{199mut} is a DAO 199W, such as human DAO 199W or DAO 199W of the same species as the non-human mammal.

The non-human mammal of the seventeenth and eighteenth aspects of the invention is preferably a rodent and more preferably a mouse.

Examples of non-human mammals with reduced or no endogenous DAO activity include the ddY mouse (Konno & Yasumura (1983) and Sasaki *et al* (1992)) and the SAM mouse strains (Yokoyama *et al,* (2001)). Thus, mice according to the eighteenth aspect of the invention can be made by introducing nucleic acid encoding human DAO or DAO^{199mut} into ddY or SAM mice by conventional transgenic methods.

Alternatively, mice according to the seventeenth or eighteenth aspect of the invention can be made by knocking-out the endogenous DAO gene in mice of any background, and introducing nucleic acid encoding human DAO or DAO^{199mut} by conventional transgenic methods.

Further preferably, the non-human mammal according to the seventeenth or eighteenth aspect of the invention has reduced or no endogenous SOD1 activity.

Additionally or alternatively, the non-human mammal according to the seventeenth or eighteenth aspect of the invention may have reduced or no endogenous HSP27 activity.

The non-human mammal according to the seventeenth or eighteenth aspect of the invention may also have either or both of human SOD1 activity or human HSP27 activity, *ie* the non-human mammal expresses or overexpresses human SOD1 or human HSP27 or both.

Non-human mammals according to the seventeenth or eighteenth aspect of the invention that have one or both endogenous copies of DAO knocked-out, or that have reduced or no endogenous DAO, HSP27 or SOD1 activity, can be made by transgenic methods. Methods for deletion of one or both copies of a gene from a non-human mammal, such as a mouse, and which reduce or eliminate the activity of the corresponding protein, are known to a person of skill in the art.

Similarly, non-human mammals according to the seventeenth or eighteenth aspect of the invention that express human DAO, or DAO^{199mut}, or that have exogenous human HSP27 or SOD1 activity, can be made by transgenic methods. Methods for insertion and expression of a human gene into a non-human mammal, such as a mouse, are known to a person of skill in the art.

Suitable techniques for making transgenic animals, and particularly transgenic mice, are well known to a person of skill in the art, and are described herein, for example, in Example 4.

It is appreciated that once non-human mammals with one of the desired

characteristics have been made, non-human mammals with two, three, four, five or six of the desired characteristics can be bred and selected for using standard breeding and selection programmes.

Thus the invention includes a non-human mammal, such as a mouse, that has:

(1) one or both endogenous copies of the DAO gene knocked-out.

(2) one or both endogenous copies of the DAO gene knocked-out, and expresses human DAO.

(3) one or both endogenous copies of the DAO gene knocked-out, and expresses DAO^{199mut}.

(4) reduced or no endogenous DAO activity (preferably), and expresses human DAO.

(5) reduced or no endogenous DAO activity (preferably), and expresses DAO^{199mut}

(6) one or both endogenous copies of the DAO gene knocked-out and has reduced or no endogenous SOD1 activity.

(7) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, and has reduced or no endogenous SOD1 activity.

(8) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, and has reduced or no endogenous SOD1 activity.

(9) reduced or no endogenous DAO activity (preferably), expresses human DAO, and has reduced or no endogenous SOD1 activity.

(10) reduced or no endogenous DAO activity (preferably), expresses

DAO^{199mut}, and has reduced or no endogenous SOD1 activity.

(11) one or both endogenous copies of the DAO gene knocked-out, and has reduced or no endogenous HSP27 activity.

(12) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, and has reduced or no endogenous HSP27 activity.

(13) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, and has reduced or no endogenous HSP27 activity.

(14) reduced or no endogenous DAO activity (preferably), expresses human DAO, and has reduced or no endogenous HSP27 activity.

(15) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, and has reduced or no endogenous HSP27 activity.

(16) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous SOD1 activity, and has reduced or no endogenous HSP27 activity.

(17) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, has reduced or no endogenous SOD1 activity, and has reduced or no endogenous HSP27 activity.

(18) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, and has reduced or no endogenous HSP27 activity.

(19) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous SOD1 activity, and has reduced or no endogenous HSP27 activity.

(20) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut} and has reduced or no endogenous SOD1 activity, and has reduced or no endogenous HSP27 activity.

(21) one or both endogenous copies of the DAO gene knocked-out, and has human SOD1 activity.

(22) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, and has human SOD1 activity.

(23) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, and has human SOD1 activity.

(24) reduced or no endogenous DAO activity (preferably), expresses human DAO, and has human SOD1 activity.

(25) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, and has human SOD1 activity.

(26) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous SOD1 activity, and has human SOD1 activity.

(27) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, has reduced or no endogenous SOD1 activity, and has human SOD1 activity.

(28) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, and has human SOD1 activity.

(29) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous SOD1 activity, and has human SOD1 activity.

(30) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, and has human SOD1 activity.

(31) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(32) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(33) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, and has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(34) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(35) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(36) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(37) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, reduced or no endogenous SOD1 activity, and has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(38) one or both endogenous copies of the DAO gene knocked-out,

expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(39) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(40) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut} and has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, and has human SOD1 activity.

(41) one or both endogenous copies of the DAO gene knocked-out, and has human HSP27 activity.

(42) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, and has human HSP27 activity.

(43) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, and has human HSP27 activity.

(44) reduced or no endogenous DAO activity (preferably), expresses human DAO, and has human HSP27 activity.

(45) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, and has human HSP27 activity.

(46) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous SOD1 activity, and has human HSP27 activity.

(47) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, has reduced or no endogenous SOD1 activity, and has human HSP27 activity.

(48) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, and has human HSP27 activity.

(49) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous SOD1 activity, and has human HSP27 activity.

(50) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, and has human HSP27 activity.

(51) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(52) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(53) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, and has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(54) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(55) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(56) one or both endogenous copies of the DAO gene knocked-out, has

reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(57) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, reduced or no endogenous SOD1 activity, and has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(58) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(59) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(60) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut} and has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, and has human HSP27 activity.

(61) one or both endogenous copies of the DAO gene knocked-out, has human SOD1 activity, and has human HSP27 activity

(62) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, has human SOD1 activity, and has human HSP27 activity.

(63) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, has human SOD1 activity, and has human HSP27 activity.

(64) reduced or no endogenous DAO activity (preferably), expresses human DAO, has human SOD1 activity, and has human HSP27 activity.

(65) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, has human SOD1 activity, and has human HSP27 activity.

(66) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous SOD1 activity, has human SOD1 activity, and has human HSP27 activity.

(67) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, has reduced or no endogenous SOD1 activity, has human SOD1 activity, and has human HSP27 activity.

(68) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, has human SOD1 activity, and has human HSP27 activity.

(69) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous SOD1 activity, has human SOD1 activity, and has human HSP27 activity.

(70) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, has human SOD1 activity, and has human HSP27 activity.

(71) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(72) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(73) one or both endogenous copies of the DAO gene knocked-out,

expresses DAO^{199mut}, and has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(74) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(75) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut}, has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(76) one or both endogenous copies of the DAO gene knocked-out, has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(77) one or both endogenous copies of the DAO gene knocked-out, expresses human DAO, reduced or no endogenous SOD1 activity, and has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(78) one or both endogenous copies of the DAO gene knocked-out, expresses DAO^{199mut}, has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(79) reduced or no endogenous DAO activity (preferably), expresses human DAO, has reduced or no endogenous SOD1 activity, has reduced or no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

(80) reduced or no endogenous DAO activity (preferably), expresses DAO^{199mut} and has reduced or no endogenous SOD1 activity, has reduced or

no endogenous HSP27 activity, has human SOD1 activity, and has human HSP27 activity.

A nineteenth aspect of the invention provides a method of screening for an agent that protects against late-onset neurodegenerative disease, the method comprising:

(a) providing at least one test and at least one control non-human mammal according to the seventeenth or eighteenth aspect of the invention,

(b) administering a test agent to the test mammals, and

(c) assessing at least one characteristic of the test and control non-human mammals,

wherein an improvement in the at least one characteristic of the test mammal relative to the control mammal indicates that the test agent protects against late-onset neurodegenerative disease.

Thus the invention includes a method of screening for an agent that protects against ALS, comprising:

(a) providing at least one test and at least one control non-human mammal according to the seventeenth or eighteenth aspect of the invention,

(b) administering a test agent to the test mammals, and

(c) assessing at least one characteristic of the test and control non-human mammals,

wherein an improvement in the at least one characteristic of the test mammal relative to the control mammal indicates that the test agent protects against ALS.

Thus the invention includes a method of screening for an agent that protects against AD, comprising:

(a) providing at least one test and at least one control non-human mammal according to the seventeenth or eighteenth aspect of the invention,

(b) administering a test agent to the test mammals, and

(c) assessing at least one characteristic of the test and control non-human mammals,

wherein an improvement in the at least one characteristic of the test mammal relative to the control mammal indicates that the test agent protects against AD.

The invention also includes a method of screening for an agent that protects against PD, comprising:

(a) providing at least one test and at least one control non-human mammal according to the seventeenth or eighteenth aspect of the invention,

(b) administering a test agent to the test mammals, and

(c) assessing at least one characteristic of the test and control non-human mammals,

wherein an improvement in the at least one characteristic of the test mammal relative to the control mammal indicates that the test agent protects against PD.

The test agent identified by the method may protect against the toxic effects of DAO^{199mut}.

It is further appreciated that step (c), assessing at least one characteristic of the test and control mammals, is usually an ongoing analysis, and, in addition to being performed after steps (a) and (b), may also comprise initially assessing the at least one characteristic prior to step (b).

Preferably, the method of screening for a test agent further comprises (d) providing D-amino acids to the non-human mammal, typically in the diet.

The test agent identified by this method may protect against DAO abnormality, or against the accumulation of D-amino acids.

Typically, the at least one characteristic is selected from survival, ageing, neurodegeneration, behaviour, apoptotic and non-apoptotic cell death, and enzyme activities.

Suitable assays and methods for performing this aspect of the invention are known in the art, and are described herein, for example, in Example 4.

The invention includes an agent that protects against late-onset neurodegenerative disease identified by the methods of the nineteenth aspect of the invention.

We have also sequenced a second candidate gene, thioredoxin reductase (TXNRD1), from the FALS region defined by linkage analysis between D12S306 and D12S79 in affected individuals from FALS pedigrees without SOD1 mutations. We detected 23 single nucleotide polymorphisms (SNPs) in TXNRD1, including 8 which showed high frequencies of 31-71% of chromosomes tested. Some of the SNPs occured significantly more frequently in FALS compared to control cases whereas other SNPs occurred significantly more frequently in control cases as opposed to in FALS cases (Table 6).

SNPs that occur significantly more frequently in FALS compared to control

cases may define chromosome 12 alleles that are linked to or associated with late-onset neurodegenerative disease, or linked to or associated with an increased tendency to develop late-onset neurodegenerative disease, or linked to or associated with an increased rate of progression or severity of a late-onset neurodegenerative disease. Conversely, SNPs that occur significantly more frequently in control cases as opposed to in FALS cases may define chromosome 12 alleles that are protective against late-onset neurodegenerative disease, or may define chromosome 12 alleles that are associated with a protective effect against late-onset neurodegenerative disease.

Specifically, the following nucleotides were observed to occur more frequently in FALS compared to control cases: T at position 99182 (63.2% vs. 35.7%); A at position 99184 (63.2% vs. 38.1%); A at position 100938 (39.5% vs. 7.2%); C at position 103317 (76.3% vs. 59.5%); C at position 120276 (76.3% vs. 59.5%); and G at position 122443 (44.7% vs. 9.5%).

The following nucleotides were observed to occur more frequently in control cases compared to FALS cases: C at position 99182 (64.3% vs. 36.8%); T at position 99184 (61.9% vs. 36.8%); T at position 100938 (92.8% vs. 60.5%); T at position 103317 (40.5% vs. 23.7%); T at position 120276 (40.5% vs. 23.7%); and C at position 122443 (90.5% vs. 55.3%).

Nucleotide positions are taken from the nucleotide sequence of the locus 17454675 sequence feature view gi|17455675:c349115-21034 (NCBI).

A twentieth aspect of the invention provides a method of determining an increased likelihood of a patient developing a late-onset neurodegenerative disease, or of determining an increased likelihood of a patient having an increased rate of progression or increased severity of a late-onset neurodegenerative disease, the method comprising analysing DNA from the patient to determine whether the patient has T at position 99182, A at position

99184, A at position 100938, C at position 103317, C at position 120276, and/or G at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has an increased likelihood of developing a late-onset neurodegenerative disease, or an increased likelihood of having an increased rate of progression or increased severity of a late-onset neurodegenerative disease.

The invention includes a method of determining an increased likelihood of a patient developing ALS, or an increased likelihood of a patient having an increased rate of progression or increased severity of ALS, the method comprising analysing DNA from the patient to determine whether the patient has T at position 99182, A at position 99184, A at position 100938, C at position 103317, C at position 120276, and/or G at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has an increased likelihood of developing ALS, or an increased likelihood of having an increased rate of progression or increased severity of ALS.

The invention includes a method of determining an increased likelihood of a patient developing PD, or an increased likelihood of a patient having an increased rate of progression or increased severity of PD, the method comprising analysing DNA from the patient to determine whether the patient has T at position 99182, A at position 99184, A at position 100938, C at position 103317, C at position 120276, and/or G at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has an increased likelihood of developing PD, or an increased likelihood of having an increased rate of progression or increased severity of PD.

The invention includes a method of determining an increased likelihood of a

patient developing AD, or an increased likelihood of a patient having an increased rate of progression or increased severity of AD, the method comprising analysing DNA from the patient to determine whether the patient has T at position 99182, A at position 99184, A at position 100938, C at position 103317, C at position 120276, and/or G at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has an increased likelihood of developing AD, or an increased likelihood of having an increased rate of progression or increased severity of AD.

A twenty-first aspect of the invention provides a method of determining a reduced likelihood of a patient developing a late-onset neurodegenerative disease, or an increased likelihood of a reduced rate of progression or severity of a late-onset neurodegenerative disease in a patient, the method comprising analysing DNA from the patient to determine whether the patient has C at position 99182, T at position 99184, T at position 100938, T at position 103317, T at position 120276 and/or C at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has a reduced likelihood of developing a late-onset neurodegenerative disease, or an increased likelihood of a reduced rate of progression or severity of a late-onset neurodegenerative disease.

The invention includes a method of determining a reduced likelihood of a patient developing ALS, or of determining an increased likelihood of a reduced rate of progression or severity of ALS in a patient, the method comprising analysing DNA from the patient to determine whether the patient has C at position 99182, T at position 99184, T at position 100938, T at position 103317, T at position 120276 and/or C at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at

these positions is an indication that the patient has a reduced likelihood of developing ALS, or an increased likelihood of a reduced rate of progression or severity of ALS.

The invention includes a method of determining a reduced likelihood of a patient developing PD, or of determining an increased likelihood of a reduced rate of progression or severity of PD in a patient, the method comprising analysing DNA from the patient to determine whether the patient has C at position 99182, T at position 99184, T at position 100938, T at position 103317, T at position 120276 and/or C at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has a reduced likelihood of developing PD, or an increased likelihood of a reduced rate of progression or severity of PD.

The invention includes a method of determining a reduced likelihood of a patient developing AD, or of determining an increased likelihood of a reduced rate of progression or severity of AD in a patient, the method comprising analysing DNA from the patient to determine whether the patient has C at position 99182, T at position 99184, T at position 100938, T at position 103317, T at position 120276 and/or C at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has a reduced likelihood of developing AD, or an increased likelihood of a reduced rate of progression or severity of AD.

All of the documents referred to herein are incorporated herein, in their entirety, by reference.

The invention will now be described in more detail with the aid of the following Figures and Examples.

Figure 1

Haplotype analysis of the 12q21-24 region in family X. Allele data for 16 markers in the 12q21-24 region are shown, inferred genotypes are indicated by italics. The names of the makers are listed on the far left hand side of each generation within the family. Haplotypes associated with the disease are indicated by thick solid "boxing". Cross-over events are indicated by a change in the "boxing". Both males and females are indicated by a diamond symbol, affected individuals are represented by a solid black diamond, obligated carriers by a solid grey diamond and individuals of unconfirmed status with a half filled grey diamond.

Figure 2

Multipoint linkage analysis of chromosome 12 from D12S99 to D12S97. The lower (and darker) line represents the parametric LOD score, and the upper (and lighter) line represents the non-parametric LOD score (NPL).

Figure 3

A) A graphical representation of exons 5 to 7 of DAO and the intervening introns. An expansion of the genomic sequence (SEQ ID No. 2) including the exonic and flanking intronic sequence of exon 6 is shown underneath. Intronic sequences are in lower case, exonic sequences are in upper case. Point mutations are indicated by bold italics with the second of the two italicised nucleotides indicating the alternative nucleotide.

B) Automatic sequence electrographs showing part of the genomic sequence of human D-amino acid oxidase (SEQ ID No. 3). The upper electrograph shows the heterozygote point A-G intron 5 polymorphism, the lower electrograph shows the "normal" sequence.

C) Automatic sequence electrographs showing part of the genomic sequence of human D-amino acid oxidase (SEQ ID No. 4). The upper electrograph shows the heterozygote C-T exon 6 point mutation resulting in an Arg 199 Trp amino acid sequence alteration, the lower electrograph shows the "normal" sequence.

Figure 4.

A) DAO cDNA sequence (SEQ ID No. 5) showing the boundary between exons 4 and 7. The localisation of the primers used for the amplification of exon 6 is shown by arrows pointing in a 5' to 3' direction. The arrow above the published sequence corresponds to the complementary or forward primer, the arrow below the published sequence corresponds to the reverse complement or reverse primer. The heterozygote point mutation (C to T) is shown in italics.

B) First strand cDNA was obtained from total RNA extracted from spinal cord or motor cortex from either control, obligate carrier or affected ALS individuals. The cDNA was amplified by PCR using primers specific to human DAO exons 4 and 7 to yield a 296 base pair (bp) product which was electrophoreses on a 2% agarose (w/v) 1x TBE gel for 2 hours at 100V. Lane 1 contains the 900 bp control cDNA PCR product from control RNA supplied with the cDNA Cycle Kit for RT-PCR First-Strand cDNA Synthesis for PCR (MS2 bacteriophage, Invitrogen). Lanes 2 ,3, 4, and 5 used total RNA from control spinal cord, control motor cortex, obligate ALS carrier spinal cord and ALS spinal cord respectively. Lanes 2, 4 and 5 yielded the expected 296 bp cDNA PCR product. No detectable cDNA PCR product was detected from total RNA extracted from control motor cortex, lane 3.

C) To prove the presence of RNA and hence cDNA in all of the human cDNA samples used in B, the cDNA was amplified using primers specific to

the human house keeping gene Glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Lane 1 containing cDNA derived from MS2 bacteriophage (Invitrogen) contains no product. Lanes 2 to 5 show a strong band of relatively equal intensity showing the presence of RNA and hence cDNA in all of the samples.

Figure 5.

A) Ribbon representation of DAO from Porcine kidney showing the FAD co-factor (PDB:1AA8, MMDB:5815).

B) A magnified region of Porcine kidney DAO showing the amino acids in the active site of the enzyme. The amino acids involved in the active site in Pig are Tyr228 and His307 which are preserved in human, pig, mouse, rat, rabbit and several lower organisms. The location of the amino acids from the region of the active site of DAO from *Rhodotorula gracilis* are also indicated (Leu51, Phe242, Arg283, Gly313 and Leu316 from pig). Arg199 the site of the mutation (R199W) is also indicated.

C) Comparison of the amino acid sequences of DAO between human (SEQ ID No. 1) and four other mammalian organisms mouse (SEQ ID No. 6), rabbit (SEQ ID No. 7), rat (SEQ ID No. 8) and pig (SEQ ID No. 9) (accession numbers: human NP_001908, mouse NP_034148, rabbit JX0132, rat NP_446078, pig OXPGDA).

Figure 6

The effect of serum-withdrawal (0-48h) on ND7 stable cell lines expressing SOD1 or SOD1 mutations in the presence of all-trans-retinoic acid. Total cell death was assessed using the trypan blue exclusion assay in ND7 cells expressing vector cassette (vector control) or expressing the wild-type SOD1 gene, or the G93A mutant or G93R mutant SOD1 gene. The values represent

the mean of 5-6 independent determinations and error bars indicate the standard error of the mean. ANOVA analysis showed that the wild-type and G93R values were significantly different from the ND7 cell controls ($p < 0.05$). * indicates a significant ($p < 0.05$) decrease (34%) in cell death with wild-type SOD1 compared with vector control at 24h and a 19% decrease at 48h. A significant ($p < 0.05$) increase (36%) in cell death was also observed with the G93R mutant compared to vector at 24h. ** indicates a significant ($p < 0.005$) decrease (51%) in cell death with wild-type expressing SOD1 virus compared with G93R mutant at 24h..

Figure 7

Apoptotic cell death in ND7 stable cell lines expressing SOD1 or SOD1 mutations following serum-withdrawal (0-48h) in the presence of all-trans-retinoic acid. Apoptotic cell death was assessed using the TUNEL labelling method in ND7 cells expressing vector cassette (vector control) or expressing the wild type SOD1 gene, G93A mutant or G93R mutant gene. The values represent the mean of 5-7 independent determinations and error bars indicate the standard error of the mean. * indicates a significant ($p < 0.02$) increase (71%) in apoptotic cell death with G93R mutant compared with vector control at 24h. ** indicates a significant ($p < 0.001$) decrease (64%) in apoptotic cell death with wild-type SOD1 compared with vector control at 24h. ANOVA analysis showed that the wild-type and G93R values were significantly different from the controls.

Figure 8

Effect of treatment with staurosporine (1uM) on cell death (as assayed by trypan blue exclusion) in control ND7 cells or cells expressing wild type SOD or either of the two mutants (G93A or G93R). Values are the mean of five to six independent determinations carried out in triplicate whose standard error is

shown by the bars. Cell death was significantly different ($p < 0.05$) in SOD1–wild type (SOD) and G93A and G93R cell lines compared to control ND7 cells (ANOVA). Student's t-tests were carried out at each time point and * and ** indicate that values were significantly different from the ND7 control cells with $p < 0.05$ and $p < 0.01$ respectively

Figure 9

Effect of treatment with γ-interferon (panel A) or ischaemia/reperfusion (panel B) on cell death (as assayed by trypan blue exclusion) in control ND7 cells or cells expressing wild type SOD1 or either of the two mutants (G93A or G93R). Values are the mean of five to six independent determinations carried out in triplicate whose standard error is shown by the bars. (A) Cell death was significantly different ($p < 0.04$) in SOD1–wild type (SOD) and G93A and G93R cell lines compared to control ND7 cells (ANOVA). * and ** indicate that values were significantly different from the ND7 controls with $p < 0.01$ and $p < 0.001$ respectively (Student's t-test).

(B) Cell death was significantly different ($p < 0.05$) in SOD1–wild type (SOD) and G93A and G93R cell lines compared to control ND7 cells (ANOVA). * and ** indicate that values were significantly different from the ND7 controls with $p < 0.01$ and $p < 0.005$ respectively (Student's t-test).

Figure 10

SOD1 expression in HSV-infected BHK cells. Western blot analysis was carried out with a polyclonal antibody to human SOD1 using extracts from BHK cells infected with SOD1 wild-type (wt) virus (lane 1), SOD1 G93A virus (lane 2), SOD1 G93R virus (lane 3), lacZ virus (lane 4) or control uninfected cells (lane 5). The positive controls were a human white blood cell (WBC) sample (lane 6) and anti-sense virus (lane 7). The cells were lysed,

lysates were separated on 15% SDS-PAGE and transferred onto nitrocellulose paper and immunostained. The protein product corresponding to rodent SOD1 (R-SOD) in lanes 1-5 and 7 is a result of the cross-reactivity of the anti-human SOD1 polyclonal antibody with endogenous rodent SOD1. Expression of the human SOD1 (H-SOD) is observed in the positive control white blood cell sample (lane 6) and is seen after infection with the wild-type and mutant SOD1 recombinant HSV (lanes 1, 2 and 3). The higher molecular weight proteins seen at 30kDa in lanes 2 and 3 are unreduced SOD1 dimers.

Figure 11

Effect of infection with HSV vectors expressing SOD1 on ND7 cell death at time 0 and 24h following transfer to serum-free medium in the presence of all-trans- retinoic acid. Cell death was assessed using the trypan blue assay. ND7 cells were either mock infected (no virus) or infected with lacZ recombinant HSV-1 vector viruses (control) or viruses expressing wild type SOD1 or G93R mutant SOD1. The values represent the means of 4 independent determinations and error bars indicate the standard error of the mean. * indicates a significant ($p < 0.02$) increase in cell survival with the SOD1 virus compared with the vector control.

Figure 12

Western blots showing the tissue expression of the hHSP27 gene in three independent mouse lines. Immunoblots of total cellular proteins probed with an antibody directed against the HA-tag which is contiguous with the human HSP27 transgene. (-) represents protein samples from wild-type animals and (+) represents protein samples from animals expressing the hHSP27 transgene.

Figure 13

Hippocampal expression of hHSP27 in three independent transgenic mouse

lines. Immunohistochemistry was carried out using an antibody to the HA-tag which is contiguous with hHSP27. The left hand panels show expression in CA1 (A-D) and the right hand panels show expression in CA3 region of hippocampus (E-H) for wild-type (A, E), hHSP27(18) (B,F), hHSP27(64) (C,G) and hHSP27(59) (D,H). Arrows indicate labelled cells and arrow heads point to dendritic labeling. Scale bar = 20 (m.

Figure 14

Expression of hHSP27 in cardiac tissue in three independent transgenic mouse lines. Photomicrographs of hearts from 3 independent lines (B-D) and a control litter-mate (A) immunostained using an HA antibody and counterstained with haematoxylin. Expression was widespread throughout the heart. Variation in intensity between myocytes is a reflection of the angle of sectioning. Scale bar = 20 (m.

Figure 15

Expression of hHSP27 in transgenic animals. This is compared with the expression of mouse HSP25 which is not present in control animals but is detected in both wild-type and transgenic animals receiving an ip injection of kainate which produces a substantial induction of mouse HSP25. hHSP27 mRNA (A-D), mHSP25 mRNA (E-H) and GFAP mRNA (I-L) in horizontal brain sections from wild-type (A, C, E, G, I, K) and hHSP27(18) (B, D, F, H, J, L) mice killed 24 h after kainate (C, D, G, H, K, L) or PBS (A, B, E, F, I, J). hHSP27 mRNA is expressed exclusively in hHSP27(18) mice (B, D) and absent in PBS (A) and kainate (C) treated wild-type mice. mHSP25 mRNA is expressed specifically in kainate injected mice (G, H). GFAP mRNA is upregulated in kainate injected mice (K, L). Scale bar = 2 mm.

Figure 16

Representative in situ film autoradiographs showing expression of hHSP27 mRNA in horizontal (A, B, C) and coronal (D, E, F) sections from three independent mouse lines, hHSP27(18) (A, D), hHSP27(64) (B, E) and hHSP27(59) (C, F). There is widespread expression of hHSP27 mRNA throughout the brain. Note the intense signal for hHSP27 mRNA in dentate gyrus and CA1-3 subfields of the hippocampus in all three hHSP27 mouse lines. The greatest variation in expression levels between the three lines appears to be in the thalamus, striatum and cortex.

dg, dentate gyrus; CA1, CA2 and CA3, respective hippocampal fields; EnCx, entorhinal cortex, PiCx, piriform cortex; Th, thalamus; cc, corpus callosum; St, striatum. Scale bar = 2 mm.

Figure 17

Bright field photomicrographs of the CA3 region of the hippocampus of wild-type (A), hHSP27(18) (B), hHSP27(64) (C) and hHSP27(59) (D) mice showing mRNA expression of the hHSP27 transgene. Strong labeling is seen over pyramidal cells of the CA3 region in sections from all three mouse lines (B, C, D). hHSP27 mRNA was not detected in CA3 cells in wild-type (Wt) mouse sections (A). Scale bar = 20 (m.

Figure 18

Cellular localisation of hHSP27 mRNA in striatum (A, B), cerebellum (C, D), cortex (E, F) and corpus callosum (G, H) in sections from Wt mice (A, C, E and G), hHSP27(18) mice (B, H), hHSP27(64) mice (F) and hHSP27(59) mice (D). hHSP27 mRNA is abundant in striatum (B) and cortex (F) of hHSP27 transgenic mice. Note the intense labeling of Purkinje cells in the cerebellum (arrows in D) and the moderate labeling of glial cells in corpus callosum (arrows in H) in sections from mice expressing the hHSP27 transgene.

Sections from Wt mice do not show hHSP27 mRNA labeling. Scale bar = 20 (m.

Figure 19

Time course of seizure severity in Wt and hHSP27(18) mice following KA (25 mg/kg, i.p.). Maximal seizure score achieved during 30 min intervals was recorded for each animal over a period of 4 h, and the mean seizure score for each group (Wt or hHSP27(18)) plotted against time after kainate injection. Seizure scores are significantly reduced in hHSP27(18) mice at 60-180 min after KA compared to Wt mice. Inset: dose response to kainate (20 mg/kg, n=18; 25 mg/kg, n=39; 30 mg/kg, n=4) in Wt mice. Data points beyond 150 min for the 30 mg/kg group are not shown due to the high mortality rate at this dose. Data were analysed using non-parametric statistics (* $p<0.05$; ** $p<0.01$; *** $p<0.005$; Mann Whitney U-test). Data are shown as mean (SEM for ease of presentation. Seizure severity scores are described in the text.

Figure 20

Haemotoxylin-eosin stained sections of the hippocampal CA3 pyramidal cell layer 7 days following vehicle (PBS) or kainic acid (25mg/kg) administration (KA) to wild-type (Wt) or hHSP27(18) transgenic animals.

Normal cell distribution in CA3 is evident in sections from Wt-PBS (A), hHSP27(18)-PBS (B) and hHSP27(18)-KA (D) animals. Note the marked loss of pyramidal neurones in the CA3 region of the hippocampus in the KA treated wild-type mice (C) but not in transgenic mice (D). Eosinophilic-stained neurones are present only in the kainate-treated wild-type mice (arrow in C). Scale bar = 50 (m. The inset shows zones (I-IV) within hippocampus analysed for cell counting.

dg, dentate gyrus; CA1 and CA3, respective hippocampal fields

Figure 21

High magnification of haemotoxylin-eosin stained sections of the hippocampal CA3 pyramidal cell layer 7 days following vehicle (PBS) or kainic acid (25mg/kg) administration (KA) to wild-type (Wt) and hHSP27(18) transgenic animals.

High magnification sections (X 400) of the CA3 subfields show the extent of degeneration in KA-treated wild type mice (C and E). H & E staining clearly shows neurons with eosinophilic cytoplasm and large, round, basophilic clumps (arrow points to three in C and four in E). There are also signs of perineuronal vacuolation in the KA-treated tissue (arrowhead points to three in D, two in E and three in F). Glial cells in D are indicated by asterisks. Scale bar = 20 (m.

Figure 22

Neuronal cell density in the hippocampus of wild-type and hHSP27(18) transgenic mice following kainic acid (25mg/kg KA) administration intraperitoneally.

Histogram values (with SEMs shown by error bars) are means of neuronal cell density (cell number/mm2) in zones I-IV (defined in Figure 20) of the hippocampus in wild-type animals treated with PBS vehicle (Wt-PBS, n=3) or kainic acid (Wt-KA, n=6) or transgenic animals treated with PBS (hHSP27(18)-PBS, n=7) or kainic acid (hHSP27(18)-KA, n=6). Cell loss was most marked in zone III of the Wt animals treated with kainic acid compared to their PBS treated controls. * indicates that the value is significantly lower than the vehicle treated control group ($p<0.05$), Kruskal-Wallis non-parametric ANOVA test followed by Mann-Whitney U-test.

Figure 23

Reduced cardiac infarct size in hHSP27(18) transgenic mice following ischaemia.

Values are means of % infarction in Langendorff mouse hearts after 10 min stabilisation followed by 30 min ischaemia and 30 min reperfusion with SEMs shown by error bars. The study demonstrates that the mice with over-expression of human HSP27 developed a mean infarct size of 36.39 % while the wild type controls had a mean of 42.49 %, a significant difference of 6.1 % ($p < 0.006$).

Example 1: Linkage of FALS to Chromosome 12 and identification of a mutation in the DAO gene

Introduction

A 5-15cM genome linkage screen was performed on extended UK/European FALS pedigrees to identify FALS-associated loci and genes. Following the genome screen of FALS families lacking SOD1 mutations a maximum multipoint LOD score of + 4.1 ($p<0.004$) at D12S78 was obtained on chromosome 12q22-23 using Genehunter version 1.1 localising the area of linkage to a 20cM interval between the markers D12S1706 and D12S354.

D-amino acid oxidase (DAO), a candidate gene in this region, was sequenced in at least one affected individual from each family showing potential linkage to chromosome 12. A disease-associated mutation was detected in exon 6 transforming a C to T in codon 199 (Figure 3A). This substitution resulted in a coding change R199W which was shown to be significantly associated with disease in this family using the Fischer's exact test ($p < 0.0048$), and was absent from unaffected individuals in this family. The activity of the R199W mutated protein extracted from spinal cord was shown to be severely

depressed.

Methods

Patients.

A diagnosis of ALS was confirmed clinically in affected individuals with evidence of both upper and lower motor neuron involvement. Bulbar signs were also present. In the index case, the patient presented with weakness in one hand and arm with small muscle wasting. The age at onset was 40 y and the duration of illness was 21 months. A similar rapid disease progression was seen in other affected family members and one case was confirmed histologically following autopsy. The average age of death for 6 affected individuals where information was available was 46.6 y (range 42-56 y).

Extraction of DNA from blood

DNA was extracted from whole blood or the buffy coat layer using a Qiagen Blood and Tissue DNA extraction kit. Briefly, 200 µl samples were mixed with 200 µl "AL" buffer and 25 µl protease K (20 mg/ml) and incubated at 70°C for 10 min. Ethanol (210 µl) was added, the samples mixed and placed on Qiagen spin columns and centrifuged for 1 min at 13,000 rpm. The column was washed twice in "AW" buffer (500 µl) and centrifuged for 1 minute at 13,000 rpm. DNA was then eluted with 200 µl buffer for 10 min, followed by centrifugation for 2 min at 13,000 rpm.

Genotyping

Following an initial genome screen using the Human Genome Mapping Project (HGMP) linkage mapping set, a subset of families indicated putative linkage to chromosome 12. Further mapping was carried out using a total of thirty three CA-repeat microsatellite markers spanning a region of 104.37 cM

on chromosome 12. The order of the microsatellite markers was determined according to the Marshfield genetic map of chromosome 12 (http://research.marshfieldclinic.org/genetics/). One primer from each oligonucleotide marker was labelled at the 5' end with either FAM, HEX, TET or NED fluorochromes. PCR reactions were carried out in a total volume of 15 µl and contained the following reaction components: 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0) and 0.1% Triton® X-100) (Promega) 1.0 to 2.5 mM $MgCl_2$ (Invitrogen) depending on the particular microsatellite, 100 µM dNTP (Amersham Pharmacia Biotech), 0.5 µM forward and reverse appropriate primer, 50 ng genomic DNA and 0.4 U Platinum Taq DNA polymerase (Invitrogen). The annealing temperatures of the PCR reactions varied from 50 to 62°C depending on the microsatellite marker. Typical cycling conditions were 94°C for 4 min, 55°C for 30 s and 72°C for 30 s for 1 cycle followed by 94°C for 1 min, 55°C for 30 s and 72°C for 30 s for 33 cycles. A final cycle of 94°C for 1 min, 55°C for 30 s and 72°C for 2 min was used to complete the amplification reaction. 5 µl of the samples were then mixed with 1 µl 5 X orange agarose loading buffer (15% Ficoll (Sigma), 0.05% Orange G (Sigma)) and electrophoresed on a 2% agarose (w/v) 1 X TBE gel at 100 V for approximately 1 h to check for the presence of a PCR product. PCR samples were then pooled together on the basis of expected size and fluorescent label used. Electrophoresis of the pooled samples was performed on the ABI 310 Genetic Analyzer. Information concerning peak size of products, allele assignment and allele frequency was determined using the ABI software package Genotyper 2.5.

Table 1A. STS markers used in linkage analysis.

STS marker	Genbank ID.	Genetic Position (cM)*	No. of Alleles	Heterogeneity
D12S99	Z16984	12.60	11	0.83
D12S77	Z16443	20.27	13	0.87

D12S358	Z24363	26.23	11	0.76
D12S364	Z24574	30.60	14	0.86
D12S62	M83621	32.48	10	-
D12S87	Z16641	51.99	9	0.79
D12S368	Z24632	66.03	8	0.80
D12S83	Z16592	75.17	10	0.80
D12S43	-	80.52	7	-
D12S92	Z16894	83.19	9	0.75
D12S81	Z16574	93.69	11	0.81
D12S88	Z16709	94.49	10	0.84
D12S95	Z16932	96.09	6	0.77
D12S1716	Z51825	101.45	7	0.74
D12S1706	Z50957	104.12	11	0.88
D12S1671	Z53634	104.12	8	0.77
D12S306	Z23480	105.18	10	0.84
D12S1607	Z52446	107.86	10	0.75
D12S78	Z16444	111.87	15	0.91
D12S338	Z24104	111.87	9	0.76
D12S1597	Z52265	114.28	11	0.85
D12S353	Z24248	115.18	10	0.77
D12S330	Z23349	116.08	15	0.73
D12S84	Z16602	117.81	10	0.83
D12S1645	Z53129	119.55	12	0.77
D12S1646	Z53130	121.84	7	0.71
D12S354	Z24250	123.77	6	0.73
D12S79	Z16516	125.31	12	0.88
D12S366	Z24599	133.33	8	0.80
D12S1349	Z52094	139.61	11	0.75
D12S342	Z24144	144.83	11	0.73
D12S1675	Z53716	150.70	8	0.74
D12S97	Z16960	160.68	12	0.88

Table 1B. STS markers used in linkage analysis.

STS Marker	Forward primer sequence (5'- 3')	Reverse primer sequence (5'- 3')
D12S99	GGCAGAAGTGCCTGGG (SEQ ID No. 10)	TCGAGGGTGCAGGTGG (SEQ ID No. 11)
D12S77	GAAGGGCAACAACAGTGAA (SEQ ID No. 12)	CTTTTTTTTCTCCCCCACTC (SEQ ID No. 13)
D12S358	GCCTTTGGGAAACTTTGG (SEQ ID No. 14)	GCACAGATGAGATCCCGT (SEQ ID No. 15)
D12S364	CCAGTCCTGAACAGGC (SEQ ID No. 16)	CCCTGGAAGTCCCATC (SEQ ID No. 17)
D12S62	ATCTTGAAGCCCTGTGCAAG (SEQ ID No. 18)	CAGAGATTTCTTAAATGCCT (SEQ ID No. 19)
D12S87	CACTAGGTGATGCTGGACAT (SEQ ID No. 20)	CTGCACAAACACTTGAAACA (SEQ ID No. 21)
D12S368	GCAACACCTTTGTGATGAAAAT (SEQ ID No. 22)	AGTCTGCACAGCCTGTCC (SEQ ID No. 23)
D12S83	TTTTTGGAAGTCTATCAATTTGA (SEQ ID No. 24)	TAGCAGAGAAAGCCAATTCA (SEQ ID No. 25)
D12S43	AATGTCCTTGTACTTAGGAT (SEQ ID No. 26)	CACTTAATATCTCAATGTATAC (SEQ ID No. 27)
D12S92	TGCCCAAGTTGCAGATG (SEQ ID No. 28)	GTCAGGATTCCCCAGAAAA (SEQ ID No. 29)
D12S81	CTATTCCAGATGAGGGGGTC (SEQ ID No. 30)	TCAAATCATAAGGGTATCAGAAATT (SEQ ID No. 31)
D12S88	TGCACCACAGCATACCAGTA (SEQ ID No. 32)	AATGAACAGCAAAAACTAAGGGA (SEQ ID No. 33)
D12S95	AAGGTGCAATGGGCTA (SEQ ID No. 34)	CAACTGTGTGTGTTTATATGTG (SEQ ID No. 35)
D12S1716	ATTGCATTATGTATTTCTGGTG (SEQ ID No. 36)	GCCTGCCCTCAAGTAG (SEQ ID No. 37)
D12S1706	CCTATGATTTCCCATCAAGTTT (SEQ ID No. 38)	ATTATTAGGAGAGCCCTGGG (SEQ ID No. 39)
D12S1671	CTCTGAGATTTAGACCAGAAAGCAC (SEQ ID No. 40)	TAGGCATAGTACATGGGTGTCC (SEQ ID No. 41)

D12S306	GTGCTAAATGCACTGTGGC (SEQ ID No. 42)	TCCCAAGTATCCGGGAC (SEQ ID No. 43)
D12S1607	AGCTGAGATCATGCCACTG (SEQ ID No. 44)	TTGGTGAGCCCTGAAGAA (SEQ ID No. 45)
D12S78	CTTTGCAGCACCATGTATTT (SEQ ID No. 46)	ACTGCTGGCTTTAACAGAAA (SEQ ID No. 47)
D12S338	CTGGCTGTTGGCTGGA (SEQ ID No. 48)	AAAATGGGAGGTCACTTCTAAT (SEQ ID No. 49)
D12S1597	AGACTGCCTGAGCCTGG (SEQ ID No. 50)	ACCTGATTTTAGTTGAATGCCC (SEQ ID No. 51)
D12S353	AATTCATTGGGAGGGCA (SEQ ID No. 52)	TGGCAAATCGGAGAGC (SEQ ID No. 53)
D12S330	CAGGGGACTATAATCATGCC (SEQ ID No. 54)	AGCTCCAGACATGTGTATTCTATG (SEQ ID No. 55)
D12S84	GCTTACAGTAGGTGCTTAATAAATG (SEQ ID No. 56)	TGTCTCTAGGCTAATGGCTT (SEQ ID No. 57)
D12S1645	ACCACATACCTGGCTGTTAC (SEQ ID No. 58)	GGTTCAAGACCTCCCAAA (SEQ ID No. 59)
D12S1646	ACCACTCCATTGCTGGC (SEQ ID No. 60)	GCTGGGTAAGAACCTCTGC (SEQ ID No. 61)
D12S354	GGTGGTTCTGGGTCAGAT (SEQ ID No. 62)	GGTTTCCTAATTTCAAGTCAA (SEQ ID No. 63)
D12S79	TTGGACTGAACTGAGATGCC (SEQ ID No. 64)	TATGTGCACCCAGACTACCA (SEQ ID No. 65)
D12S366	CTCCTGTCTCGCTTCAG (SEQ ID No. 66)	GCTCTCACCAGGCAATTACTAT (SEQ ID No. 67)
D12S1349	CCCAAGAGTGACATTGGC (SEQ ID No. 68)	GCATGGCTGGAGCATAAG (SEQ ID No. 69)
D12S342	CGCTCTCACAGTTCTGGAGG (SEQ ID No. 70)	GCCAAGGCAGAGTTTTGGAC (SEQ ID No. 71)
D12S1675	TGCTGTTGCTGCTGTAATAAATC (SEQ ID No. 72)	GAATCGCTTGAACCCAGG (SEQ ID No. 73)
D12S97	GCTCGAACTCCTCAGTGAAG (SEQ ID No. 74)	AGACTCCTCATCGTGGAAAA (SEQ ID No. 75)

Linkage Analysis of genotyping data

Multipoint and 2-point analysis of genotyping data were performed using Genehunter version 1.1 and the MLINK program of the FASTLINK version 4.1p software package was used to calculate LOD scores. Individuals confirmed either clinically and/or neuropathologically with familial amyotrophic lateral sclerosis (FALS) were entered as affected, unaffected individuals at risk (< 80 years). Some pedigrees were too large for the Genehunter program to analyse and so were trimmed until they could be analysed. The analysis was carried out under the model that FALS was inherited in an autosomal dominant manner. The frequency of the mutant allele was taken as 0.0001. Simple counting estimates were used to determine marker allele frequencies from at least 52 individuals.

Amplification of DAO

The 10 exons plus 5' UTR of DAO were amplified from genomic DNA using primers (Sigma-Genosys) annealing to the flanking regions of DAO. PCR reactions were carried out in a total volume of 100 µl containing the following reaction components: 1 X PCR buffer (20 mM Tris-HCl (pH 8.0), 50 mM KCl) (Invitrogen), 100 µM dNTP (Amersham Pharmacia Biotech), 0.5 µM forward and reverse appropriate primers, 100 ng genomic DNA and 2 U Platinum Taq DNA polymerase (Invitrogen). The concentration of $MgCl_2$ (Invitrogen) varied for each exon. Exons U, 1, 4, 5 and 8 used 1.0 mM $MgCl_2$, exons 2, 3, 6, 7, 9, and 10 used 1.5 mM $MgCl_2$. Primers used in the reaction were designed from the nucleotide sequence data obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (NT_009660, Homo sapiens chromosome 12 [gi: 16163473] reverse complement strand (graphical view)) and are listed in Table 2. The annealing temperatures for the exons

varied from 58 to 65°C. A typical cycling condition for the amplification of DAO was 94°C for 4 min, 58°C for 30 s and 72°C for 30 s for 1 cycle followed by 94°C for 1 min, 58°C for 30 s and 72°C for 30 s for 33 cycles. A final cycle of 94°C for 1 min, 58°C for 30 s and 72°C for 2 min was used to complete the amplification reaction.

Table 2. Primer sequences and their locations used in the amplification and screening of DAO. The location of the DAO primers were taken from the nucleotide sequence of NT_009660, homo sapiens chromosome 12 [gi:16163473] reverse complement strand (graphical view).

Primer Name	Sequence	Position
Exon U for	CCCCTGTCCTGTGCCTCTC (SEQ ID No. 76)	31231-31213
Exon U rev	CTGGATGTTTGAGAGGCTGGA (SEQ ID No. 77)	30566-30586
Exon 1 for	GATATTCCTTCCTCCTAGGG (SEQ ID No. 78)	26102-26081
Exon 1 rev	GCGTGAACCACCACGCCT (SEQ ID No. 79)	25595-25612
Exon 2 for	GGCATTTGGGGAGTTGCAG (SEQ ID No. 80)	23647-23629
Exon 2 rev	GGAGTCTGTTGCCTACGAG (SEQ ID No. 81)	23255-23273
Exon 3 for	GGCCAGACTGGTCTGGA (SEQ ID No. 82)	21628-21612
Exon 3 rev	GGGTAGCCTCTCTGAAGGA (SEQ ID No. 83)	21239-21257
Exon 4 for	CCTTGCATTGAGCTCAAAACC (SEQ ID No. 84)	20901-20881
Exon 4 rev	CAGCGGTGATCAGGACCAA (SEQ ID No. 85)	20513-20531
Exon 5 for	GTGGTCATAGGAAGTGCTGG (SEQ ID No. 86)	18294-18275
Exon 5 rev	GAATAGGTGAGTGCTTCTGG (SEQ ID No. 87)	17759-17778
Exon 6 for	GGAGCCTCATAACAGCCTAT (SEQ ID No. 88)	16790-16771
Exon 6 rev	GATCAAGAGGAAATCCTGAAAG (SEQ ID No. 89)	16395-16406

Exon 7 for	GCATTTACATGTGTGTACTGG (SEQ ID No. 90)	14203-14183
Exon 7 rev	GGGCAATAAGAGTGAAACTCT (SEQ ID No. 91)	13684-13705
Exon 8 for	GGCAATGTTAGTGGTGGTGG (SEQ ID No. 92)	12537-12521
Exon 8 rev	GGCCAGACAGGATTCATTAG (SEQ ID No. 93)	11956-11975
Exon 9 for	GCTGAGAAATGGTAATAGTGATG (SEQ ID No. 94)	11860-11839
Exon 9 rev	GAGCAGGTCCTTAACCATCAT (SEQ ID No. 95)	11280-11300
Exon 10 for	CAACTGTTCCAGGGGGTCC (SEQ ID No. 96)	10685-11666
Exon 10 rev	CTAGTTCAGTGCCTGACCTC (SEQ ID No. 97)	9783-9802

Purification of PCR products by gel extraction

The PCR products (100 µl) were mixed with 20 µl 5 x orange agarose loading buffer (15% Ficoll (Sigma), 0.05% Orange G (Sigma)) and separated on a 2% agarose (w/v) (VWR International) 1 X TBE (VWR International) gel at 100V for approximately 2 h. The DNA bands were visualised using a long wavelength UV light source (302 nm) and the appropriate DNA band was then cut from the gel and placed into a 1.5ml microcentrifuge tube using a scalpel. The DNA was extracted from the agarose gel using the NucleoTrap Gel Extraction kit (Clontech). The protocol in brief was as follows: The agarose slice containing the DNA band was weighed and 300 µl of NT1 buffer was added for every 100 mg of agarose. 10 µl of NucleoTrap matrix was then added to the tube containing the sample and vortexed. The sample was then heated for 10 min at 50°C with regular vortexing every 2 min. After 10 min the NucleoTrap matrix was pelleted by centrifugation for 30 s at 10,000 rpm. Two washes using the NT2 buffer and two washes using NT3 buffer of the NucleoTrap matrix were then performed by re-suspension of the NucleoTrap matrix in the appropriate wash buffer followed by centrifugation for 30 s at 10,000 rpm. The NucleoTrap matrix (Clontech) was allowed to air dry for 15 min before it was re-suspended in 20 µl distilled H_2O.

Purification of PCR products by spin columns

The PCR products (100 µl) were purified using the Nucleospin Extraction kit (Clontech). The protocol in brief was as follows: The PCR samples were mixed with 400 µl NT2 buffer, loaded onto a Nucleospin column and centrifuged for 1 min at 13,000 rpm. Two 700 µl NT3 buffer washes were then carried out by passing the NT3 buffer through the Nucleospin column and centrifuge for 1 min at 13,000 rpm. The PCR products were then eluted off the columns in 50 µl distilled H_2O. The samples were then ethanol precipitated and resuspended in 30 µl H_2O.

DNA Sequencing

Purified PCR products were sequenced in both the forward and reverse directions. Reactions were carried out using the ABI Prism™ BigDye™ terminator kit (Applied Biosystems). Briefly, 50 ng of purified PCR product was mixed with 4 µl FS BigDye sequencing mix 3.2 pmols sequencing primer and de-ionized water to a final volume of 10 µl. The samples were cycled on an Applied Biosystems Gene Amp PCR system 9700 using the following conditions 96°C for 10 s, 50°C for 5 s and 60°C for 4 min for 35 cycles. Once the cycling reaction was complete the samples were purified by ethanol precipitation. The dry pellets were then mixed with 12 µl HiDi formamide (Applied Biosystems) prior to loading on the AB3100 (Applied Biosystems) sequencing machine.

Extraction of total RNA from the buffy coat layer

Whole blood (10 ml) was mixed with 1 ml acid citrate dextrose (1.5% citric acid, 2.5% trisodium citrate, 2% dextrose). The sample was then centrifuged at 2,500 rpm for 10 min. The plasma layer was removed to expose the buffy coat layer, which was removed and used for the extraction of total RNA. 1.6 ml RNAzol (Biogenesis) was mixed with the buffy coat in a sterile 15 ml falcon tube. The sample was homogenised with 20-25 strokes of a sterile 5 ml syringe and a 19 gauge needle. To the homogenised sample, 160 µl chloroform (VWR international) /isoamylalcohol (VWR international) at a ratio of 49:1 was added, mixed and placed on ice for 5 minutes. The sample was then centrifuged at 8,000 rpm for 15 min at 4°C. The aqueous layer was then transferred to a fresh sterile 15 ml falcon tube and placed on ice. 0.8 ml isopropanol (stored at -20°C) was mixed with the sample which was then left on ice for a minimum of 1 h. The sample was then centrifuged at 8,000 rpm for 15 min at 4°C to pellet the RNA. The aqueous layer was then removed carefully and discarded leaving the RNA pellet. The RNA pellet was

resuspended in 100 µl DEPC treated sterile distilled H_2O. 10 µl of the resuspended RNA was mixed with a further 490 µl DEPC treated sterile distilled H_2O to calculate the concentration of RNA using a UV spectrophotometer. 210 µl absolute ethanol (stored at -20°C) was added to the remaining 90 µl sample and stored at -70°C until needed.

First strand cDNA synthesis.

1st strand cDNA was obtained from total RNA using the cDNA Cycle Kit for RT-PCR First-Strand cDNA Synthesis for PCR (Invitrogen) according to the manufacturer's instructions. Briefly, 5 µg of total RNA plus 1 µl Oligo dT primer (0.2 µg/µl) in a total volume of 12.5 µl were heated for 10 min at 65°C and then placed at room temperature for 2 min. To this, 1.0 µl RNase inhibitor (10 U/µl), 4.0µl 5x RT buffer, 1.0 µl 100 µM dNTPs, 1.0 µl 80mM Sodium pyrophosphate and 0.5µl (10U/µl) AMV Reverse Transcriptase. The sample was then incubated for 60 min at 42°C followed by heat inactivation of the enzyme for 2 min at 95°C, then quickly placed on ice. The sample was then purified by performing a phenol chloroform extraction followed by ethanol precipitation. The cDNA pellet was resuspended in 20 µl sterile water. 2 µl of the 1st strand cDNA synthesis reaction was then mixed with a primer specific for DAO exon 4 in the forward (5') direction (5'-GCTGGTTCCACACAAGCCTAATTCT -3') (SEQ ID No. 98) and exon 7 in the reverse (3') direction (5'- GGGATGATGTACGGGGAATTG -3') (SEQ ID No. 99). 1 µM of both primers were added to the reaction together with 1x PCR buffer (20 mM Tris-HCl (pH 8.0), 50 mM KCl) (Invitrogen), 2.0 mM $MgCl_2$ and 1U Platinum Taq DNA polymerase (Invitrogen). Cycling conditions for the reaction were the same as for those described earlier in the method for the amplification of DAO.

Detection of DAO cDNA

The 296 bp amplified cDNA product of DAO from the various tissues together with Invitrogen's 900 bp internal cDNA control were electrophoresed on a 2% agarose (w/v) (VWR International) 1 X TBE (VWR International) gel together with a ϕX174/HaeIII DNA size marker (MBI Fermentas). Individual DNA bands were detected on the gel by staining with 0.5 μg/ml Ethidium bromide and visualised on a GelDoc 2000 system (BioRad).

Statistics

The statistical significance of the association of the Arg199Trp mutation in DAO with disease was determined by the use of the Fischer's exact test. Individuals whose status is known were used in the analysis.

Results

Chromosome 12 linkage.

A whole genome screen with microsatellite markers spaced 5-15 centimorgans apart was carried out on 13-16 families with confirmed ALS and lacking SOD1 mutations. This yielded a region with a significant multipoint LOD score of $> +3.0$ on chromosome 12. The region of this putative FALS locus was further refined by screening with additional markers. A total of 33 chromosome 12 markers were used. The major family contributing to this linkage provided DNA from 4 affected individuals and allowed partial reconstruction of 3 further individuals. Multipoint analysis was carried out without using the reconstructed genotypes and revealed a region of linkage between D12S1706 and D12S354 with a maximum LOD score in this family of + 2.94 ($p < 0.007$, non parametric LOD score (NPL score) = 5.78) at the marker D12S1645 (Figure 1). The maximum two point LOD scores determined using MLINK in this family (Table 3) were obtained for the following markers (Z_{MAX} at $\theta = 0$), D12S84 (1.54), D12S1645 (2.06) and

D12S1646 (1.66). Potential recombination events were indicated to occur in the regions flanking these markers i.e. between D12S306 and D12S79. This is indicated from the disease haplotype shown in Figure 2.

Table 3. Two-point LOD scores for FALS family X versus chromosome 12q21-24. The two-point LOD score were calculated using the MLINK program. Abbreviations used: maximum LOD score (Z_{max}), centromere (cent), q are telomere (qter).

Locus	Recombination fraction (θ)				
	0.00	0.1	0.2	0.3	Z_{MAX}
Cent					
D12S1716	0.22	0.18	0.12	0.06	0.22
D12S1706	-0.08	-0.03	-0.01	-0.00	-0.00
D12S1671	-0.24	0.27	0.27	0.16	0.27
D12S306	1.55	1.39	1.09	0.70	1.55
D12S1607	-0.89	-0.38	-0.13	-0.04	-0.04
D12S78	1.64	1.65	1.29	0.81	1.65
D12S338	-0.50	-0.074	0.02	0.04	0.04
D12S1597	-99.99	0.75	0.52	0.20	0.75
D12S353	-99.99	0.12	0.19	0.11	0.19
D12S330	-99.99	0.71	0.57	0.36	0.71
D12S84	1.54	1.28	0.90	0.47	1.54
D12S1645	2.06	1.6	1.10	0.59	2.06
D12S1646	1.66	1.28	0.88	0.47	1.66
D12S354	-99.99	0.18	0.27	0.22	0.27
D12S79	-99.99	0.88	0.89	0.60	0.89
D12S366	-99.99	0.73	0.76	0.50	0.76
Qter					

Sequence analysis and mutation screening of DAO.

The 11 exons of DAO which include exons 1-10 plus the 5'UTR exon termed "U" were initially sequenced in at least one affected individual from each family showing potential linkage to chromosome 12. A disease-associated mutation was detected in exon 6 transforming a C to T in codon 199 (Figure 3A). This substitution resulted in a coding change R199W which was shown to be significantly associated with disease in this family using the Fischer's exact test ($p < 0.0048$) and absent from unaffected individuals in this family. Exon 6 was also screened for this mutation in a total of 194 individuals (controls, sporadic ALS and FALS) in order to determine its prevalence in the general population but no further individuals carried this mutation (Table 4). No other coding alterations were found in DAO after screening all exons in 57 FALS families lacking SOD1 mutations. However a number of polymorphisms were detected, one in the 5'UTR, two in the 3'UTR, 13 were intronic and one was a non-coding exonic mutation (Ser93) and these are listed in Table 4. The most common was a previously reported polymorphism (position 25972) with a prevalence of 21% (of chromosomes) whilst the other polymorphisms showed a frequency of 0.8 - 8.3% (of chromosomes) in the FALS population. An example of an intronic mutation is shown in figure 3B. No extensive frequency analysis of these polymorphisms has been carried out in a control population. However, a preliminary study of 3 polymorphisms was carried out in FALS and controls. The intron 5 polymorphism (shown in figure 3B) was more prevalent in controls compared to FALS as was one of the intron 8 insertions (Table 4). The small numbers though did not allow valid statistical analysis. The locations of the mutation/polymorphisms, as listed in Table 4, were taken from the nucleotide sequence of NT_009660, Homo sapiens chromosome 12 [gi: 16163473] reverse complement strand (graphical view) (NCBI). Only one previously known sequence polymorphism (indicated with *, db_xref "= dbSNP:2111902") was found.

Table 4. DNA and amino acid sequence alterations for DAO. Occurrence of the mutation/polymorphisms as a percentage and as number of chromosomes in ALS, controls and combined are indicated.

Exon / Intron	Mutation / Polymorphism	Position	Percentage Occurrence in FALS (as no. of chromosomes)
Intron –1	T – G	25972*	21.18 (25/118)
Intron 1	G – A	23620	0.85 (1/118)
Exon 2	G – A (TCG – TCA) (Ser – Ser)	23409	2.54 (3/118)
Intron 3	G – A	20745	1.72 (2/116)
Intron 4	T – C	18168	4.31 (5/116)
Intron 4	G – C	18143	1.72 (2/116)
Intron 5	G – A	17896	1.72 (2/116)
Intron 5	A – G	16735	3.8 (1/28)
Exon 6	C – A (CGG – TGG) (Arg – Trp)	16593	2.2 (6/274)
Intron 7	T – C	12446	0.88 (1/114)
Intron 7	C – T	12289	3.51 (4/114)
Intron 8	G – A	12075	0.88 (1/114)
Intron 8	A – G	11746	1.01 (3/296)

Intron 8	Ins A	11600 – 11609	8.3 (21/254)
Intron 8	Ins C/T	11600 – 11601	1.2 (3/254)
Intron 9	T – C	11436	1.35 (4/296)
Intron 9	C – T	10526	2 (1/50)
Exon 10 (3' UTR)	C – A	10291	2.38 (3/126)
Exon 10 (3' UTR)	G – T	10101	3.17 (4/126)

Exon / Intron	**Polymorphism**	**Position**	**Percentage Occurrence in Screened Individuals (as a no. of chromosomes)**
			FALS SALS Controls Total
Intron 5	A – G	16735	3.8 (1/28) - 8.3 (4/48) 6.5 (5/76)
Exon 6	C – A	16593	2.2 (6/274) 0 (0/30) 0 (0/84) 2.3 (9/388)
Intron 8	Ins A	11600 – 11609	8.3 (21/254) - 9.5 (4/42) 8.4 (25/296)
Intron 8	Ins C/T	11600 – 11601	1.2 (3/254) – 4.8 (2/42) 1.7 (5/296)

DAO transcript analysis

The effect of the R199W mutation and associated variations on transcript processing was examined on spinal cord and motor cortex tissue available from individual 3.7 (Figure 1), a control and a sporadic ALS case. The cDNA was amplified using primers within exons 4 and 7 of DAO (Figure 4A) which yielded a 296bp PCR product. This PCR product was present in all three spinal cord samples (lanes 2 ,4 and 5 of Figure 4B) indicating the presence of DAO mRNA transcripts in this tissue. However, there was no detectable PCR product in the sample obtained from motor cortex (lane 3, Figure 4B) despite similar cDNA loading as indicated by the level of the human house keeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). A PCR product was detected of approximately equal intensity in all of the human tissue samples (lanes 2 to 5, Figure 4C) but not in the MS2 bacteriophage sample (lane 1, Figure 4C).

Measurement of DAO enzyme activity

DAO enzyme activity was assayed, using the method described below, from the spinal cord of individual 3.7 who is heterozygous for the R199W mutation, 4 controls (mean age 53 years [range: 20 – 87 years] and mean post mortem interval 27.4h [range: 14.5 - 41h]) and 7 additional ALS cases (mean age 67 years [range: 49-89 years] and mean post mortem interval 15.3h [range: 5 – 30h]). Human motor cortex and rat spinal cord samples were also assayed.

Tissue from human spinal cord and motor cortex and rat spinal cord was homogenised in 0.02 M sodium pyrophosphate pH 8.3 containing 1.4×10^{-5} M FAD (total volume 600 µl), centrifuged for 1 min at 4°C and the supernatant removed for DAO enzyme assay determination (Nagata Y *et al*, 1988, "Two spectrophotometric assays for D-amino acid oxidase: for the study of distribution patterns" *Int J Biochem.* **20(11)**: 1235-8). Enzyme activity was

assayed at 37°C in a total volume of 3 ml containing 0.05 M D-serine in 0.02 M sodium pyrophosphate containing 0.0065% dimethoxybenzidine, 100 µl of 1 mg/ml peroxidase and 200 µl tissue extract. Absorbance changes at 436 nm were recorded for 1 hr and used to determine enzyme units/mg (Worthington Biochemical Coroporation). Total protein was determined in the tissue homogenate using the method of Lowry *et al* (Lowry O *et al,* 1951, *J.B.C.* **193**: 265-275).

Enzyme activity (units/mg protein) in control and ALS spinal cord were 1.501 $\pm$ 0.213 x 10^{-3} (mean value $\pm$ SEM) and 1.721 $\pm$ 0.295 x 10^{-3} respectively compared to a similar value of 1.473 x10^{-3} units/mg in rat spinal cord. There was no significant difference between values in control and ALS spinal cord. Enzyme activity of individual 3.7 expressing the R199W mutation was measured twice using two different tissue samples and found to be markedly reduced, being 0.065 x 10^{-3} units/mg protein. No enzyme activity was detected in motor cortex which was consistent with the low levels of DAO mRNA found in this tissue.

Discussion

We have demonstrated a point mutation R199W in the open reading frame of the DAO gene in all affected individuals in an extended family with FALS. The substitution (CGG to TGG) changes the amino acid from arginine to tryptophan which is close to the active site, would be expected to have steric and charge effects and severely reduces the activity of the mutated protein.

The amino acid sequence of human DAO shows a high degree of homology with mouse, rabbit, rat and pig (80% identical with mouse, 83% identical with rabbit, 80% identical with rat and 84% identical with pig). A 3D model of pig DAO is shown in Figure 5A. The active site region containing the key residues such as Tyr228 and His307 is shown in more detail in Figure 5B. It

is predicted by similarity of the amino acid sequences that these same two amino acids are also the amino acids involved in the active site for all five of these organisms (Tyr228 and His307 for human, rabbit and pig and Tyr227 and His306 for mouse and rabbit). The disease associated mutation detected in this study occurs in codon 199 transforming an arginine into a tryptophan. As can be seen from Figure 5B, Arg199 lies in the active site close to the FAD residue and between Tyr228 and His307. This amino acid is highly conserved between all five organisms implicating the potential importance of this amino acid to the overall structure of the active site of the DAO protein (Figure 5C).

DAO protein sequence comparison between mammalian and lower organisms such as the yeast *Schizosaccharomyces pombe*, fungi *Rhodotorula gracilis*, *Trigonopsis variabilis*, *Fusarium solani* and bacteria *Streptomyces coelicor* show a much reduced amount of homology, 26 to 32% (data obtained from the NCBI). The Tyr228, Arg199 and His307 from the human DAO protein sequence were again conserved across all of these organisms (Pilone 2000). High resolution x-ray crystallographic structure analysis of DAO from *Rhodotorula gracilis* showed the amino acids to be involved in the active site in some manner (binding a D-amino acid or water) to be Asn54, Tyr223, Tyr238, Arg285, Ser335 and Gln339 (Umhau et al 2000) of these only Tyr228 and Arg283 have been conserved between *Rhodotorula gracilis* and human. The human and pig alternatives for the other amino acids (Leu51, Phe242, Gly313 and Leu316) are still present in the active site region and have been labelled in Figure 5B.

D-amino acids accumulate during the ageing process and have been detected in the neuritic plaques of Alzheimer's disease cases. A role for DAO in the ageing process was suggested from the murine model of ageing known as the senescence-accelerated mouse (SAM) which was developed from the AKR/J mouse strain (Takeda *et al* 1981). These mice develop senescence-related

signs such as systemic amyloidosis, immune responsiveness, hair loss and periophthalmic lesions. More recently a missense mutation has been identified in senescence-prone (SAMP) strains in the DAO gene at codon 181 causing a Gly to Arg transformation (Yokoyama *et al* 2001). However, Yokoyama *et al* also identified the identical DAO mutation in senescence resistant SAMR mouse strains. This findings, together with similar DAO activities in the cerebral cortex of SAMP and SAMR mice, led Yokoyama *et al* to "deny the possibility of a direct relationship between the reduction in the activity of DAO and ageing".

The Gly to Arg mutation at position 181 is also found in mutant mice of the ddY strain which lack DAO activity and show a characteristic high level of D-alanine excretion in their urine (Sasaki et al 1992). As can be seen from Figure 5, this amino acid lies close to the active site adjacent to residues involved in FAD binding.

More recently, evidence has suggested that D-serine one of the most abundant D-amino acids serves a physiological role as a co-agonist at the glycine site of the NMDA receptor. DAO is found in glial cells in close proximity to the neuronal distribution of NMDA receptors and attenuates NMDA transmission (Snyder and Kim 2000). Studies using DAO knock-out mice has shown that formalin induced nociceptive pathways are indeed enhanced in these animals (Wake *et al* 2001) suggesting a role for CNS DAO in regulating D-serine neuromodulation.

In vitro studies on cultured rats astrocytes have shown that there is a higher level of expression in astrocytes from the cerebellum than that from the cerebral cortex. It was also found that DAO was expressed at higher levels in type 1 compared to type 2 astrocytes (Urai *et al* 2002).

The current evidence therefore points to a potential physiological role for

DAO in regulating levels of D-serine and hence modulating NMDA receptor function and also in the metabolism of D-amino acids that might otherwise accumulate with toxic effects. Prior to the present invention, however, there was no evidence to suggest that DAO might be involved in late-onset neurodegenerative diseases, such as ALS, AD or PD.

The region between D12S95 and D12S330 also contains the ataxin 2 gene (D12S78-D12S79) which is abnormal in spinocerebellar ataxia type 2 (SCA2) and overlaps with the loci for two neuromuscular disorders which are also clinically distinct from FALS, (Imbert et al. 1996) scapuloperoneal spinal muscular atrophy (SPSMA) and spinal muscular atrophy of the lower limbs (SMAL). SPSMA is characterised by laryngeal palsy in the new-born, followed by progressive lower limb weakness and contractures in the first and second decade and is localised to a 19cM interval between D12S338 and D12S366 (Isozumi et al. 1996). SMAL is characterised by a congenital non-progressive muscular atrophy of the lower extremities and is localised to an 11cM interval between D12S78 and D12S1646 (van der Vleuten et al. 1998). Telomeric to the region of FALS linkage lies the locus for spinal muscular atrophy type 4, an adult onset lower motor neurone disease also known as distal hereditary motor neuropathy type II (distal HMN II) which is characterised by spinal motor neurone loss but shows no upper motor neurone features (Timmerman et al. 1996). This region (D12S86 - D12S340) was significantly excluded in this FALS study (Multipoint LOD score ranging between -2.5 and -3.5 across this region) and not further investigated.

Example 2: Identification of Single Nucleotide Polymorphisms in Thioredoxin Reductase linked FALS.

Introduction

A second candidate gene, thioredoxin reductase (TXNRD1), from the FALS

region defined by linkage analysis between D12S306 and D12S79 was sequenced in affected individuals from FALS pedigrees without SOD1 mutations. No coding mutations were detected in any controls or FALS cases. However 23 single nucleotide polymorphisms SNPs were detected, including 8 which showed high frequencies of 31-71% of chromosomes tested. Some of the SNPs were observed to occur significantly more frequently in FALS compared to control cases, whereas other SNPs occurred significantly more frequently in control cases as opposed to in FALS cases.

Methods

All methods are as described in Example 1, except as described below.

Amplification of TXNRD1

The 13 exons plus 3 potential exons of TXNRD1 were amplified from genomic DNA using primers (Sigma-Genosys) annealing to the flanking regions of TXNRD1. PCR reactions were carried out in a total volume of 100 µl containing the following reaction components: 1x PCR buffer (20 mM Tris-HCl (pH 8.0), 50 mM KCl) (Invitrogen), 100 µM dNTP (Amersham Pharmacia Biotech), 0.5 µM forward and reverse appropriate primers, 100 ng genomic DNA and 2 U Platinum Taq DNA polymerase (Invitrogen). The $MgCl_2$ (Invitrogen) concentration varied for each exon. 1.0 mM $MgCl_2$ was used for exons 11 and 13, 1.5 mM $MgCl_2$ was used for all other exons. Primers used in the reaction were designed from the nucleotide sequence data obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (nucleotide sequence of the locus 17454675 sequence feature view gi|17455675:c349115-210344) are listed in Table 5. The annealing temperatures for the exons varied from 54 to 64°C. A typical cycling condition for the amplification of TXNRD1 was 94°C for 4 min, 58°C for 30 s and 72°C for 30 s for 1 cycle followed by 94°C for 1 min, 58°C for 30

s and 72°C for 30 s for 33 cycles. A final cycle of 94°C for 1 min, 58°C for 30 s and 72°C for 2 min was used to complete the amplification reaction.

Table 5. Primer sequences and their locations used in the amplification and screening of TXNRD1. The location of the TXNRD1 primers were taken from the nucleotide sequence of the locus 17454675 sequence feature view gi|17455675:c349115-210344. Primers for the potential TXNRD1 exon (indicated with a *) were taken from Osborne and Tonissen, BMC Genomics 2001, 2:10.

Primer Name	Sequence	Position
Exon 1 for	CAGGGCTGCACGAGGAGT (SEQ ID No. 100)	74195-74212
Exon 1 rev	CGTCGCCGCCCCATTTAAT (SEQ ID No. 101)	74988-74969
Exon 2 for	GACTCCAAGCTTGATTGTGAG (SEQ ID No. 102)	76032-76052
Exon 2 rev	GGGAGGGAAAGAGGAAGGG (SEQ ID No. 103)	76618-76600
Exon 3 for	GTTGTTTTACTTATGTAGAGG (SEQ ID No. 104)	98563-98583
Exon 3 rev	GGCACAAGACCCTTATTATCT (SEQ ID No. 105)	99238-99218
Exon 4 for	GGAATTATGTGAGAGAAGTCTA (SEQ ID No. 106)	100503-100524
Exon 4 rev	CCTATATATGCTAGATTA (SEQ ID No. 107)	101129-101109
Exon 5 for	GGATGATGTTACCGTGTTTGG (SEQ ID No. 108)	103112-102132
Exon 5 rev	GCAAATGCAATTAGTACTTCCTT (SEQ ID No. 109)	103748-103726
Exon 6 for	CAGGGGATCTGTCTTGTATT (SEQ ID No. 110)	106235-106254
Exon 6 rev	CCAGGCGGTCTGGTCCA (SEQ ID No. 111)	106797-106781
Exon 7 for	GGAGGTATGGTAAAGTGTAAG (SEQ ID No. 112)	106755-106775

Exon 7 rev	GTGATACTCTGTGTAGCAAGA (SEQ ID No. 113)	107356-107336
Exon 8 for	CTGACTAATATCAGAAGTTACAC (SEQ ID No. 114)	108370-108392
Exon 8 rev	CTACAAATAAGAACAAAGCTATATA (SEQ ID No. 115)	109049-109025
Exon 9 for	GGGATGGTAGTGTGCTTTCT (SEQ ID No. 116)	113522-113541
Exon 9 rev	GACGGGGTTTCACCACGTTG (SEQ ID No. 117)	114170-114161
Exon 10 for	GGATAGGTTTATTAAGGCATT (SEQ ID No. 118)	114561-114581
Exon 10 rev	GCAGGTGGATCACCTGAGG (SEQ ID No. 119)	115191-115173
Exon 11 for	CTTCCAGTGCCATTCACATTA (SEQ ID No. 120)	115649-115669
Exon 11 rev	CTGACCTGGTGATCTGCCT (SEQ ID No. 121)	116427-116409
Exon 12 for	GTGTGTCATTGTTGATAACATCAG (SEQ ID No. 122)	119725-119748
Exon 12 rev	GTATTTAGGGGTAAAGGAGAAT (SEQ ID No. 123)	120364-120343
Exon 13 for	GCTTTTAGTGAATAATGCCAAAGG (SEQ ID No. 124)	122242-122265
Exon 13 rev	GGGGCAGCAGGCAGTGCA (SEQ ID No. 125)	123278-123261
Exon 3b for*	GTAGGTATGTTGGAGGAGGG (SEQ ID No. 126)	97006-97025
Exon 3b rev*	GATCAGCCTGGCCAACGG (SEQ ID No. 127)	97902-97885
Exon 15 for*	GTTTCACCGTGTTAACCAGG (SEQ ID No. 128)	127399-127418
Exon 15 rev*	GAGGCAGACAGTATCAATTAG (SEQ ID No. 129)	128120-128100
Exon 16 for*	GGTTGACAAGCACCTGTGG (SEQ ID No. 130)	136551-136569
Exon 16 rev*	GACAACGAGAATAACAAAATCCA (SEQ ID No. 131)	137257-137235

Sequence analysis and mutation screening of TXNRD1.

The sequence data base indicates the existence of 13 exons in TXNRD1 but more recently it has been suggested from cDNA sequence analysis that three further exons exist, exon 3b, 15 and 16 (Osborne and Tonissen 2001). All of these 16 exons were sequenced in 19 FALS cases and most exons were also sequenced in 21 control individuals. No coding mutations were detected in any controls or FALS cases. However 23 single nucleotide polymorphisms were detected, one of which has already been reported (Table 6). These SNPs in contrast to those found for DAO included 8 which showed high frequencies of 31-71% of chromosomes tested. The remaining SNPs were less common with frequencies of 5-16% of chromosomes tested. The locations of the polymorphisms, as shown in Table 6, were taken from the nucleotide sequence of the locus 17454675 sequence feature view gi|17455675:c349115-21034 (NCBI). A previously known sequence polymorphism (indicated by * in Table 6, (JBC vol. 276, p30542-30551, 2001 figure 1B) was found. Potential exons of TXNRD1 (indicated by **) described by Osborne and Tonissen BMC Genomics 2001, 2:10 (http://www.biomedcentral.com/1471-2164/2/10).

Table 6. DNA and amino acid sequence alterations for TXNRD1. Occurrence of the mutation/polymorphisms as a percentage and as number of chromosomes in ALS, controls and combined are indicated.

Exon / Intron	Mutation / Polymorphism	Position	% Occurrence in FALS (as no. of chromosomes)	% Occurrence in controls (as no of chromosomes)	Total % Occurrence
Intron -1	C – G	74295*	49.5 (15/38)	40.5 (17/42)	40 (32/80)
Intron -1	C – G	74355	7.9 (3/38)	23.8 (10/42)	16.25 (13/80)
Intron 3	C – T	99182	63.2 (24/38)	35.7 (15/42)	48.8 (39/80)
Intron 3	T – A	99184	63.2 (24/38)	38.1 (16/42)	50 (40/80)
Intron 4	A – G	100909	47.4 (18/38)	42.9 (18/42)	45 (36/80)
Intron 4	A – T	100938	60.5 (23/38)	92.8 (39/42)	77.5 (62/80)
Exon 5	C – T CTC –> CTT (Leu – Leu)	103317	23.7 (9/38)	40.5 (17/42)	32.5 (26/80)
Intron 5	A – G	103622	0 (0/38)	9.5 (4/42)	5 (4/80)
Intron 5	G – T	103649	52.6 (20/38)	33.3 (14/42)	42.5 (34/80)
Exon 7	T – G GGT –> GGG (Gly – Gly)	107068	2.6 (1/38)	-	2.6 (1/38)
Intron 7	A – G	107177	2.6 (1/38)	-	2.6 (1/38)
Intron 8	T – C	113563	10.5 (4/38)	-	10.5 (4/38)

Intron 8	C – A	113659	7.9 (3/38)	-	7.9 (3/38)
Intron 9	A – T	114781	5.3 (2/38)	-	5.3 (2/38)
Intron 11	G – C	116274	7.9 (3/38)	-	7.9 (3/38)
Intron 11	T – C	119815	5.3 (2/38)	-	5.3 (2/38)
Intron 12	C – T	120276	23.7 (9/38)	40.5 (17/42)	32.5 (26/80)
Intron 12	G – C	122443	55.3 (21/38)	90.5 (38/42)	73.8 (59/80)
Exon 3b**	A – G	97594	7.9 (3/38)	-	7.9 (3/38)
Intron 15**	A – G	127876	71.1 (27/38)	-	71.1 (27/38)
Intron 16** (3' UTR)	C – T	136669	7.9 (3/38)	-	7.9 (3/38)
Intron 16** (3' UTR)	C – T	137021	15.8 (6/38)	-	15.8 (6/38)
Intron 16** (3' UTR)	C – T	137034	5.3 (2/38)	-	5.3 (2/38)

Interestingly, it was observed that the representation of particular SNPs was higher in one group than in the other. The alternative nucleotide from the published sequence for the two SNPs in intron 3 at positions 99182 (c-t) and 99184 (t-a) were observed to occur more frequently in FALS compared to control cases. Whereas the reverse was true for the SNPs at positions 100938 (intron 4) (a-t), 103317 (exon 5) (c-t), 120276 (intron 12) (c-t) and 122443 (intron 12) (g-c), in particular the SNPs at positions 100938 and 122443 which had a occurrence of over 90% in control cases compared to 60 and 55% in FALS cases (Table 6).

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Example 3: Neuroprotective effects of copper/zinc-dependent superoxide dismutase (SOD1) against a wide variety of death-inducing stimuli and proapoptotic effect of FALS mutations.

Abstract

Superoxide dismutase plays a key role in cell protection against the damaging effects of superoxide. Mutations in the copper/zinc dependent intracellular form of superoxide dismutase (SOD1) are associated with a subset of cases of familial amyotrophic lateral sclerosis (FALS). In this study we have investigated the effects of over-expressing wild-type SOD1 and two mutant forms of SOD1 found in FALS, G93A and G93R, on cell survival using stably transfected neuronal cells. G93R is associated with early age of onset and severely reduced erythrocyte SOD1 enzyme activity. Over-expression of wild-type SOD1 in ND7 cells significantly enhanced cell survival and reduced apoptosis after serum deprivation. Conversely, cells expressing the G93R mutation of SOD1 exhibited significantly increased cell death and increased number of TUNEL-positive cells, having a more profound effect than G93A

SOD1 expressing cells, thus reflecting the relative clinical severity of these mutations. The effects of three further apoptotic and non-apoptotic death-inducing paradigms were investigated, hypoxia with reperfusion, staurosporine and gamma interferon induced cell death. With each paradigm, cell death was significantly reduced by overexpression of wild-type SOD1 and increased by overexpression of the SOD1 mutations G93A and G93R. We further used these SOD1 constructs to develop a virus expressing either wild type SOD1 or the SOD1 mutant G93R and found a similar protective effect against serum withdrawal following infection with an HSV vector expressing wild-type SOD1 which offers a potential tool for neuroprotective gene delivery *in vivo*.

Introduction

There is considerable evidence that oxidative stress and free radical mediated reactions involving superoxide, hydroxyl and peroxynitrite anions participate in the mechanism of nerve cell death in cerebral ischaemia and Parkinson's disease (PD) [34]. Important enzymes involved in the detoxification of superoxide free radicals are the mitochondrial manganese-dependent and the cytosolic and extracellular copper/zinc-dependent forms of superoxide dismutases (SOD) which convert the superoxide free radical anion into hydrogen peroxide which is further detoxified by enzymes such as catalase and glutathione peroxidase. The importance of SOD in handling free radicals during oxidative stress is well established, for example in protection against reperfusion damage in ischaemic brain [15, 19, 24, 37]. A role for reactive oxygen species such as superoxide in neuronal apoptosis has also been implicated as increased levels are detected early after activation of apoptosis and SOD1 injection has been shown to delay apoptosis [13, 14]. Furthermore mutations in copper/zinc dependent SOD (SOD1) are associated with a subset of cases of familial motor neurone disease/ amyotrophic lateral sclerosis

(FALS) [33]. High levels of SOD1 are indeed found in motor neurones and nigral neurones which may reflect the specific demands of these cell populations [2].

Transgenic mice overexpressing FALS causing SOD1 mutations (G93A, G93R, G37R and G85R) develop progressive paralysis with motor neurone loss [e.g. 18], thus providing an valuable mouse model of disease although the loss of upper motor neurones typical of the human form of the disease is never seen in these transgenic mice. The age of onset for the G93A mutation (3-10 months) was shown to be largely dependent on the transgene copy number [8, 9]. Surprisingly some FALS mutations, for example A4V which is responsible for a severe form of the disease did not cause motor neurone disease in mice and recently it was shown that overexpressing G93R only in neurones did not result in motor deficits indicating the importance of other factors in initiating the disease [29].

The development of disease in SOD1 mutant mice is attributed to a gain of function and not to reduced enzyme activity as endogenous SOD1 activity is evident and mice which are null for SOD1 do not show motor neurone degeneration [32]. Several lines of study have been developed to determine the property of the mutant SOD1 causing the toxic gain of function which include increased tyrosine nitration, peroxidation, excitotoxicity, SOD1 aggregation, copper toxicity and reduced Zn^{2+} affinity (see reviews by Cleveland and Rothstein [1] and Beckman et al [5]). Mutant forms of SOD1 possess differing degrees of dismutase activity but the reduced affinity for Zn^{2+} affects their catalytic activity leading to superoxide generation from oxygen [6]. The zinc deficiency [1] could also occur in sporadic cases of ALS and hence underlie this aetiology of this form of the disease where a modest zinc deficiency has already been reported [12].

Evidence of apoptosis is also seen in transgenic mice overexpressing FALS

SOD1 mutations from the elevated levels of caspase-1 and caspase-3 [23, 30] and from the observation that neuroprotection can be obtained by crossing these SOD1 transgenics with mice overexpressing Bcl-2 [21]. Experiments in cell culture further substantiate the pro-apoptotic properties of FALS associated SOD1 mutations [31].

In the present project we studied the effects of SOD1 and SOD1 mutants found in FALS in cell culture on a wide variety of death-inducing stimuli both apoptotic and non-apoptotic in order to characterise their mode of action. We chose two mutant forms of SOD1, G93A, which has been widely reported [7] and a second mutation, G93R, which has only been reported in a single family [25, 25, 27]. This mutation is of particular interest as it causes a distinctive and consistent early age of onset and we were interested to determine whether clinical severity associated with these two mutations was reflected by their mode of action at the cellular level. In addition, in view of the potential benefits arising from the neuroprotective properties of SOD1 which could be applied to a number of neurological conditions we have also developed a viral vector for SOD1 and investigated its neuroprotective properties on cell survival *in vitro*.

Methods

SOD1 cDNA construct

Human fibroblast SOD1 cDNA obtained from the ATCC (61646) was used in this study. Initially the G and C tails on either side of the SOD1 insert were removed. In order to do this, PCR was carried out with the forward primer designed with an Xba1 restriction enzyme site just preceding the start codon and the reverse primer with an EcoR1 site at the end of the insert to enable cloning of the fragment. The SOD1 cDNA was amplified by PCR. The 545bp PCR product was checked by sequence analysis and a polyA tail added

prior to the introduction of the gene into a pR20.5 viral vector replacing the LacZ reporter gene.

Site directed mutagenesis

Two SOD1 mutants associated with FALS were also prepared in parallel by site directed mutagenesis. These have a single base mutation at codon 93 of the amino acid coding sequence. One has a base causing a glycine to alanine substitution (G93A) and the other a glycine to arginine substitution (G93R). The latter mutation produces early onset of disease and is associated with reduced enzyme activity. The altered site II *in vitro* mutagenesis system (Promega) was used and mutated plasmid isolates were screened by sequencing.

SOD1 and SOD1 mutant stable cell lines

The effects of overexpressing SOD1 or the two SOD1 mutants in stable transfected neuronal cells after serum deprivation, staurosporine administration, hypoxia with reperfusion and exposure to gamma interferon were investigated in order to characterise the mechanism of mutant mediated cell death and wild-type mediated neuroprotection.

Human wild type SOD1 or SOD1 mutant cDNA was inserted into the mammalian expression vector pCDNA3neo under control of a CMV promoter and transfected into a rat neuroblastoma/sensory hybrid cell line (ND7 cells) using the calcium phosphate method. Stable cell lines were produced, clones having been selected using neomycin. Cell death and apoptotic cell death was measured in these cell lines after serum deprivation (0, 24 and 48h), using trypan blue exclusion and the TdT-mediated biotin-dUTP nicked-end labelling (TUNEL) techniques respectively. Staurosporine was applied at a concentration of 1uM and cell counts were taken every 2 hours for up to 8

hours. Interferon gamma was used at a concentration of 100ng/ml and cell counts were taken at 0, 24 and 48 h. Each experiment was analysed in triplicate and 3-7 separate experiments were carried out for each study.

Cloning of SOD1 gene into disabled viral vector

Human fibroblast SOD1 cDNA obtained from the ATCC (61646) was inserted into a disabled, non-virulent strain (1764) of HSV (ICP27 deleted). Initially the SOD1 gene was inserted into a shuttle plasmid pR20.5 (constructed by Dr R. Coffin). This vector also contains a Green Fluorescent Protein (GFP) reporter gene whose expression which is driven by the CMV promoter allows distribution of virus to be monitored. The SOD1 gene is driven by the RSV promoter with the latent associated transcript LAT P2 region (from HSV-1) between the two promoters to gain long term expression of both transgenes. The vector contains vhs flanking regions allowing homologous recombination into the virion host shutoff protein gene (vhs) in the disabled HSV-1 viral construct and further disables the virus. Essential genes are inserted into an essential gene in the HSV-1 genome which in this case is the vhs gene. This ensures that no transfer of transgenes occurs between a disabled viral vector and any wild-type virus that it may encounter. This virus containing the SOD1 insert was grown on a ICP27 expressing cell line thereby complementing the defect and allowing growth in culture. Recombinant virus was plaque purified by isolation of green virus plaques visualised by fluorescence microscopy from a background of untransformed white virus. Recombinant SOD1 transgene insertion was confirmed by Southern blot analysis and expression of protein verified by Western blotting.

Calcium phosphate transfection

A standard calcium phosphate transfection method was employed to introduce HSV-1 DNA and the pR20.5 expression vector into the mammalian cell line

(baby hamster kidney cells BHK) for recombination into the virus genome. Complementing BHK cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and 100units/ml penicillin and streptomycin. Cells were transfected at 70% confluence, shocked with dimethylsulphoxide after 7 hours, and then harvested after being incubated for 4-5 days. Harvested virus was titred onto a monolayer of complementing BHK cells and recombinants were visualised by fluorescence microscopy.

Purification of recombinant virus

Viral recombinants were then purified by picking green fluorescent plaques. Each plaque was freeze thawed and used to directly infect a pre-plated out monolayer. A 2 day cycle of picking and infecting was continued until pure, that is, all plaques were green. Once pure a large scale virus culture can be prepared in Coring 850cm^2 roller bottles in which complementing cells are first grown until approximately 70% confluent then infected with 2 x 10^6 plaque forming units of virus per bottle. The infected roller bottles are incubated at 31°C and 0.5 rpm for 3-5 days or until cytopathic effect was observed. Harvested cells were spun out at low speed to remove cell debris. Recombinant virus was then pelleted from supernatant, sonicated and stocks at 1 x 10^9 pfu prepared.

Western blotting

Expression of the SOD1 gene in virus was detected by Western analysis 100ug of total protein was run on a 15% SDS-polyacrylamide gel electrophoresis and then electroblotted on to a Hybond C nitrocellulose membrane. Reduced SOD1 protein was detected at 18kDa by probing the filter with a rabbit polyclonal SOD1 antibody (from Dr. Yoram Groner). This is not a human-specific antibody. It recognises SOD1 from a number of species including endogenous SOD1 in the BHK cells in which virus is grown. The human and

hamster SOD1 protein can be resolved, on this high percent gel, by size the latter giving a 16kDa band. A human white blood cell protein sample was run in parallel as a positive control to confirm band size.

Statistical analysis

Analysis of cell survival was carried out by ANOVA followed by repeated Student t-tests.

Results

Effect of over-expression of SOD1 and SOD1 mutants on cell viability and apoptosis

The effects on cell viability of overexpressing SOD1 or SOD1 containing FALS mutations in stably transfected neuronal cells was investigated. Human wild type SOD1 or SOD1 mutant cDNA was inserted into the mammalian expression vector pCDNA3neo under control of a CMV promoter and transfected into a rat neuroblastoma/sensory hybrid cell line (ND7 cells). Expression of SOD1 was detected by western blotting in ND7 cells and PC12 cells. Cell death and apoptotic cell death were measured in these cell lines after serum deprivation (0, 24 and 48h). Serum withdrawal was carried out in the presence of all trans retinoic acid which induces high levels of apoptosis without significant differentiation and hence has been widely used as a model for inducing apoptosis in ND7 cells [36].

Total cell death was assessed using the trypan blue exclusion assay in ND7 cells expressing either the vector cassette (vector control) or expressing the wild-type SOD gene, G93A or G93R mutant gene. Cell death was significantly attenuated in stable cell lines over-expressing wild-type SOD1, being decreased by 34% at 24h ($p< 0.05$) and by 19% at 48h ($p<0.035$) compared to the vector control. Conversely, cells expressing the G93R

mutation showed a significantly enhanced cell death which was increased by 36% at 24h compared to a vector transfected control cell line ($p<0.04$) and increased by 110% compared to the wild-type expressing cell line ($p<0.005$). Cells expressing the G93A mutation were not significantly different from the vector control cell line but cell death was significantly increased ($p < 0.05$) compared to the wild-type expressing cells at 24h (Figure 6).

Apoptotic cell death after serum withdrawal was assessed using the TUNEL labelling method in ND7 cells expressing the vector cassette (vector control) or expressing the wild-type SOD1 gene, G93A mutant or G93R mutant gene. Cells overexpressing wild-type SOD showed a significantly reduced number of apoptotic cells at 24h, being decreased by 64% ($p<0.001$) at 24h whereas the G93R mutation caused a 71% increase in apoptotic cells ($p<0.02$) at 24h compared to vector control. At this time point the number of apoptotic cells was 5 times greater in the G93R cell lines compared to the wild-type SOD1 expressing cells. These results from neural cell lines indicate that overexpressing wild type SOD1 inhibits/delays apoptosis while the G93R mutation was proapoptotic (Figure 7).

Effect of over-expression of SOD1 or SOD1 mutants on other death-inducing stimuli

Having established that wild type SOD1 had a protective effect against serum deprivation whereas the mutants had a damaging effect, we next wished to investigate the effect of the wild type SOD1 and the mutants on the response to other death-inducing stimuli. We therefore carried out a time course analysing the response of the different cell lines to treatment with staurosporine which has been shown to induce apoptotic death in a variety of different neuronal and non-neuronal cell types. In these experiments (Figure 8) the G93R mutant clearly enhanced the degree of cell death produced by staurosporine (2-8 hours) and a similar although less dramatic effect of the

G93A mutant was also observed. In contrast, the wild type SOD1 showed a significant protective effect both when compared to the effect of over-expressing the mutant and also compared to the control cells containing only empty expression vector. Hence, as with the effect of serum removal, the mutants enhanced the damaging effects of staurosporine whereas the wild type protein produced a protective effect.

Similar results were also observed when the different cell types were treated with γ-interferon which also induces cell death in a variety of neuronal and non-neuronal cell types. As for the other stimuli, an enhanced cell death was observed in the cells over-expressing particularly the G93R mutant and to a lesser extent with the G93A mutant, whereas the cells over-expressing the wild type were significantly protected compared to the mutant cells (Figure 9a).

In view of the role of SOD in dealing with free radicals formed in cells, it was of particular interest to investigate the response of our cells to a period of simulated ischaemia followed by re-oxygenation. Each of the cell lines was therefore exposed to a 4-hour period of simulated ischaemia followed by 24-hours of reoxygenation. The results of this experiment (Figure 9b) indicated that as before, over-expression of both the mutants significantly enhanced the degree of cell death observed, with the G93R mutation having a stronger effect than the G93A mutation. Cells transfected with the wild type SOD1 showed significantly reduced cell death compared to the cells expressing either of the two mutants confirming that the mutants had a particularly damaging effect in the response to simulated ischaemia/reoxygenation as with the other stimuli.

The establishment of a modified virus capable of expressing superoxide dismutase

Having demonstrated using cell lines, that over-expression of wild type SOD1 was able to have a protective effect against a variety of damaging stimuli, we wished to develop a system for manipulating SOD1 expression efficiently both in cultured cells and ultimately *in vivo*. A non-virulent form of Herpes simplex virus (HSV) was selected as being most appropriate to do this as it naturally establishes asymptomatic, long lived, latent infections in neuronal cells and has already been successfully used to deliver genes to neuronal cells *in vivo* and to modify neuronal function both in our laboratory and by others [22]. The human SOD1 gene was inserted into a non-virulent strain (1764) of Herpes simplex virus (HSV), which has been used to safely and effectively deliver a gene to both the central and peripheral nervous system [3, 36]. Delivery of a further disabled virus which had a deletion of a HSV immediate early gene ICP27 essential for viral replication has also been successful in the heart [4]. Most cytopathic effects exhibited by HSV vectors have been attributed to immediate early gene expression [20]. Thus deleting ICP27 prevents lytic replication and makes the virus avirulent *in vivo* but requires complementation for growth in culture. Further disablement is achieved by deleting non-essential HSV-1 genes ICP34-5 and inserting an inactivating insertion in VMW65 which abolishes its ability to trans-activate immediate early genes but allows encoding of its essential structural protein.

The SOD1 gene was inserted into a shuttle plasmid pR20.5 which also contained a Green Fluorescent Protein (GFP) reporter gene, expression of which is driven by the CMV promoter allowing distribution of virus to be monitored. The SOD-1 gene is driven by the RSV promoter with the latent associated transcript LAT P2 region (from HSV-1) between the two promoters to gain long term expression of both transgenes. The vector contains vhs flanking regions allowing homologous recombination into the virion host shutoff protein gene (vhs) in the disabled HSV-1 viral construct and thus further disabling the virus as it is an essential gene. This ensures that no

transfer of transgenes occurs between a disabled viral vector and any wild type virus which it may encounter.

Following infection of cultured ND7 cells with the viruses, recombinant SOD1 transgene insertion was confirmed by Southern blot analysis and the expression of protein verified by Western blotting (Figure 10). The protein product corresponding to human SOD1 was present in the three SOD1 infected cells and the white blood sample extract whereas the smaller rodent protein was present in all four cell lines including the vector control. The higher molecular weight proteins seen at 30kDa in lanes 2 and 3 are likely to be unreduced SOD1 dimers.

Neuroprotective effect of viral delivery of SOD1

Cell death after infection with HSV vectors was assessed by trypan blue exclusion at time 0 and 24h after transfer to serum-free medium in the presence of all-trans-retinoic acid. ND7 cells were either mock-infected (no virus) or infected with lacZ recombinant HSV-1 vector viruses (control) or expressing wild-type or G93R mutant virus. The SOD1 infected cells showed a significant reduction in cell death of 40% ($p < 0.02$) compared to the vector control in keeping with the results obtained with the cell lines. The virus expressing the G93R mutant form of SOD1 induced the same level of cell death as the control virus (Figure 11) suggesting that it had no effect on this system and further studies will be required to investigate the reasons for this.

Discussion

In this report, we have demonstrated that the disease-associated SOD1 mutants G93A and G93R enhance the level of cell death observed in a neuronal cell line exposed to a variety of apoptotic stimuli including serum removal, treatment with staurosporine or γ-interferon or exposure to simulated

ischaemia followed by reperfusion. This extends the study of Rabizadeh *et al.*, (1995) who showed that the SOD1 mutations G37R and A4V associated with ALS converted SOD1 from an anti-apoptotic gene to a proapoptotic gene. Moreover, in our study a particularly marked effect was observed with the G93R mutation compared to the G93A mutation paralleling the much more severe disease phenotype and early onset observed in ALS patients with this mutation. Although the G93R mutation has only been reported in a single family [10, 25, 27], it is associated with a particularly early age of onset being 36 years (4 individuals with a range of age of onset of 29-41 years). This compares with the mean age of onset for all families with SOD1 mutations in our UK cohort which is 48.8 years (ranging from 24 to 72 years) [11, 27]. Erythrocyte SOD1 enzyme activity was also markedly reduced in individuals possessing this mutation compared to all other mutations that we have studied

In contrast, several studies previously demonstrated a protective effect of the wild type SOD1 against neuronal apoptosis [17, 31] and we have shown that this is the case in our system also. Thus, the wild type SOD1 protein was able to protect our neuronal cell line against a wide variety of apoptotic stresses including serum removal, ischaemia/reperfusion or treatment with staurosporine or γ-interferon. Hence, in a wide variety of different situations the wild type SOD1 is protective whilst the mutations G93A or G93R convert the protein into a pro-apoptotic form. This is in contrast to our findings with the Parkinson's disease-associated mutations of α-synuclein and the wild type form of the protein [38]. Thus, we have previously shown that two disease-associated mutations of α-synuclein result in enhanced sensitivity to serum removal or simulated ischaemia when they are over-expressed in ND7 cells. In contrast however, over-expression of the wild type α-synuclein results in a protective effect against serum removal but produces a damaging effect, albeit less than observed with the mutants, when cells are exposed to simulated ischaemia. Hence, in the case of α-synuclein the wild type protein appears to

produce a protective effect against some stresses but not others, whereas the Parkinson's disease-associated mutations have a pro-apoptotic effect in cells exposed to a wide variety of different stimuli. In contrast, in the case of SOD1, the wild type protein appears to have a widespread protective effect whereas the mutants have a similarly widespread damaging effect.

The protective effect of SOD1 against a number of different damaging stimuli, led us to try and develop a virus vector system which was able to effectively deliver SOD1 to cells in culture and ultimately to the intact animal to produce a protective effect. In the experiments described here, we have indeed demonstrated that a virus vector can be used to over-express SOD1 in cultured ND7 cells and to produce a protective effect against serum removal. This parallels the result of Ghadge *et al.*, [16] who used replication deficient recombinant adenoviruses to deliver human wild type or mutant SOD genes (A4VV148G) into primary neurones or PC12 cells and demonstrated a pro-apoptotic effect of the mutant SOD1 proteins and an anti-apoptotic effect of wild type SOD1.

The ability of our viruses to effectively deliver specific genes *in vivo* [28, 35] suggests that it may be ultimately possible to enhance neuronal survival for therapeutic benefit by delivering SOD1 with a viral vector. In the meanwhile, our results provide a comprehensive view of the effect of wild type and mutant SOD1 on the responses of neuronal cells to apoptotic stimuli. Thus, we demonstrate that disease-associated mutants of SOD1 enhance the degree of cell death observed in response to a wide variety of different stimuli with the severity of this effect correlating with the severity of the disease caused by the specific mutation. In contrast, over-expression of wild type SOD1 has an anti-apoptotic effect protecting cells from a wide variety but not all damaging stimuli.

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Example 4: Heat shock protein 27 overexpression in transgenic animals has a protective effect in both the heart and the brain

Summary

The 27kDa heat shock protein (HSP27) has a potent ability to increase cell survival in response to a wide range of cellular challenges. We have shown that HSP27 overexpression protects neuronal cells against both apoptotic cell death and oxidative stress whereas HSP70 only protects against oxidative stress in this tissue. In order to investigate the mode of action of HSP27 in vivo, we have developed three independent transgenic lines which express human HSP27 (hHSP27) at high levels in a wide range of tissues and have tested them in two well established models of cytotoxicity, firstly, an in vivo model of apoptosis produced by kainic acid injection in which cell death occurs in hippocampus and secondly, cardiac ischaemia using the Langendorff isolated heart perfusion model. Our results demonstrate for the first time the marked protective effects of HSP27 overexpression in vivo which significantly reduces kainate-induced seizure severity and reduces neuronal cell death in the CA3 region of hippocampus and reduces myocardial infarct size following exposure to ischaemic conditions. These studies clearly demonstrate that HSP27 has a major neuroprotective effect in the CNS in keeping with its properties demonstrated in culture and in common with HSP70 plays a substantial role in reducing cardiac damage produced by an ischaemic insult.

Introduction

The heat shock proteins (HSPs) are a family of proteins originally identified as being upregulated in response to elevated temperature but now a wide range of cellular stresses such as hypoxia, ischaemia, glutamate and heavy metals have been shown to induce HSPs (1-6). HSPs consist of a family of highly conserved proteins grouped according to their molecular size, the high molecular weight proteins, 110kDa, 90kDa, 70-72kDa, 55-60kDa and the small HSPs which include HSP27, ubiquitin, alphaA- and alphaB-crystallin and related species. Although highly conserved across species, variation in protein size occurs for example, the 27kDa human HSP27 has a corresponding isoform of 25kDa in rodents referred to as HSP25. HSPs are both constitutively expressed and also are induced in response to stressful stimuli e.g. HSP27 and HSP70. Rapid induction of HSP expression is mediated by specific heat shock factors (HSF1-4) which regulate transcription (7).

The HSPs play a key role in cellular defense systems acting as protein chaperones facilitating protein folding and the removal of aberrant proteins. These properties have been shown to contribute to the enhanced cellular survival produced following preconditioning stimuli in which a subthreshold stimulus is used to raise endogenous heat shock protein levels prior to the main stimulus. Primary neuronal cultures are protected by prior exposure to mild heat or ischaemic stress before subsequent more severe heat or ischaemic stress or exposure to glutamate (8-10). In cardiac tissue, a mild heat shock also protects against a subsequent thermal or ischaemic stress (11).

The effects of heat shock can be mimicked by overexpression of HSPs alone. Both the ND7 immortalised neuronal cell line, which is derived from dorsal root ganglia neurones, and primary cultures of dorsal root ganglion neurones can be protected against subsequent ischaemic or thermal stress by overexpression of HSP70 (12-15) or HSP90 (90kDa HSP)(13, 15). In cardiac cells or primary cultures of cardiac cells, overexpression of HSP70 protects

against subsequent thermal or ischaemic stress (16-18) but HSP90 only protects against thermal stress and does not protect against ischaemic stress.

Marked differences are seen for each HSP in their tissue and cellular specificity and their response to different insults, for example, seizure activity is associated with a rapid HSP70 induction in hippocampal neuronal populations whereas HSP27 induction is primarily in glial cells and of longer duration (19). Similarly, whereas prior heat shock protects ND7 neuronal cells and primary neurones against apoptosis due to withdrawal of serum or NGF (20, 13), overexpression of HSP70 or HSP90 is not protective. Overexpression of HSP27 in ND7 neuronal cell lines does however protect not only against subsequent exposure to heat shock and simulated ischaemia but most importantly to stimulation of apoptosis by serum withdrawal in the presence of all trans retinoic acid (21). Similarly, in primary dorsal root ganglion cells, infection with Herpes simplex virus (HSV) vectors expressing HSP27 or 70 protects against heat shock and simulated ischaemia whilst only expression of HSP27 protects against apoptosis induced by NGF withdrawal (21). HSP27 also reduces the extent of apoptosis in monocytic cells exposed to DNA damaging agents (22) and fibrosarcoma cells exposed to Fas-induced apoptosis or staurosporine (23). Overexpression of HSP27 in cardiac myocytes using an adenovirus vector also protects against simulated ischaemia (24). Infection of neonatal cardiac cells using an HSP27 expressing HSV vector protects cells not only against heat shock and ischaemia but also minimises the extent of apoptosis induced by serum withdrawal or treatment with ceramide (25).

Through our previous studies we were thus able to show that transfection with HSP27 conferred protection against apoptosis in neuronal cells which was not evident with HSP70 although both HSPs were protective against heat shock and hypoxia. This finding prompted us to develop transgenic mouse lines

overexpressing the human HSP27 (hHSP27) gene in order to determine whether evidence could be obtained in vivo for protection against apoptotic and ischaemic insults in CNS and cardiac tissue. No such transgenic mouse has been previously reported for HSP27 although transgenic mice overexpressing HSP70 have been generated. Studies in vivo of myocardial ischaemia in mouse lines overexpressing HSP70 using a constitutively active promoter have found reduced infarct volume compared to controls (26), improved ATP stores (27) and improved contractile recovery (28). Studies of cerebral ischaemia using HSP70 transgenic lines have not yielded consistent results, whilst some authors have shown reduced cerebral infarcts (29) others do not show reduced infarct size following permanent cerebral focal ischaemia although astrocyte and hippocampal but not neuronal cultures from these animals show protection against oxygen-glucose deprivation and glutamate toxicity (30). Clearly the inducible HSPs have cytoprotective properties and the implications are that HSP27 may be even more important than HSP70 in the nervous system.

In order to carry out further elucidation of the cytoprotective properties of HSP27 and to determine whether in vivo protection can be demonstrated by HSP27, we generated transgenic mouse lines in which wild type human HSP27 (hHSP27) was expressed at high levels in cardiac and CNS tissue. Three independent lines were generated in order to confirm that protective effects could be replicated and to ensure that the effect was due to the HSP itself rather than due to the particular site of integration. These independent transgenic lines were then tested in the CNS using the in vivo administration of kainic acid to induce seizures and hippocampal cell death and in the Langendorff isolated perfused heart model to investigate the effects of protection against cardiac ischaemia (31, 32). The kainic acid model is a well established experimental model of limbic seizures in which systemic kainic acid targets kainate receptors, a subset of ionotropic glutamate receptors,

resulting in hyperexcitation and causing cell death in a localised region of the brain where these receptors are abundant, the CA3 region of hippocampus (33, 34). Although the mode of cell death includes both apoptotic and necrotic components it provides a useful model to induce apoptotic cell death in vivo (35-38).

The results obtained show protection in both tissues showing that HSP27 has an important role in neuroprotection as predicted from earlier cell culture studies in addition to its role in protection against oxidative stress in cardiac tissue in common with other HSPs.

Experimental procedures

Establishment of transgenic mouse lines expressing human HSP27

Transgenic mice were created using a transgene containing human HSP27 cDNA with a chicken beta actin promoter and CMV enhancer (pCAGGS; Ref. 39). In order to track expression of the transgene a Hemagglutinin (HA) tag was placed contiguous with the HSP 27 cDNA sequence. The linearised transgene was microinjected into pronuclei of fertilised eggs from C57BL10 X CBA/Ca mice which were subsequently transferred to pseudo pregnant recipients. This procedure was carried out in the Imperial College Gene Targeting Unit. At 3-4 weeks after birth, a small tail biopsy was taken from pups and genomic DNA screened for the integration of the transgene using PCR. Founders revealed by this screening were used to establish independent transgenic lines by breeding to wild-type F1 hybrid (C57BL10 X CBA/Ca) mice. Following successful transmission a range of tissues were screened by Western blot and immunohistochemistry to confirm CNS and cardiac expression. Over-expression of human HSP27 did not appear to produce any obvious effects on development or survival in mice from all three lines generated. Histological comparisons of brain and cardiac tissue from the

different mouse lines revealed no observable morphological differences when compared with corresponding tissue from their respective wild-type littermates, although no detailed (quantitative) studies were carried out as a part of this study.

Experimental Model of in vivo apoptosis using kainate

Mice were injected intraperitoneally (i.p.) with kainic acid (KA, Tocris UK) (20-30 mg/kg) or vehicle (phosphate buffered saline [PBS]; 1 ml/kg). For dose response studies the following kainate doses were used with wild-type (wt) mice: 20 mg/kg, n=18; 25 mg/kg, n=39; 30 mg/kg, n=4. A dose of 25mg/kg was chosen for behavioural studies as it produced the most consistent response (hHSP27(18), n=35; wt, n=39; hHSP27(64), n=30; wt, n=25). Animals were monitored continuously for 4h for the onset and extent of seizure activity. The seizures were scored using a modified scale devised by Racine (40): 1, behavioural arrest, staring spells; 2, head bobbing, gnawing; 3, unilateral forelimb clonus; 4, bilateral forelimb clonus; 5, severe seizures with loss of postural control; 6, seizure-induced death. Animal care and procedures carried out followed Home Office guidelines and an ethical review committee approved protocol.

For in situ hybridisation studies, animals were killed at 24 h after KA or vehicle or were untreated. Brains were rapidly removed and frozen on dry ice. Sections were cut (10 (m) on a cryostat (Bright Instruments), thaw mounted onto Superfrost slides (BDH) and stored at ñ80 (C until use. For histology and neurone counts, animals were killed at 7 days after kainate injection (hHSP27(18), n=6, wild-type, n=6) or vehicle (hHSP27(18), n=7, wild-type, n=3) and brains were removed and processed as above.

Langendorff heart perfusion model

We have used a well established Langendorff heart perfusion model (31, 32, 41, 42), to examine the effects of mice overexpressing the hHSP27 transgene to ascertain their ability to withstand periods of lethal ischaemia followed by reperfusion. Mice expressing hHSP27(18) and wild-type littermates were anaesthetised and anti-coagulated by co-administration of 60 mg/kg sodium pentobarbitone and 100 IU heparin (i.p.). With the demonstration of deep anaesthesia the heart was excised and immersed in ice-cold buffer. The aorta was cannulated and the heart transferred to a Langendorff apparatus and retrogradely perfused at a constant pressure of 110 mm Hg with Krebs Henseleit buffer oxygenated with 95%O2 / 5%CO2 gas mixture (pH 7.4). A water filled latex balloon connected to a hydrostatic pressure transducer and coupled to a recorder was inserted into the left ventricle through an incision in the left atrial appendage and inflated to set an end diastolic pressure of 5-10 mm Hg. Coronary flow was measured by timed collection of effluent measured over 1 min at regular intervals. A temperature probe inserted into the right ventricle enabled maintenance of normothermia (37 (C) throughout the experimental protocol. Hearts were paced at the electrical threshold at 600 beats per minute throughout stabilisation and for the latter 20 min of the 30 min reperfusion period with electrodes in the left atria and on the aortic cannula. In the event of an arrhythmia, the rhythm was recaptured by a transient increase in the pacing threshold.

Following stabilisation global ischaemia was induced for 30 min. The heart was then reperfused for 30 min following which triphenyltetrazolium chloride was infused to distinguish viable from necrotic tissue. The hearts were than frozen, sliced and fixed in formalin. The infarct size as a percentage of the left ventricle was determined on magnified video images digitised to allow planimetry as previously described (31, 32, 41, 42).

In Situ Hybridisation

Specific antisense oligonucleotides were synthesised (Sigma-Genosys Ltd) for use in the in situ hybridisation studies. For detection of Hemagglutinin tag mRNA, a 27 mer oligonucleotide, 5' agcgtaatctggaacatcgtatgggta 3' was used. For detection of human HSP27 mRNA, two probes (A and B) were used which were based on the human HSP27 sequence (43). Probe A, is a 33 mer oligonucleotide complimentary to nucleotides 540-572 and probe B is a 33 mer oligonucleotide complimentary to nucleotides 593-625. Both human HSP27 probes and the Hemagglutinin tag probe yielded identical patterns of expression. For mouse HSP25 mRNA, three probes (probes 1, 2 and 3) were used which are based on the mouse HSP25 sequence (44). Probe 1 is a 34 mer oligonucleotide complimentary to nucleotides 358-391, probe 2, is a 33 mer oligonucleotide complimentary to nucleotides 497-529 and probe 3 is complimentary to nucleotides 550-582. All three HSP25 probes yielded identical patterns of expression. For mouse glial fibrillary acidic protein (GFAP) mRNA, a 45 mer oligonucleotide complimentary to nucleotides 1119-1163 of the mouse GFAP gene (45) was used. Oligonucleotide probes were 3' end-labelled using terminal deoxynucleotidyl transferase (Promega) and [35S]dATP (Amersham Pharmacia Biotech).

Cryostat sections were processed and in situ hybridisation carried out as previously described (19). In brief, slides were fixed in 4% paraformaldehyde in PBS (4 (C) for 10 min, rinsed in PBS for 2x 5 min and then treated with 0.25% acetic anhydride in 0.1M triethanolamine/0.9% NaCl for 10 min. Following dehydration in increasing concentrations of ethanol, the sections were delipidated in chloroform for 5 min, rinsed in ethanol and allowed to air dry. Sections were then hybridised overnight at 42 (C with 1-2 x 106 c.p.m of labelled probe in 100(l of hybridisation buffer (5x Denhardt's [0.02% Bovine Serum Albumin, 0.02% Ficoll and 0.02% Polyvinylpyrrolidone], 4x standard saline citrate [SSC], 50% formamide, 10% dextran sulphate, 200 (g/ml polyadenylic acid, 200 (g/ml sheared single-stranded salmon sperm DNA, 50

mM phosphate buffer and 20 mM dithiothreitol). The following day, sections were stringently washed (1x SSC at room temperature for 30 min, 1x SSC at 55 (C for 30 min, 0.5x SSC at 55 (C for 30 min, 0.1x SSC at 55 (C for 30 min and 0.1x SSC at room temperature), dehydrated in an ascending series of ethanols and air dried. When dry, sections were exposed to X-ray film (BioMax MR, Eastman Kodak Co) for 7-14d and developed. Sections were then dipped in photographic emulsion (Ilford K5) and exposed for 1-6 weeks at 4 (C, developed and counter-stained with toluidine blue for light microscopic analyses.

Analysis of neuronal cell density in hippocampus.

Serial coronal sections of the brain were cut at 10μM, stained with hematoxylin-eosin, dehydrated through a series of alcohols, cleared in xylene and coverslipped. Histological analysis of cell morphology and cell counts in the hippocampus were confined to approximately -1.94 mm to -2.46 mm posterior to bregma (46). The Nikon Eclipse E800 microscope used for this study was equipped with a motor-driven stage to traverse the x- and y-axes. To define the boundaries of the hippocampus, slides were viewed with a 10X objective. The entire hippocampus was then captured by a JVC 3-CCD colour video camera and the image was tiled onto a colour monitor screen. A geometrical Area of Interest (0.036 mm2) was placed in the CA1 and CA3 regions of the hippocampus. This procedure was standardized in order to sample the same areas of these regions. Digital images were analysed by the Image-Pro Plus 4.1 program (Data Cell). Morphological criteria of the normal pyramidal cells were established in the control mice based on size (length) and cellular staining. To prevent counting glial cells and degenerating neurones, all counting was thresholded at a minimal cell length of 0.009 pixels. The mean neurone count was obtained from 3 sections per animal. Cell counts were averaged and neuronal cell density was expressed in terms of

morphologically normal nuclei per square field of 0.036 mm2. Differences in neuronal cell densities between all the treatment groups were assessed using the Kruskal-Wallis nonparametric ANOVA test followed by Mann-Whitney U-test (InStat v2.01).

Western blot Analysis

Tissues were snap-frozen in liquid nitrogen and protein was extracted by homogenisation in lysis buffer (75 mM Tris pH 6.8, 20 % [v/v] glycerol, 10 % [w/v] SDS, 100 mM dithiothreitol, 0.002 % [w/v] phenylmethylsulphonylfluoride) supplemented with protease inhibitors. The proteins were separated on a 10 % Tris-HCL SDS-PAGE gel (Bio-Rad) at 100 V for 2 h, and then transferred onto a polyvinyl difluoride membrane (HybondP, Amersham Pharmacia Biotech) for 2 h at 250 mA. Western blot analysis was carried out using either antibodies directed against hHSP27 (1:1000)(Stressgen) or the HA-tag (1:500)(Santacruz), followed by horseradish peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h respectively. Detection was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturers instructions.

Immunocytochemistry

Brain and heart samples were frozen in liquid nitrogen cooled isopentane, sectioned at 10 (m, and thaw mounted onto glass slides. The sections were fixed for 20 min at room temperature with 4 % formalin prior to blocking with a 3 % hydrogen peroxidase solution, avidin/biotin kit (Dako Ltd), and 3 % milk for 10 min respectively. Antibodies to either hHSP27 (1:1000)(Stressgen) or the HA-tag (1:100)(Santacruz) were applied at room temperature, followed by incubation of biotinylated secondary antibody (Dako Ltd), and streptavidin-ABC-horseadish peroxidase complex (DAKO) for 30 min each. Immunoreactivity was visualised by addition of diaminobenzidine

(Sigma). The tissue sections were counter stained with heamatoxylin, dehydrated and mounted for microscopical analysis.

Statistical analysis

Seizure score data were analysed by Mann-Whitney U-test. Fisher's exact test was used to compare mortality rates. Significance level was set at 0.05 for all comparisons.

Results

Establishment of transgenic mouse lines expressing human HSP27

Three independent lines (18, 59 and 64) of transgenic mice with similar patterns of expression of the human HSP27 transgene were generated. All three lines showed widespread expression in brain, spinal cord, heart, muscle, liver, kidney, lung and pancreas. This was shown in Western blots using antibodies specific for human HSP27 encoded by the transgene (hHSP27)(data not shown) or the HA tag linked to it (Figure 12). All transgenic lines showed normal reproductive patterns and longevity and behaviour was not noticeably different from wild-type littermates.

Transgene expression in brain and heart.

Immunocytochemistry was carried out on the three independent mouse lines expressing the transgene using the same two antibodies. Using the HA tag antibody, labelled neuronal cell bodies were detected throughout the brain, being particularly abundant in the hippocampus, cerebral cortex, cerebellum, striatum, thalamus and subventricular nuclei. Extensive labelling of fibrous networks and small cells was also seen suggesting expression in glial cells.

A similar distribution of expression of hHSP27 was seen for all three mouse

lines (18, 59 and 64). In CA1, CA3 and the hilus, cell bodies and their projections were strongly labelled (Figure 13). Extensive axonal and dendritic labelling was seen in CA1 for lines 18 and 64 and similarly in CA3 for lines 59 and 64. In the dentate gyrus, transgene expression occurred in granule cells as well as the interneurones lining the internal surface of the granule cell layer which were labelled using a *GAD67 antibody in adjacent sections (data not shown).*

In cardiac tissue, transgene expression in myocytes was widespread in all three independent lines as demonstrated by immunohistochemistry using the HA antibody counterstained with haematoxylin (Figure 14).

Distribution of expression of human HSP27 (hHSP27) transgene mRNA in brain.

The expression of the hHSP27 transgene in the three mouse lines showing CNS expression of HSP27 protein from Western blot and immunohistochemistry was also characterised by in situ bybridisation (ISH). Two HSP27 oligonucleotide probes (probes A and B) directed against the human sequence and not reactive with mouse (43) were tested together with a probe for the HA tag sequence which was contiguous with the HSP27 transgene. All three probes produced identical RNase-A sensitive patterns of expression in brain sections from unstressed hHSP27(18) mice, while no signal was detected in sections from unstressed- and kainate-stressed wild-type mice (HSP27-probe B, data shown in Figure 15A-D; HSP27-probe A and HA tag probe, data not shown). Probe B was used for further characterisation of CNS expression of the HSP27 transgene.

To validate further the specificity of these probes for the HSP27 transgene, three additional probes (probes 1, 2 and 3) directed to the endogenous inducible mouse HSP25 gene (44) were synthesised. All three probes (1, 2

and 3) yielded identical hybridisation signals in brain sections from kainate-stressed hHSP27(18) and kainate-stressed wild type mice that greatly contrasted with the hybridisation pattern for the HSP27 transgene (probe 1 data shown in Figure 15G, H; probe 2 and 3, data not shown). Furthermore, the HSP25 mRNA signal was absent in mouse brain sections from unstressed hHSP27(18) mice (Figure 15E), unstressed wild type mice (Figure 15F) and RNase-A treated sections (data not shown), thus confirming the species specificity of the probes. Therefore, the transgenic mice clearly contain greatly elevated levels of HSP27 mRNA which is present in the absence of stress.

In kainate-treated mice, HSP25 mRNA expression was induced intensely in thalamus, moderately in hippocampus and at low levels in the cerebral cortex (Figure 15G, H) being predominantly localised to glial cells (data not shown). Induction of HSP25 appeared to be similar in both control and transgenic animals indicating no suppression of endogenous HSP25 induction by the transgene.

A molecular marker of brain injury in response to kainate, GFAP induction, was used to substantiate the behavioural response to kainate. GFAP mRNA expression was upregulated following kainate administration to a similar extent in both wt and hHSP27(18) mice (Figure 15K, L) compared to PBS treated control mice (Figure 15I, J). High expression of GFAP mRNA was seen in hippocampus, entorhinal cortex and in septal nuclei, while moderate to low levels were observed in corpus callosum and thalamus (Figure 15K, L).

Expression of hHSP27 mRNA was extensive throughout cerebral cortex, CA1 and CA3 of hippocampus, dentate gyrus, striatum and some thalamic regions (e.g. ventro-posterior lateral and medial thalamic nuclei). Moderate levels of expression were seen in lateral and medial habenula nucleus, dorsal lateral geniculate and lateral septal nucleus. Little expression was seen in white

matter (Figure 16). There was some variation in the level of expression between lines, for example in line 18, expression was highly abundant in cerebral cortex, hippocampus, striatum and cerebellum whereas for lines 59 and 64, a moderate but more widespread expression was seen throughout the brain.

Cellular labeling of hHSP27 mRNA

Analyses of emulsion dipped sections at the light microscopic level revealed a predominantly neuronal expression for hHSP27 mRNA in the dentate granule cells, CA1, CA3 (Figure 17) and hilar cells of the hippocampus, striatum (Figure 18B) and cerebral cortex (Figure 18F) in all three lines. Intense labelling was seen in Purkinje cells of the cerebellum (Figure 18D) and moderate glial labelling was observed in the corpus callosum (Figure 18H) of all mice expressing the hHSP27 transgene. Small cells with the characteristic appearance of glial cells which were positive for HSP27 were also seen in hippocampal regions (data not shown).

Behavioural effects of kainate administered i.p.

For the present study we have concentrated on a single dose of kainate (25 mg/kg, i.p.) that produces a consistent and maximal effect in our hands. Higher doses increase mortality rate and lower doses (20 mg/kg or lower) are less consistent, with fewer animals developing seizures (Figure 19, inset). Animals were observed for 4 h following kainate administration to allow an evaluation of short term effects of epileptogenesis, seizure activity and tissue was examined at 24 h and 7 days to characterise gene expression and to investigate morphological effects of cell death, respectively. Wild-type animals injected with kainate exhibited behavioural abnormalities as early as 15 min after administration of kainate, notably behavioural arrest and staring spells. This was followed by facial clonus and masticatory seizures within 30-

60 min. Seizure behaviour then progressed to forelimb clonus, rearing and loss of balance within 1-2 h after injection with kainate. Most animals developed status epilepticus but this varied in duration. In some animals brief episodes (1-2 min) of generalised tonic-clonic seizures were observed. This behavioural profile in response to kainate injection is consistent with previous studies in the mouse (47-51).

The time course of response to kainate injection (i.p.) was markedly different in transgenic animals, hHSP27(18) mice overall exhibited much milder seizures throughout most of the 4 h observation period following kainate administration (Figure 19). Further analysis showed mean seizure scores were significantly reduced in kainate treated hHSP27 (18) mice at 60 min ($p<0.01$), 90 min ($p<0.05$), 120 min ($p<0.005$), 150 min ($p<0.005$) and 180 min ($p<0.05$) compared to wild-type animals (Mann-Whitney U-test, Figure 19). Seizure scores for a second transgenic line, hHSP27(64) mice were analysed in two separate experiments and were also shown to be significantly lower than wild-type mice following kainate injection ($p< 0.05$), and there appeared to be a delay in time to maximum seizure in hHSP27(64) mice, but this property did not reach statistical significance (data not shown). No significant behavioural changes were observed in vehicle treated animals.

hHSP27 overexpression also had a significant effect on reducing mortality in the 2 independent lines tested. The mortality rate for hHSP27(18) mice was 18.4% and for hHSP27(64) mice was 16% after kainate treatment which was significantly reduced compared to wild-type littermates (38.5%, $p<0.05$; 40%, $p<0.05$, respectively, by Fisher's exact test).

Cell morphology following kainate administration

Typical morphological changes were seen at the light microscopic level following kainate administration (i.p.) which included pyramidal cell loss in

CA3 of the hippocampus with the appearance of vacuoles, atrophic changes, cell shrinkage and chromatin condensation. These effects were seen in kainate treated wild-type animals but were much reduced in the kainate-treated transgenic line (Figure 20). The CA3 region showed signs of neuronal degeneration, with some of the neurones exhibiting eosinophilic cytoplasm and basophilic chromatin clumping (Figure 21 C and E). These features are characteristic of the light microscopy appearance of apoptotic neurons, although no attempt was made to confirm this type of cell death. There were also signs of perineuronal vacuolation, evident in sections from all kainate-treated mice (Figure 21 D, E, F).

Quantitative analysis of neuronal loss at 7 days following kainate injection was carried out by automated cell counting of haematoxylin-eosin stained sections in four zones within the CA1 and CA3 regions of the hippocampus in four animal groups, PBS (n=3) and KA-treated (n=6) wild-type animals and PBS (n=7) and KA-treated (n=6) transgenic animals (hHSP27(18)). Neuronal cell density for each group is shown in Table 7. Quantitative analysis of the neuronal cell density showed a significant loss of 33.1% ($p<0.05$) in CA3 (zone III) in kainate-treated wild-type mice but this was not significant in other regions analysed. (Table 7, Figure 22). There was no significant cell loss in CA1, in agreement with previous reports of kainate neurotoxicity in mice (52, 53). In kainate-treated transgenic animals, cell loss was much attenuated and was not significantly different from the respective vehicle-treated control group.

Table 7. Neuronal cell density in KA treated wild type and hHSP27(18) transgenic mice. Values are means (± SEM) for neuronal cell density (cell number/mm2) of zones I-IV of the hippocampus in wild type (WT) and hHSP27(18) transgenic mice treated with kainic acid (25 mg/kg, i.p.) or phosphate-buffered saline (PBS: 1 ml/kg, i.p.). Morphometric analysis of haemotoxylin-eosin-stained sections revealed a significant loss of pyramidal neurones in zone III of the WT animals. * indicates that the value is significantly lower than the vehicle-treated control ($p<0.05$, Mann-Whitney *U*-test).

Zone	Group	Neuronal cell density (Mean + SEM)
Zone I (CA3-medial)	Wt-PBS	2472.2 ± 313.1
	Wt-KA	2018.5 ± 269.9
	hHSP27(18)-PBS	1805.6 ± 137.7
	hHSP27(18)-KA	2356.5 ± 104.5
Zone II (CA3- arc)	Wt-PBS	3509.3 ± 386.4
	Wt-KA	2958.4 ± 503.9
	hHSP27(18)-PBS	2964.3 ± 252.5
	hHSP27(18)-KA	3583.3 ± 373.2
Zone III (CA3-lateral)	Wt-PBS	1805.5 ± 55.6
	Wt-KA	1208.4 ± 160.2*
	hHSP27(18)-PBS	1575.4 ± 171.6
	hHSP27(18)-KA	1495.4 ± 47.8
Zone IV (CA1)	Wt-PBS	3138.9 ± 121.1
	Wt-KA	3222.2 ± 249.6
	hHSP27(18)-PBS	3059.5 ± 124.2
	hHSP27(18)-KA	3162.0 ± 124.7

Myocardial Infarction.

Having established that the HSP27 transgenic animals demonstrated a clear neuroprotective effect in keeping with earlier cell culture studies, we investigated whether these animals also showed cardiac protection against ischaemia with reperfusion. Infarct size was measured in Langendorff perfused mouse hearts following 30 min of ischemia and 30 min reperfusion. The extent of infarct size was shown to be significantly limited ($p< 0.006$) to 36.4% in animals expressing hHSP27(18) compared to 42.5% in control litter-mates.

Discussion

In this study we have generated three independent transgenic mouse lines which express human HSP27 at high levels in a wide range of tissues which include brain, spinal cord, heart, skeletal muscle, lung, liver, pancreas and kidney. A detailed examination of the distribution patterns present in brain and heart confirmed the cellular localisation within these tissues and provided a firm basis for the assay of the cytoprotective properties of HSP27 overexpression in vivo. In the brain hHSP27 mRNA and protein was abundant within neurones of the cerebral cortex, hippocampus, striatum, cerebellum and thalamic nuclei. In addition glial expression was also evident. All three lines demonstrated a similar and robust pattern of expression of human HSP27 which was distinguishable from the expression of endogenous mouse HSP25 at the mRNA level by the use of selective oligonucleotide probes and both at the protein and mRNA level by use of an antibody or oligonucleotide probe specific to the HA tag sequence which is contiguous with the hHSP27 cDNA sequence.

Our previous studies in cell culture indicated that HSP27 possessed a unique property amongst heat shock proteins tested to date of being able to protect

neuronal cells in culture against apoptotic cell death (21). It was therefore of particular interest to use an in vivo model in which apoptotic cell death is known to occur as evidenced from morphological and molecular markers (47). Hence we used the i.p. administration of kainate to test the neuroprotective properties of hHSP27 overexpression in the transgenic lines generated. This model is well established in several laboratories and leads to a characteristic generation of seizures with induction of caspase 3 in the CA3 and CA1 regions of hippocampus (54, 55, 19) followed by the appearance of apoptotic changes in these regions and cell death (37, 49, 56). We therefore compared the behavioural responses to kainate injection compared to vehicle injection in groups of two transgenic lines (line 18 and line 64). Morphological effects have been compared in parallel in line 18. A marked effect of neuroprotection was seen in both lines as evidenced from enhanced survival and significantly reduced seizure severity sustained over the major period of seizures (60-180 min).

The effect of HSP27 overexpression on cell death was examined at 7 days after kainate injection where maximal cell death occurs in the CA3 region of hippocampus. The severity of cell death usually ranges from 30ñ50% in the mouse (e.g. a 23% reduction in cell density following a dose of 34 mg/kg kainate). This level of cell loss between kainate and vehicle treated animals was seen in the control littermates being 33% ($p < 0.05$). In contrast, while cell density was similar in vehicle treated transgenics compared to controls, no significant cell loss was detected in transgenic animals after kainate administration.

In order to control for strain differences in susceptibility to kainate, breeding at all generations was always with an F1 hybrid C57BL/CBA/Ca to maintain a hybrid genetic background. This strategy was adopted to avoid the marked differences that have been reported between inbred strains in behavioural tests

involving kainate (e.g. Refs. 57 and 53). All tests were carried out between litter-mates which either carried the transgene (+ve) or lacked it (-ve) thus avoiding problems arising from use of inbred strains. Thus the reduced seizure susceptibility seen here in two independent transgenic lines is likely to have resulted from the overexpression of the human transgene.

We also show for the first time in this study the gross anatomical labeling pattern of induced endogenous mouse HSP25 mRNA in mice following kainic acid administration. Mouse HSP25 mRNA was predominantly localised to glial cells with modest labeling of neurones, particularly in the pyramidal cell layers of the hippocampus (data not shown) which reproduces the finding in the rat (19). The distribution of mouse HSP25 mRNA was similar to that for GFAP mRNA as assessed on adjacent sections by in situ hybridisation although the up-regulation of GFAP mRNA was more profound and widespread in agreement with similar studies in the rat (19). Up-regulation of GFAP has been described as a marker for neurotoxicity and aberrant neuronal activity (58, 59). The comparable pattern of expression and induction levels of HSP25 mRNA and GFAP mRNA in both transgenic and wild-type mice in response to kainic acid would suggest that mice of both genotype were subjected to a similar level of noxious stress.

The effect of overexpression of hHSP27 clearly showed a significant and rapid effect in the behavioural response to kainate injection indicating an immediate suppression of the spread of the seizure activity. This may be due to an interaction of HSP27 with components of the excitatory signaling cascade involved in seizure propagation which have not as yet been elucidated. HSP27 is known to be involved in apoptotic signaling cascades and these sites of action are likely to contribute to the longer-term effects of HSP27 to reduce overall cell death. Direct molecular interactions with two pathways leading to apoptosis, the caspase-dependent and Daxx components of Fas mediated

apoptosis (60) have been reported.

Cardiac protection

These experiments represent for the first time that overexpression of HSP27 is protective in limiting infarct volume in the heart in vivo in support of earlier studies in cultured cardiac cells (25). HSP70 overexpressing mice have also been successfully used to demonstrate a decrease in cardiac infarct size in Langendorff perfused isolated hearts after 20 min ischaemia followed by reperfusion (26) and in vivo following coronary artery occlusion for 30 min followed by reperfusion (61). A similar degree of protection has thus been demonstrated for transgenic animals overexpressing either HSP27 or HSP70 indicating that both HSPs can interact with damaging oxidative cascades either through their protein chaperone properties or other mechanisms. It is already known that HSP27 increases glutathione (reduced form)-dependent chaperone activity against misfolded or oxidised proteins (62) which could contribute to protection against reactive oxygen species. A combined effect of overexpression of both heat shock proteins may have a synergistic effect improving outcome.

In contrast to the similar protective effects of overexpression of HSP27 and 70 in cardiac tissue a differential effect is seen in the nervous system. Conflicting results have been obtained with HSP70 transgenics in *in vivo* models such as permanent focal cerebral ischaemia models where protection has been reported by Rajdev et al. (29) whilst no significant difference in infarct size assessed at 24 h was found by Lee at al. (30). In the present study a clear neuroprotective effect was seen with over expression of HSP27 in vivo. These results provide further support for the idea that HSP27 is more important than HSP70 in the nervous system.

Mechanism of cytoprotection mediated by hHSP27

HSP27 has been shown to have multiple inhibitory effects on apoptotic signaling cascades affecting both the intrinsic mitochondrial and extrinsic pathway of apoptosis. In the intrinsic mitochondrial pathway, HSP27 appears to be able to suppress cytochrome c-caspase 3-dependent apoptosis through actions at multiple sites, affecting the release of cytochrome c (63) and by binding directly to cytochrome c and inhibiting subsequent apoptosome formation, and preventing caspase 9 maturation (64). In addition, Pandey et al. (65) have also shown a direct interaction of HSP27 with caspase 3 processing inhibiting cytochrome c-dependent apoptosis in 293T and L929 cells. The cell specificity of these actions of HSP27 remains to be elucidated in more intact systems. Events upstream of cytochrome c are also known to be affected by HSP27 and could contribute to protective effects for example through the reduction of F-actin damage and through mobilisation of members of the Bcl-2 family (63). The inhibitory effect of HSP27 is specific for cytochrome c-caspase dependent apoptosis and does not affect the actions of AIF (apoptosis inducing factor) which is also released from mitochondria during apoptosis. It has been demonstrated both in vivo and in vitro that large non-phosphorylated oligomers of HSP27 are the active form of the protein responsible for the caspase-dependent anti-apoptotic effect of HSP27 (64).

In the extrinsic pathway of apoptosis, HSP27 inhibits the Daxx pathway (60) preventing c-Jun amino-terminal kinase 3 activation. Phosphorylation of HSP27 modulates activity by producing a large change in the supramolecular organisation of the protein with a shift from oligomers to dimers. In Fas-mediated apoptosis, phosphorylated dimers of HSP27 interact with Daxx, a mediator of Fas-mediated apoptosis, preventing the interaction with Fas and Ask1 and blocking Daxx mediated apoptosis (60). The Daxx-dependent pathway is distinct from that mediated by FADD which is not sensitive to

HSP27.

It will be important to determine the role of phosphorylation in mediating both the short and longer-term effects of HSP27 which we have observed in vivo. An increase in phosphorylation is detected within minutes of a cellular stress with the increased expression occurring after a few hours. The rapid stress induced phosphorylation results from stimulation of the p38 MAP kinase cascade and leads to the phosphorylation of mammalian HSP27 at 2/3 serine residues by MAPKAP kinase2/3. However, phosphorylation differentially affects the protective properties of HSP27. Thus, chaperone activity and the ability to block the mitochondrial pathway are inhibited by phosphorylation whereas the ability to block Daxx-mediated apoptosis is stimulated by phosphorylation.

These studies demonstrate for the first time in vivo an important role for HSP27 in both cardiac and CNS tissue in response to ischaemic and apoptotic injury and provide support for the unique property of HSP27 amongst HSPs to protect neurones from apoptotic cell death. Recently we demonstrated that intracerebral viral delivery of HSP27 reduced kainate-induced neuronal cell death in hippocampus in rats (66). Thus HSP27 shows enormous potential as a target for neuroprotective intervention.

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CLAIMS

1. A method of determining an increased risk of a late-onset neurodegenerative disease to a patient, the method comprising analysing a sample from the patient to determine whether the patient has a D-amino acid oxidase (DAO) abnormality, wherein the presence of a DAO abnormality is an indication that the patient has an increased risk of the late-onset neurodegenerative disease.

2. A method according to Claim 1 wherein determining an increased risk of a late-onset neurodegenerative disease to a patient comprises determining an increased likelihood of developing the late-onset neurodegenerative disease, or determining an increased likelihood of having an increased rate of progression of the late-onset neurodegenerative disease, or determining an increased likelihood of having an increased severity of the late-onset neurodegenerative disease.

3. A method according to Claim 1 wherein analysing the sample comprises determining the level of at least one D-amino acid in the sample, and wherein a D-amino acid level greater than a predetermined normal range is an indication that the patient has a DAO abnormality.

4. A method according to Claim 3 wherein the predetermined normal range is about 100 nmol/g.

5. A method according to Claim 3 wherein the D-amino acid in the sample whose level is determined is D-serine.

6. A method according to Claim 1 wherein analysing the sample comprises determining the D- to L-amino acid ratio (D/L ratio) of at least one amino acid in the sample, and wherein a D/L ratio greater than a predetermined normal value is an indication that the patient has a DAO

abnormality.

7. A method according to Claim 6 wherein the at least one amino acid in the sample whose D/L ratio is determined is serine.

8. A method according to Claim 6 wherein the predetermined normal value is about 0.15.

9. A method according to Claim 6 wherein the predetermined normal value is about 0.25.

10. A method according to Claim 1 wherein analysing the sample comprises determining the activity of DAO in the sample, and wherein an activity below a predetermined normal range is an indication that the patient has a DAO abnormality.

11. A method according to Claim 1 wherein analysing the sample comprises determining the amount of DAO in the sample, and wherein an amount of DAO below a predetermined normal range is an indication that the patient has a DAO abnormality.

12. A method according to any one of the preceding claims wherein the sample is a blood, serum, saliva, urine, cerebrospinal fluid, leukocyte or skin sample from the patient.

13. A method according to Claim 1 wherein analysing the sample comprises determining whether nucleic acid in the sample contains a mutation in a DAO gene, wherein the presence of a DAO mutation is an indication that the patient has a DAO abnormality.

14. A method according to Claim 13 wherein analysing the sample comprises amplifying DAO-encoding nucleic acid, and analysing the

amplified nucleic acid.

15. A method according to Claim 14 further comprising the prior step of reverse transcribing RNA in the sample.

16. A method according to any one of Claims 13 to 15 wherein the patient is a foetus.

17. A method according to any one of Claims 13 to 16, wherein DAO nucleic acid from the patient is compared to DAO nucleic acid of at least one relative of the patient.

18. A method according to Claim 17 wherein DAO nucleic acid from the patient is compared to DAO nucleic acid of at least one relative of the patient who has the late-onset neurodegenerative disease and to at least one relative of the patient who does not have has the late-onset neurodegenerative disease.

19. A method according to any one of Claims 13 to 18 wherein the DAO mutation changes Arg at amino acid 199 to another amino acid (DAO^{199mut}).

20. A method according to Claim 19 wherein the DAO mutation changes Arg at amino acid 199 to Trp (DAO R199W).

21. A method according to any one of the preceding claims wherein the late-onset neurodegenerative disease is amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) or Alzheimer's disease (AD).

22. A method for treating a patient with a late-onset neurodegenerative disease comprising the step of providing to the patient an effective amount of DAO.

23. A method according to Claim 22 wherein the late-onset neurodegenerative disease is ALS, PD or AD.

24. A method according to Claim 23 wherein providing DAO comprises administering DAO protein to the patient.

25. A method according to Claim 24 wherein the DAO protein is human DAO protein.

26. A method according to Claim 24 or 25 wherein the late-onset neurodegenerative disease is ALS, the method further comprising administering to the patient an agent selected from *i*) methylcobalamin, *ii*) 1-(2-naphth-2-ylethyl)-4-(3-trifluoromethylphenyl)-1,2,3,6- tetrahydropyridine, *iii*) a non-cysteine glutathione precursor, *iv*) a glutathione derivative, *v*) topiramate, or v*i*) 2-amino-6-(trifluoromethoxy)benzothiazole, or a pharmaceutically acceptable salt thereof.

27. A method according to Claim 24 or 25 wherein the late-onset neurodegenerative disease is PD, the method further comprising administering either or both of L-DOPA and a DOPA decarboxylase inhibitor to the patient.

28. A method according to Claim 24 or 25 wherein the late-onset neurodegenerative disease is AD, the method further comprising administering an acetylcholine esterase inhibitor to the patient.

29. A method according to Claim 23 wherein providing DAO comprises administering a nucleic acid that encodes DAO to the patient.

30. A method according to Claim 29 wherein the nucleic acid encodes human DAO.

31. A method according to Claim 29 or 30 wherein the late-onset neurodegenerative disease is ALS and the nucleic acid is administered to motor neurones.

32. A method according to Claim 31 wherein the nucleic acid is administered to motor neurones by injecting the nucleic acid in an adenoviral vector into muscle of the patient.

33. A method according to Claim 29 or 30 wherein the late-onset neurodegenerative disease is PD, and the nucleic acid is administered to the substantia nigra.

34. A method according to Claim 33 wherein the nucleic acid is administered to the substantia nigra by direct stereotaxic intracerebral injection.

35. A method according to Claim 29 or 30 wherein the late-onset neurodegenerative disease is AD, and the nucleic acid is administered to the basal forebrain.

36. A method according to Claim 35 wherein the nucleic acid is administered to the basal forebrain by direct injection to the basal forebrain.

37. A pharmaceutical composition comprising DAO and a pharmaceutically acceptable carrier.

38. A pharmaceutical composition comprising a nucleic acid molecule that encodes DAO and a pharmaceutically acceptable carrier.

39. DAO for use in medicine.

40. A nucleic acid molecule that encodes DAO for use in medicine.

41. A nucleic acid molecule that encodes DAO in a disabled herpes simplex viral vector for use in medicine.

42. Use of DAO in the preparation of a medicament for treating a late-

onset neurodegenerative disease.

43. Use of a nucleic acid molecule that encodes DAO in the preparation of a medicament for treating a late-onset neurodegenerative disease.

44. Use according to Claim 42 or 43 wherein the late-onset neurodegenerative disease is ALS, PD or AD.

45. DAO having any amino acid except Arg at position 199 (DAO^{199mut}).

46. DAO^{199mut} having Trp at amino acid position 199 (DAO 199W).

47. DAO^{199mut} according to Claim 45 or 46 which is human DAO^{199mut}.

48. A nucleic acid encoding DAO^{199mut} according to any one of Claims 45 to 47, or the complement thereof.

49. A vector comprising the nucleic acid of Claim 48.

50. A host cell comprising the nucleic acid of Claim 48 or the vector of Claim 49.

51. A host cell according to Claim 50 which is a mammalian neuronal cell, preferably a human neuronal cell.

52. A cell culture comprising the host cell of Claim 50 or 51.

53. A cell culture according to Claim 52 that expresses DAO^{199mut}.

54. A cell culture according to Claim 53 wherein DAO^{199mut} is DAO 199W, preferably human DAO 199W.

55. A cell culture according to any one of Claims 52 to 54 wherein expression of DAO^{199mut} is inducible.

56. A cell culture according to any one of Claims 52 to 54 wherein expression of DAO^{199mut} is transient.

57. A host cell according to Claim 50 or 51, or a cell culture according to any one of Claims 52 to 56, with reduced or no endogenous DAO activity.

58. A host cell according to Claim 50 or 51 or a cell culture according to any one of Claims 52 to 57, with reduced or no endogenous SOD1 activity, with reduced or no endogenous HSP27 activity, or with reduced or no endogenous SOD1 activity and reduced or no HSP27 activity.

59. A method of screening for a protective agent, the method comprising:

providing a cell culture according to any one of Claims 52 to 58,

providing a test agent,

expressing DAO^{199mut}, and

assessing at least one characteristic of the cells in the culture,

wherein an improvement in the at least one characteristic of the cells in the presence of the test agent indicates that the test agent is a protective agent.

60. A method according to Claim 59 wherein the protective agent protects cells against the toxic effects of DAO^{199mut}.

61. A method of screening for a protective agent according to Claim 59, further comprising providing D-amino acids to the culture.

62. A method according to Claim 61 wherein the protective agent protects cells against DAO abnormality, or protects cells against the accumulation of D-amino acids, or protects against late-onset neurodegenerative disease.

63. A method according to any one of Claims 59 to 62 wherein the at least one characteristic of the cells is selected from cell viability, cell survival, cell

growth, apoptotic and non-apoptotic cell-death, and enzyme activity.

64. A neuronal protective agent identified by the method of any one of Claims 59 to 63.

65. An experimental model for screening for a protective agent, the model comprising non-human tissue without or with reduced endogenous DAO activity.

66. An experimental model according to Claim 65 that expresses DAO^{199mut}.

67. An experimental model according to Claim 66 wherein the DAO^{199mut} is DAO 199W, preferably human DAO 199W.

68. An experimental model according to Claim 66 or 67 wherein expression of DAO^{199mut} is inducible.

69. An experimental model according to Claim 66 or 67 wherein expression of DAO^{199mut} is transient.

70. An experimental model according to any one of Claims 65 to 69 wherein the model comprises a cerebral artery occlusion, neonatal axotomy or lesions of the substantia nigra.

71. A method of screening for a protective agent, the method comprising:

providing an experimental model according to any one of Claims 65 to 70,

providing a test agent,

expressing DAO^{199mut}, and

assessing at least one characteristic of the tissue in the model,

wherein an improvement in the at least one characteristic of the tissue in the model in the presence of the test agent indicates that the test agent is a protective agent.

72. A method according to Claim 71 wherein the protective agent protects tissue against the toxic effects of DAO^{199mut}.

73. A method of screening for a protective agent according to Claim 71, further comprising providing D-amino acids to the model.

74. A method according to Claim 73 wherein the protective agent protects tissue against DAO abnormality, or protects tissue against the accumulation of D-amino acids, or protects against late-onset neurodegenerative disease.

75. A method according to any one of Claims 71 to 74 wherein the at least one characteristic of the tissue is tissue survival, apoptotic and non-apoptotic cell-death, and enzyme activity.

76. A protective agent identified by the method of any one of Claims 71 to 75.

77. A non-human mammal having one or both endogenous copies of the DAO gene knocked-out.

78. A non-human mammal according to Claim 77 that expresses human DAO, or DAO^{199mut}.

79. A non-human mammal that expresses human DAO, or DAO^{199mut}.

80. A non-human mammal according to Claim 79 without or with reduced endogenous DAO activity.

81. A non-human mammal according to any one of Claims 78 to 80

wherein the DAO^{199mut} is DAO 199W, preferably human DAO 199W.

82. A non-human mammal according to any one of Claims 77 to 81 and with reduced or no endogenous SOD1 activity, with reduced or no endogenous HSP27 activity, or with reduced or no endogenous SOD1 and with reduced or no endogenous HSP27 activity.

83. A non-human mammal according to any one of Claims 77 to 82 and having at least one of human SOD1 activity or human HSP27 activity.

84. A method of screening for an agent that protects against late-onset neurodegenerative disease, the method comprising:

providing at least one test and at least one control non-human mammal according to any one of Claims 77 to 83,

administering a test agent to the at least one test mammal,

assessing at least one characteristic of the test and control non-human mammals,

wherein an improvement in the at least one characteristic of the test mammal relative to the control mammal indicates that the test agent protects against late-onset neurodegenerative disease.

85. A method according to Claim 84 wherein the test agent protects against the toxic effects of DAO^{199mut}.

86. A method according to Claim 84, further comprising providing D-amino acids to the non-human mammal.

87. A method according to Claim 86 wherein the test agent protects against DAO abnormality, or against the accumulation of D-amino acids.

88. A method according to any one of Claims 84 to 87 wherein the at least

one characteristic is selected from survival, ageing, neurodegeneration, behaviour, apoptotic and non-apoptotic cell death, and enzyme activity.

89. An agent that protects against late-onset neurodegenerative disease identified by the method of any one of Claims 84 to 88.

90. A method according to any one of Claims 62, 74, or 84 to 88 wherein the late-onset neurodegenerative disease is ALS, PD or AD.

91. A method of determining an increased likelihood of a patient developing a late-onset neurodegenerative disease, the method comprising analysing DNA from the patient to determine whether the patient has T at position 99182, A at position 99184, A at position 100938, C at position 103317, C at position 120276, and/or G at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has an increased likelihood of developing a late-onset neurodegenerative disease.

92. A method of determining a reduced likelihood of a patient developing a late-onset neurodegenerative disease, the method comprising analysing DNA from the patient to determine whether the patient has C at position 99182, T at position 99184, T at position 100938, T at position 103317, T at position 120276 and/or C at position 122443 in the TXNRD1 gene, wherein the presence of one or more of these nucleotides at these positions is an indication that the patient has a reduced likelihood of developing a late-onset neurodegenerative disease.

93. A method according to Claim 91 or 92 wherein the late-onset neurodegenerative disease is ALS, AD or PD.

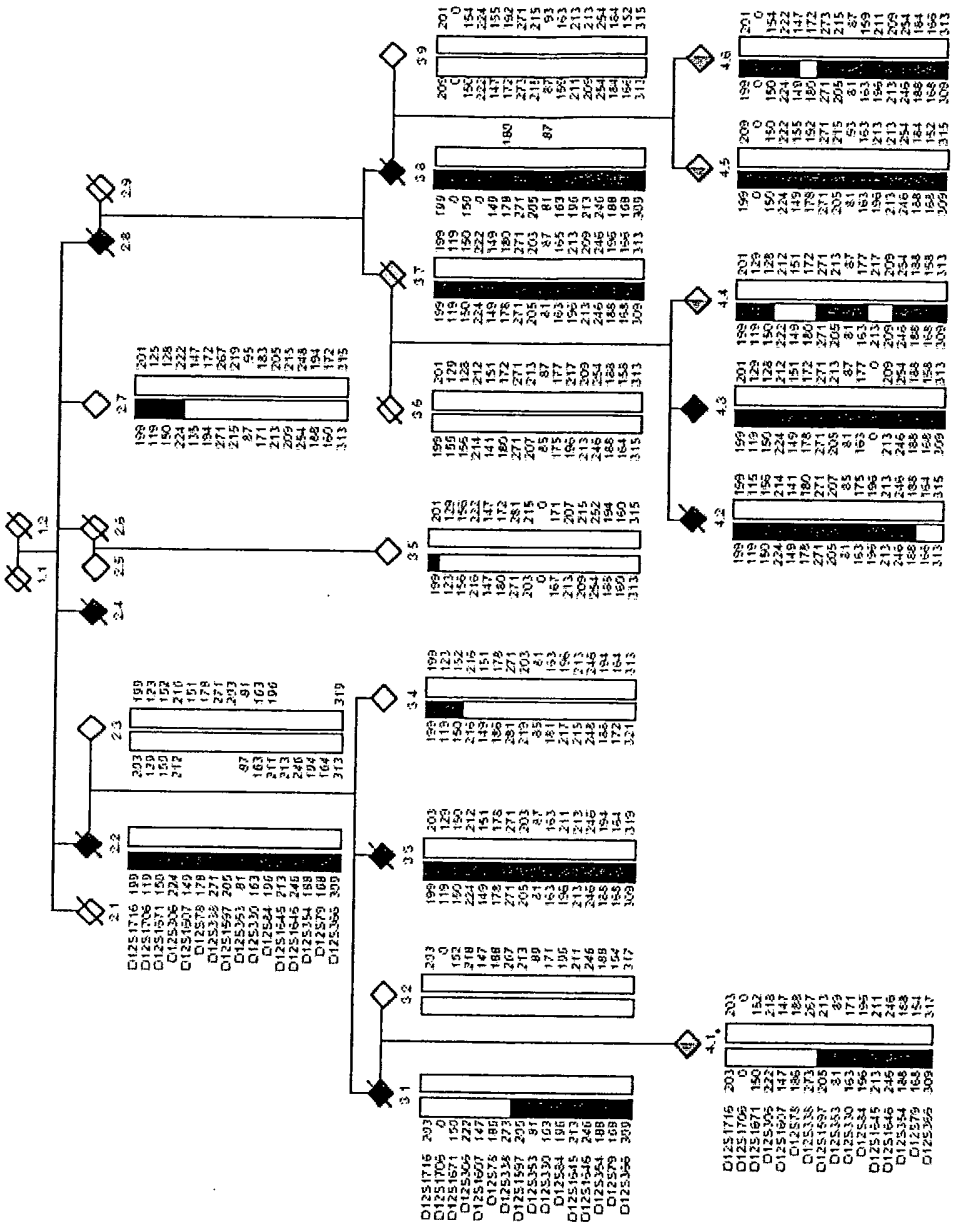


Figure 1.

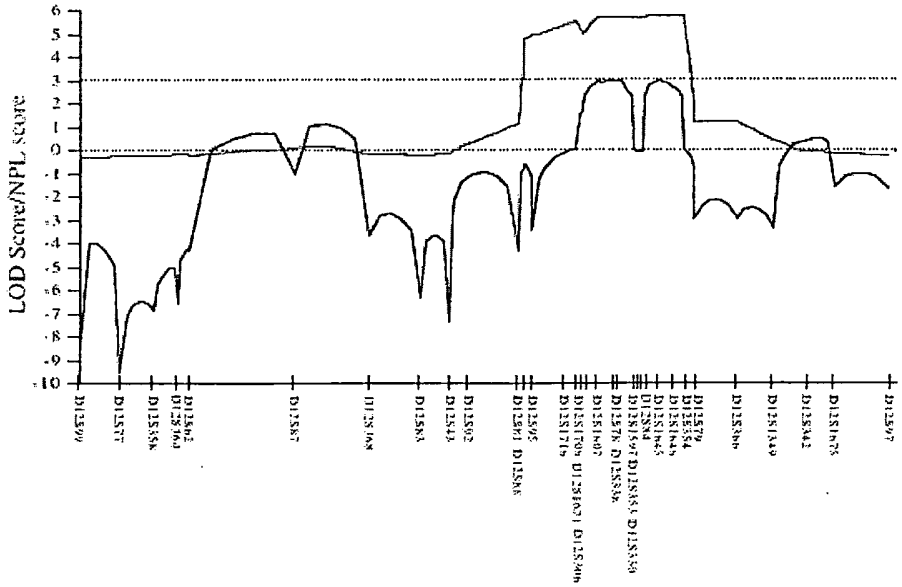


Figure 2.

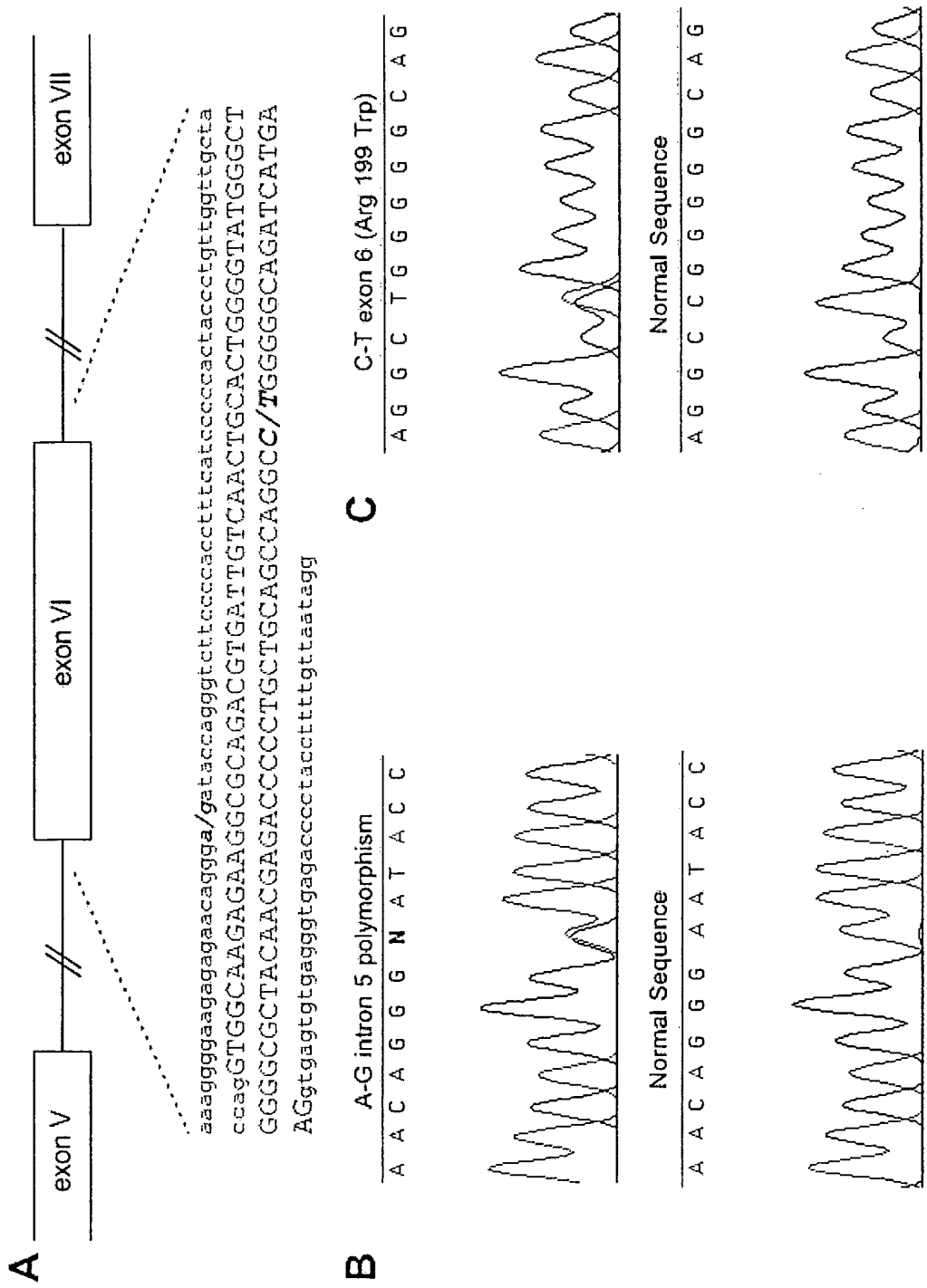

A
exon V
exon VI
exon VII
aaaggggaagagagaacaggga/gataccagggtcttccccaccttttcatccccactaccctgttggttgcta
ccagGTGGCAAGAGAAGGCGCAGACGATGATTGTCAACTGCACTGGGGTATGGGCT
GGGGCGCTACAACGAGACCCCCCTGCTGCAGCCAGGCC/TGGGGGGCAGATCATGA
AGgtgagtgtgagggtgagaccccctacctttttgttaatagg
B
A-G intron 5 polymorphism
A A C A G G G G N A T A C C
Normal Sequence
A A C A G G G G A A T A C C
C
C-T exon 6 (Arg 199 Trp)
A G G C T G G G G G C A G
Normal Sequence
A G G C C G G G G G C A G

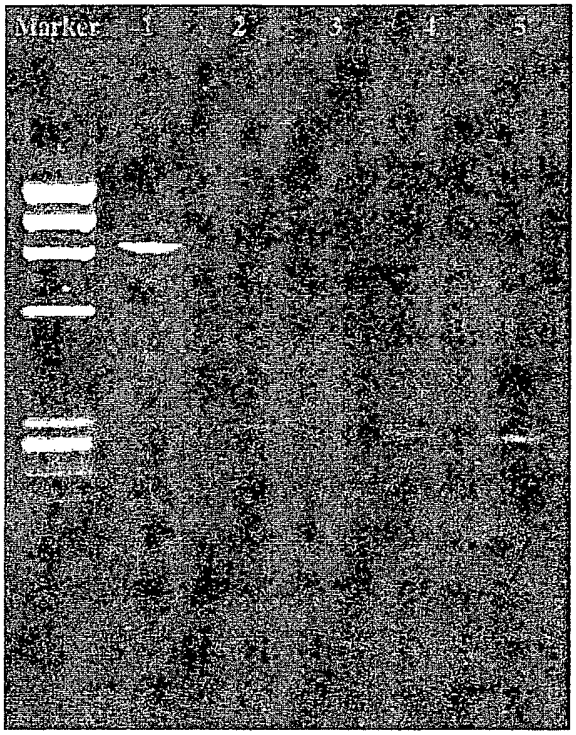
Figure 3.

A cDNA partial sequence

Exon 4 > Exon 7

```
ctatggctggttccacacaagcctaattctggagggaaagaactatctacagtgg
ctgactgaaaggttaactgagaggggagtgaagttcttccagcggaaagtggagt
cttttgaggaggtggcaagagaaggcgcagacgtgattgtcaactgcactggggt
atgggctggggcgctacaacgagacccccctgctgcagccaggcc/tgggggcaga
tcatgaaggtggacgccccttggatgaagcacttcattctcacccatgacccaga
gagaggcatctacaattccccgtacatcatcccagg
```

B

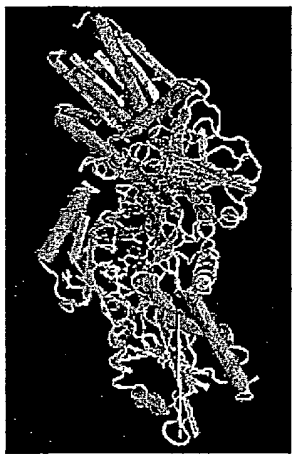


C

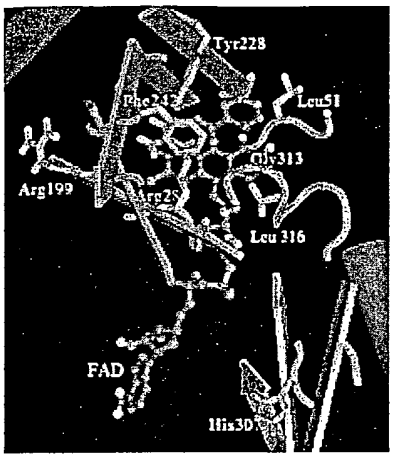


Figure 4.

A



B



C

```
                10        20        30        40        50        60
Human MRVVVIGAGVIGLSTALCIHERYHSVLQPLHIKVYADRFTPLTTTDVAAGLWQPYLSDPN   60
Mouse MRVAVIGAGVIGLSTALCIHERYHPT-QPLHMKIYADRFTPPTTSDVAAGLWQPYLSDPS   59
Rabbit MRVVVIGAGVIGLSTALCIHELYHSALQPLDMTIYADRFTPLTNTDVAAGLWQPYLSDPS   60
Rat   MRVAVIGAGVIGLSTALCIHERYHPA-QPLHMKIYADRFTPPTTSDVAAGLWQPYLSDPS   59
Pig   MRVVVIGAGVIGLSTALCIHERYHSVLQPLDVKVYADRFTPPTTTDVAAGLWQPYTSEPS   60

                70        80        90        100       110       120
Human NPQEADWSQQTFDYLLSHVHSPNAENLGLFLISGYNLFHEAIPDPSWKDTVLGFRKLTPR  120
Mouse NPQEAEWSQQTFDYLLSCLHSPNAEKMGLALISGYNLFRDEVPDPFWKNAVLGFRKLTPS  119
Rabbit NPQEADWSRQTFNHLLSHIHSPSAEKMGLALISGYNLFRKAVPDPSWKDTVLGFRKLTLR  120
Rat   NPQEAEWNQQTFDHLQSCLHSPNAEKMGLALISGYNLFRDEVPDPFWKSTVLGFRKLTPS  119
Pig   NPQEANWNQQTFNYLLSHIGSPNAANMGLTPVSGYNLFREAVPDPYWKDMVLGFRKLTPR  120

                130       140       150       160       170       180
Human ELDMFPDYGYGWFHTSLILEGKNYLQWLTERLTERGVKFFQRKVESFEEVAREGADVIVN  180
Mouse EMDLFPDYGYGWFNTSLLLEGKSYLPWLTERLTERGVNLIHRKVESLEEVARGGVDVIIN  179
Rabbit ELDMFPGYSYGWFNTSLILDGRSYLQWLTKRLTERGVKLFQRKVESFDEVAGGGVDVIVN  180
Rat   ELDMFPDYSYGWFNTSLLLEGKSYLSWLTERLTERGVKFIHRKVASFEEVVRGGVDVIIN  179
Pig   ELDMFPDYRYGWFNTSLILEGRKYLQWLTERLTERGVKFFLRKVESFEEVARGGADVIIN  180

                190       200       210       220       230       240
Human CTGVWAGALQRDPLLQPGRGQIMKVDAPWMKHFILTHDPERGIYNSPYIIPGTQTVTLGG  240
Mouse CTGVWAGALQADASLQPGRGQIIQVEAPWIKHFILTHDPSLGIYNSPYIIPGSKTVTLGG  239
Rabbit CTGVWASALQPDPLLQPGRGQIIKVDAPWVKHFIITHDPESGIYKSPYIIPGVHAVTLGG  240
Rat   CTGVWAGALQADASLQPGRGQIIQVEAPWIKHFILTHDPSLGIYNSPYIIPGSKTVTLGG  239
Pig   CTGVWAGVLQPBPLLQPGRGQIIKVDAPWLKNFIITHDLERGIYNSPYIIPGLQAVTLGG  240

                250       260       270       280       290       300
Human IFQLGNWSELNNIQDHNTIWEGCCRLEPTLKNARIIGERTGFRPVRPQIRLEREQLRTGP  300
Mouse IFQLGNWSGLNSVRDHNTIWKSCCKLEPTLKNARIVGELTGFRPVRPQVRLEREWLRFGS  299
Rabbit IPQMGNWSEGNSTDDHNTIWKGCCSLEPTLKDARIVGEWTGFRPVRPQIRLGREQLSAGP  300
Rat   VPQLGNWSELNSVHDHNTIWKSCCQLEPTLKNARIMGELTGFRPVRPQVRLERERLRFGS  299
Pig   TFQVGNWNEINNIQDHNTIWEGCCRLEPTLKDAKIVGEYTGFRPVRPQVRLEREQLRFGS  300

                310       320       330       340
Human SNTEVIHNYGHGGYGLTIHWGCALEAAKLFGRILEEKKLSRMPPSHL  347
Mouse SSAEVIHNYGHGGYGLTIHWGCAMEAANLFGKILEEKKLSRLPPSHL  346
Rabbit SKTEVIHNYGHGGYGLTIHWGCALEAAKLFGKILEEKKSSRMPPSHL  347
Rat   SSAEVIHNYGHGGYGLTIHWGCAMEAANLFGKILEEKNLSRMPPSHL  346
Pig   SNTEVIHNYGHGGYGLTIHWGCALEVAKLFGKVLEERNLLTMPPSHL  347
```

Figure 5

FIGURE 6

The effect of serum-withdrawal (0-48h) on ND7 stable cell lines expressing SOD1 or SOD1 mutations in the presence of all-trans-retinoic acid.

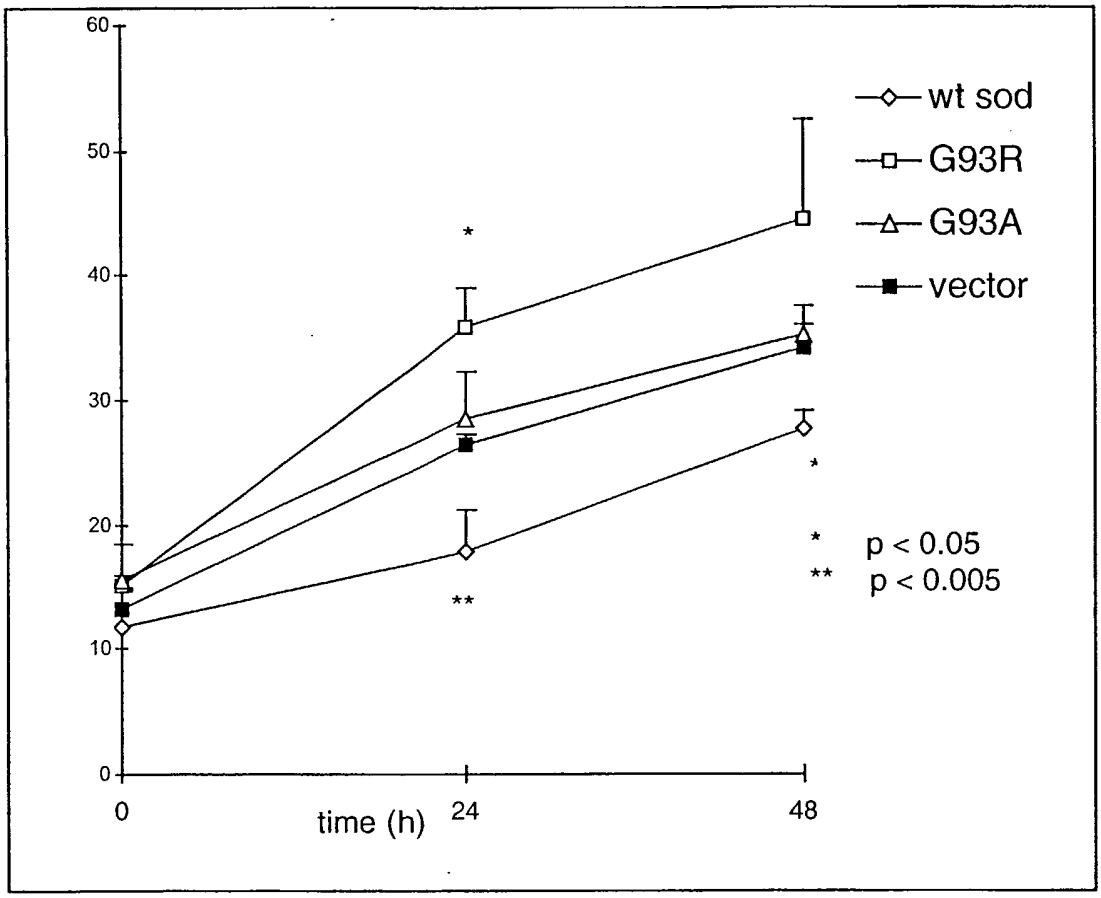


FIGURE 7

Apoptotic cell death in ND7 stable cell lines expressing SOD or SOD mutations following serum-withdrawl (0-48h) in the presence of all-trans-retinoic acid.

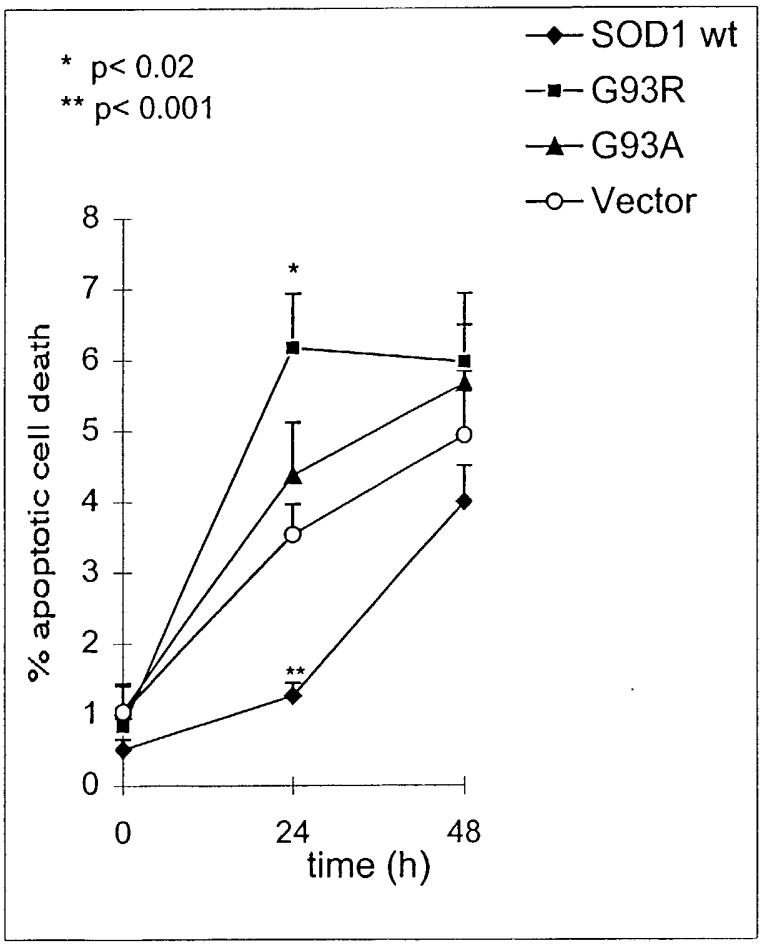


FIGURE 8

Effect of staurosporine on cell survival

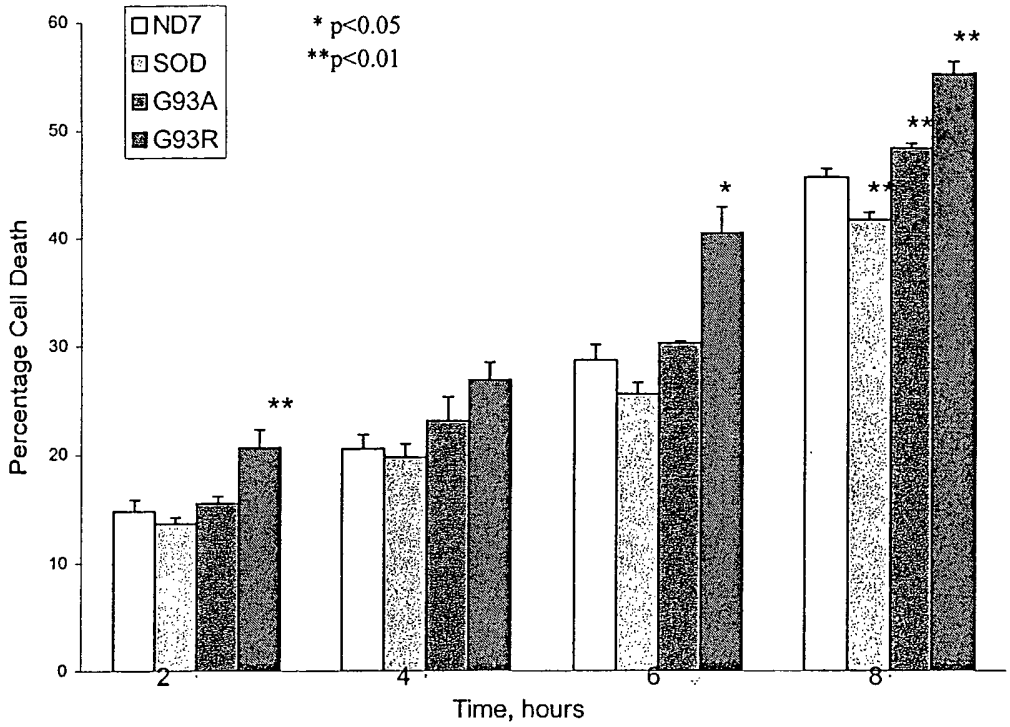


FIGURE 9A

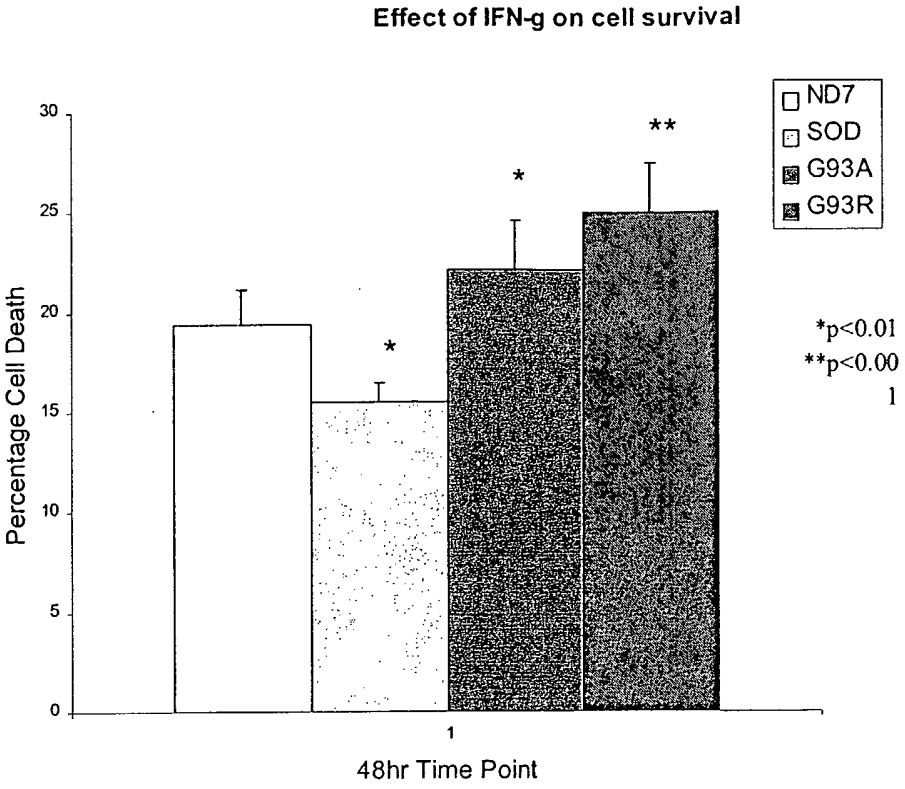

Effect of IFN-g on cell survival
ND7
SOD
G93A
G93R
*p<0.01
**p<0.00
1
Percentage Cell Death
30
25
20
15
10
5
0
*
*
**
1
48hr Time Point

FIGURE 9B

Effect of ischaemia (4h ischaemia + 24 h reperfusion) on cell survival

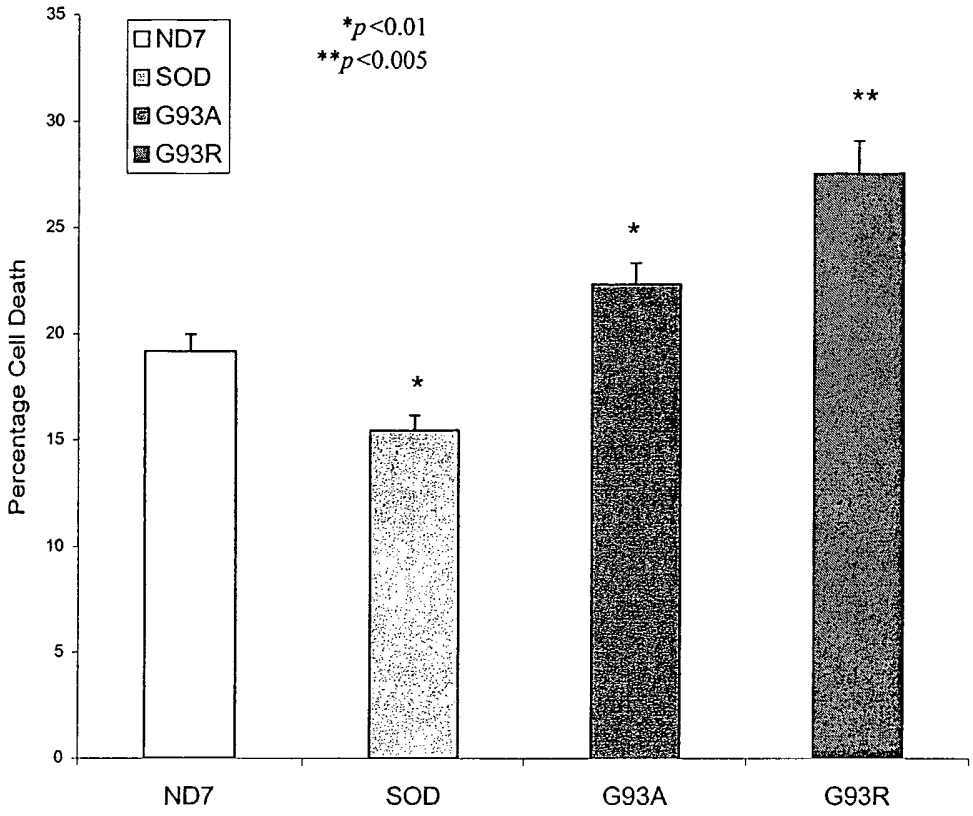
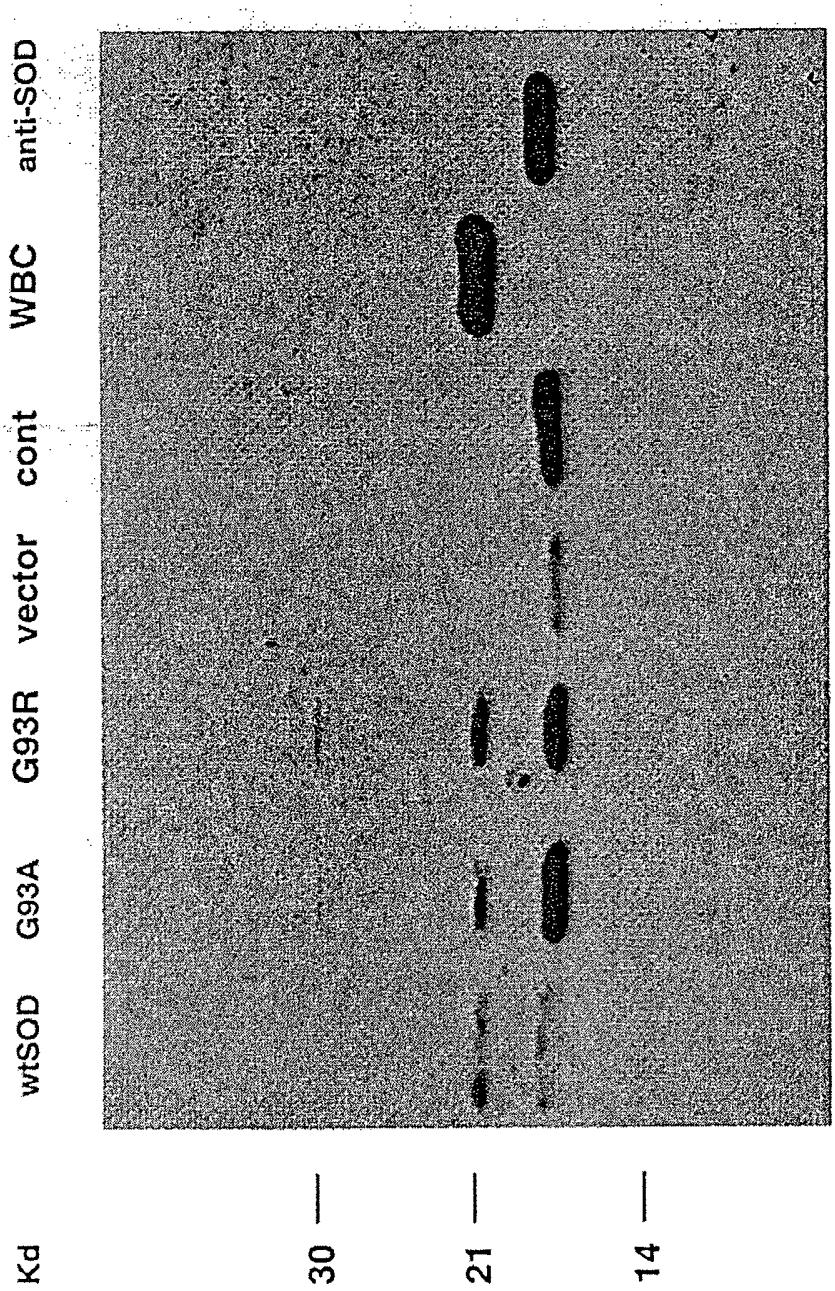


FIGURE 10



SOD1 expression in HSV-infected BHK cells.

FIGURE 11

Effect of infection with HSV vectors expressing SOD1 and the G93R SOD1 mutations on ND7 cell death at time 0 and 24h following transfer to serum-free medium in the presence of all-trans- retinoic acid.

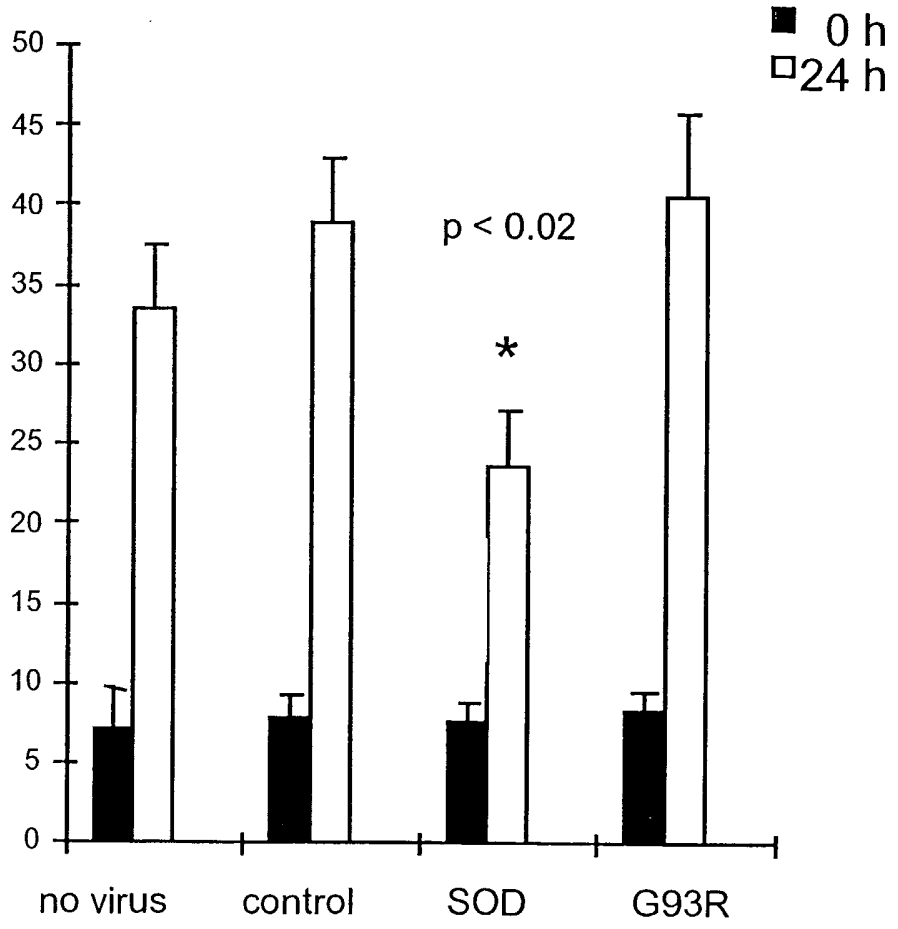


FIGURE 12

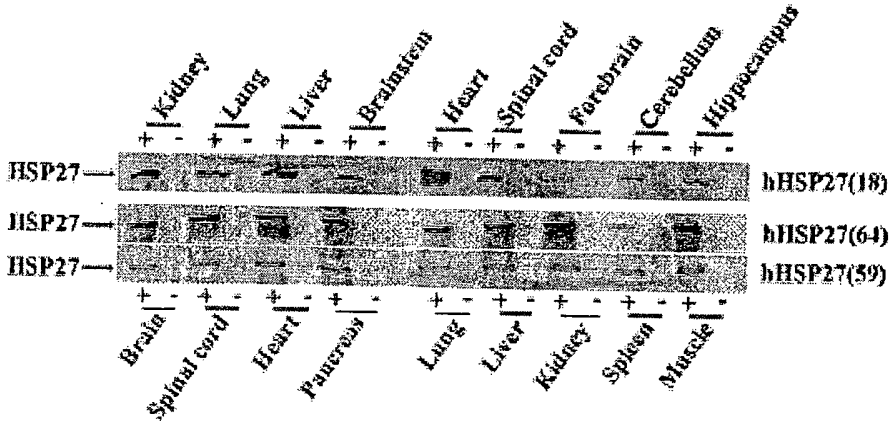

Kidney
Lung
Liver
Brainstem
Heart
Spinal cord
Forebrain
Cerebellum
Hippocampus
HSP27
HSP27
HSP27
hHSP27(18)
hHSP27(64)
hHSP27(59)
Brain
Spinal cord
Heart
Pancreas
Lung
Liver
Kidney
Spleen
Muscle

FIGURE 13

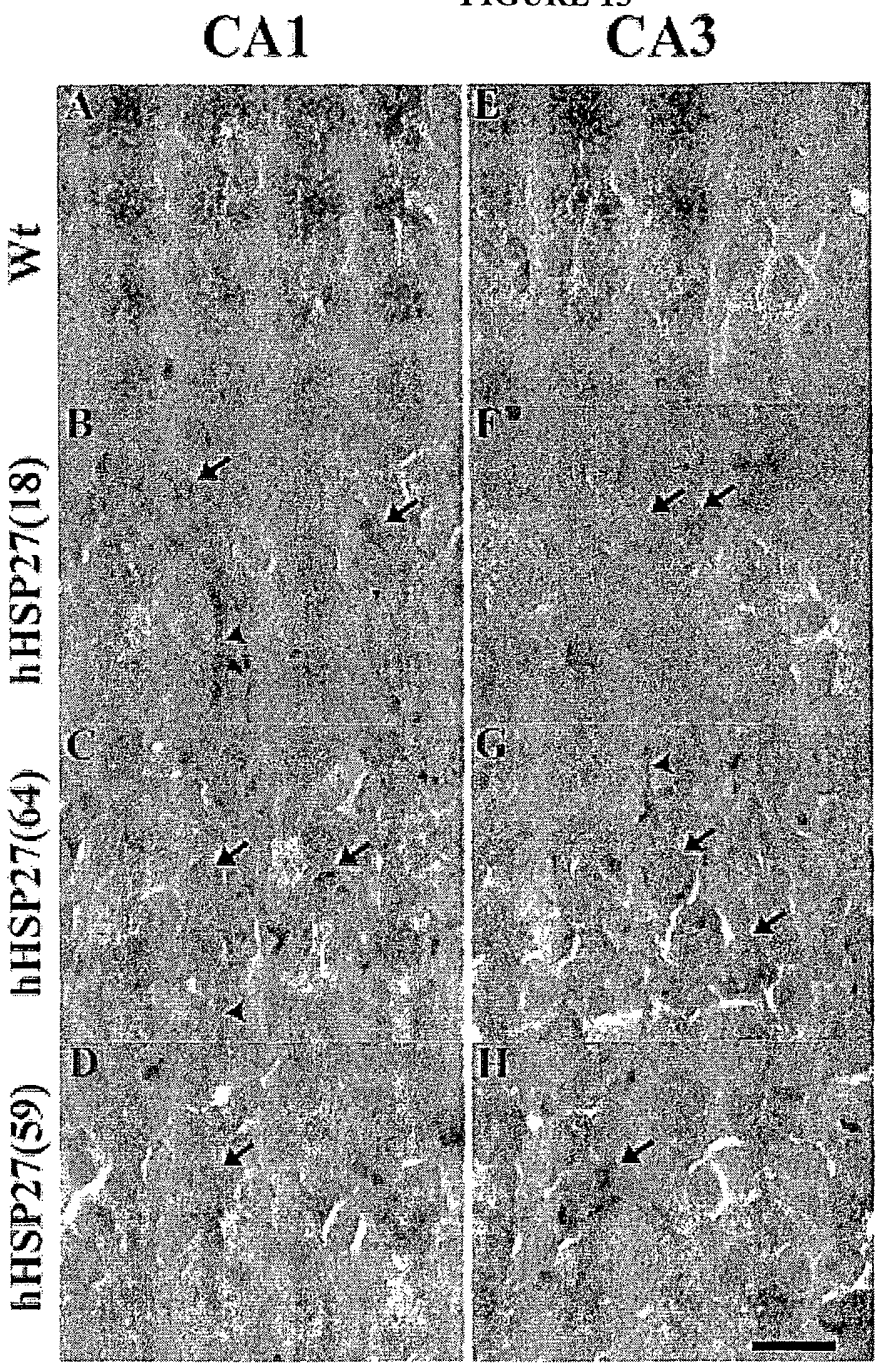


FIGURE 14

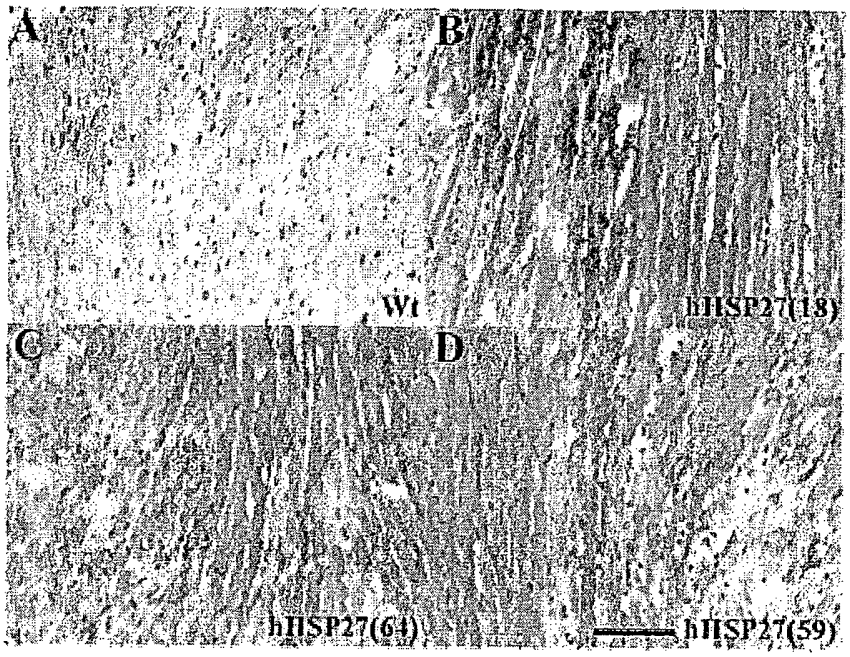


FIGURE 15

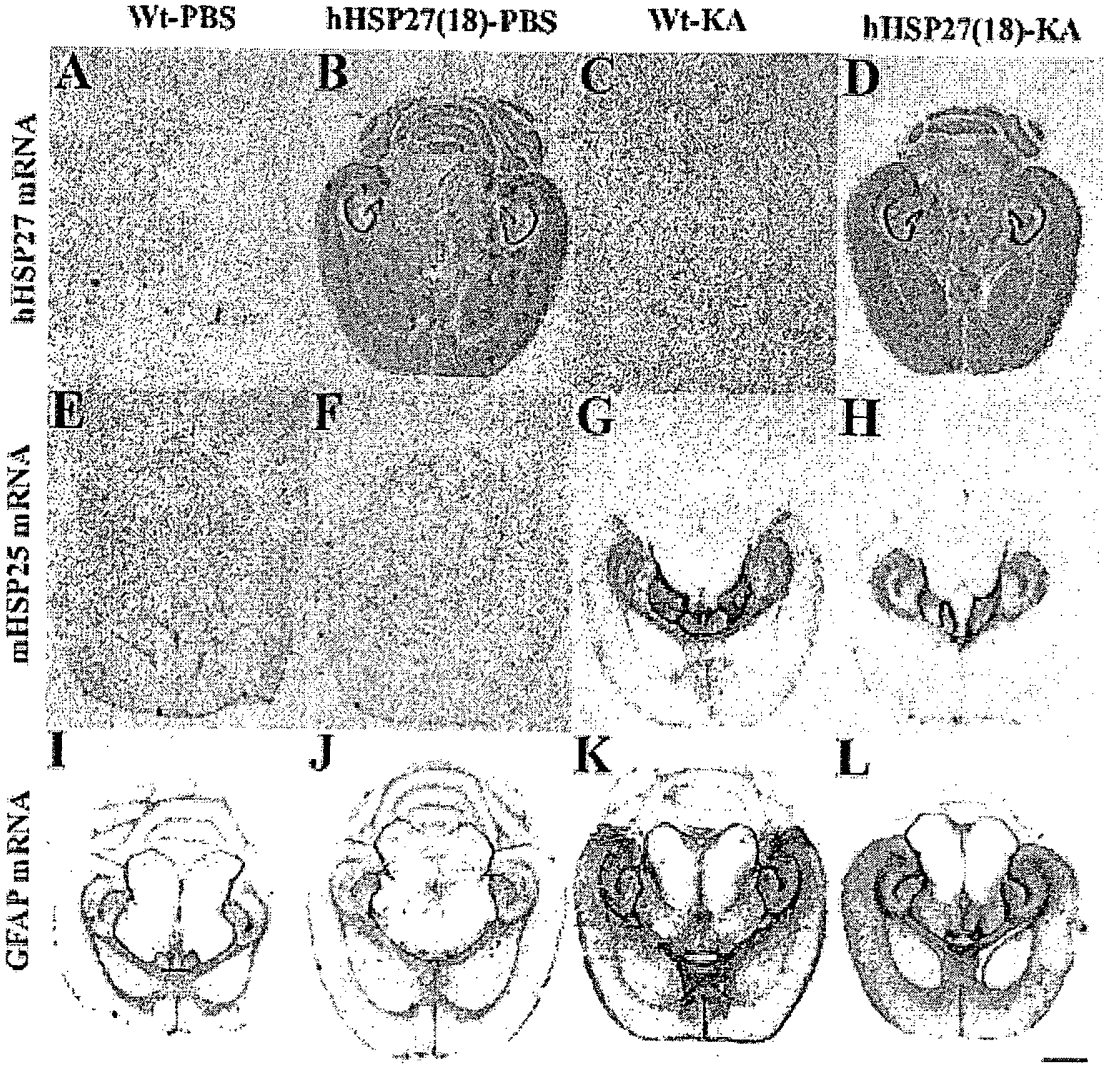

Wt-PBS
hHSP27(18)-PBS
Wt-KA
hHSP27(18)-KA
hHSP27 mRNA
mHSP25 mRNA
GFAP mRNA
A
B
C
D
E
F
G
H
I
J
K
L

FIGURE 16

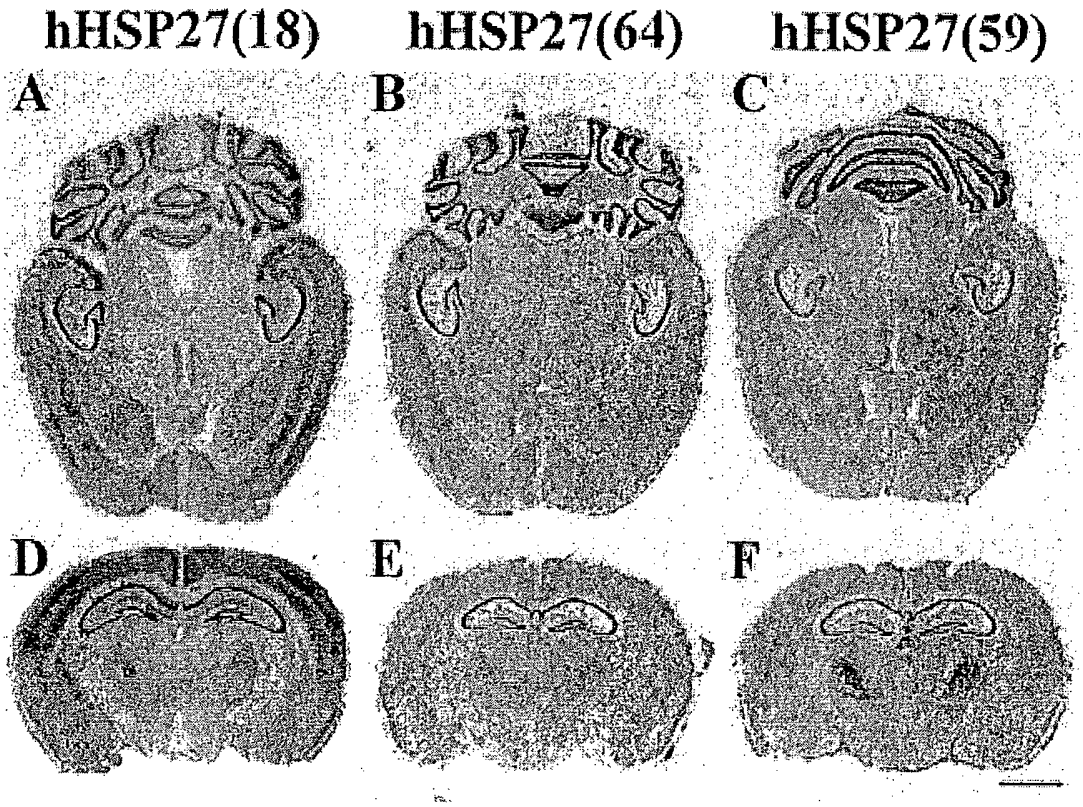


FIGURE 17

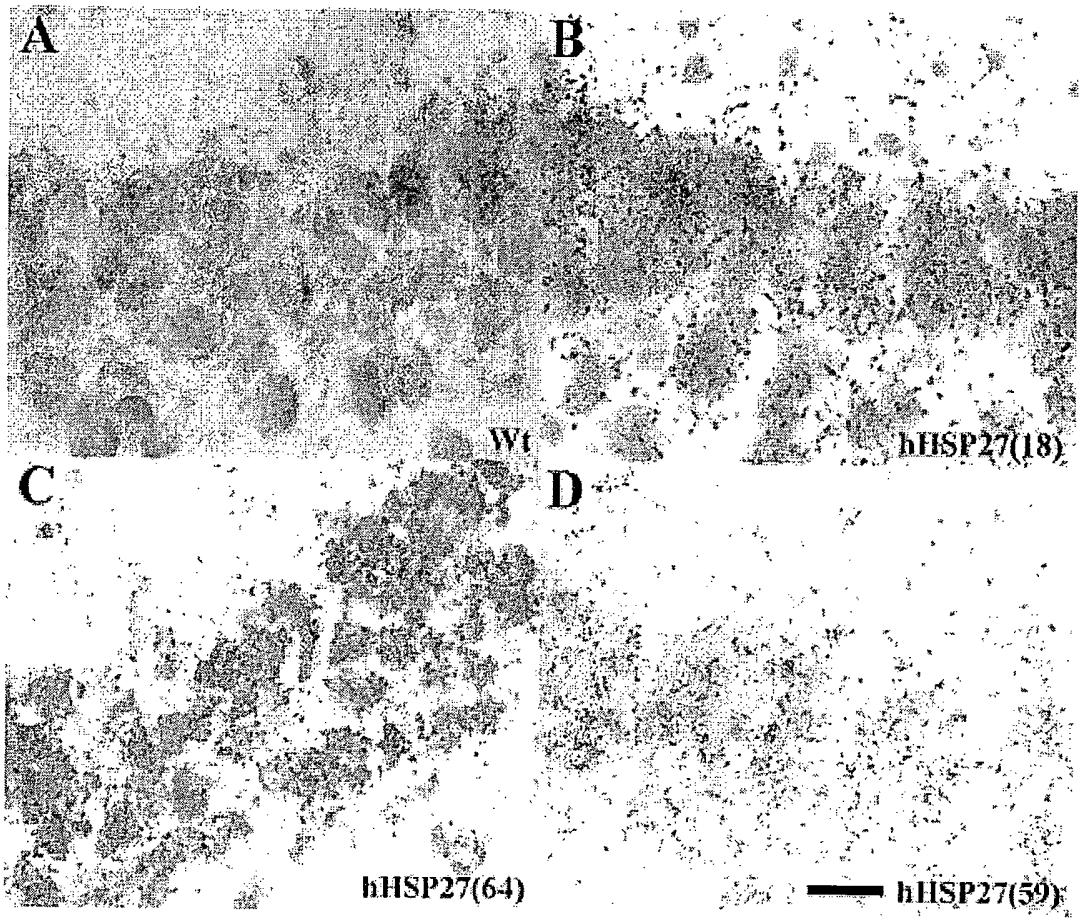


FIGURE 18

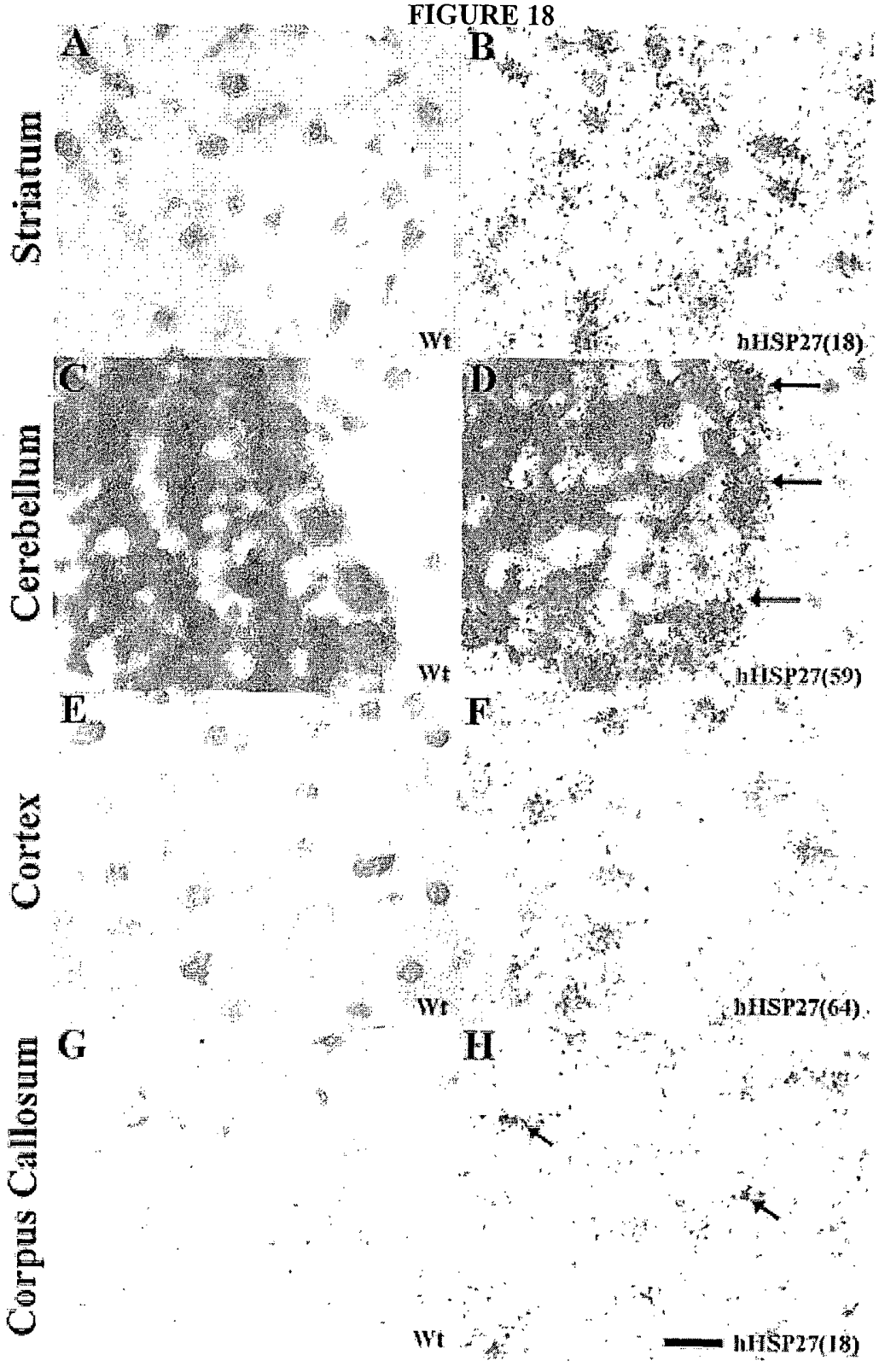

A
B
Striatum
Wt
hHSP27(18)
C
D
Cerebellum
Wt
hHSP27(59)
E
F
Cortex
Wt
hHSP27(64)
G
H
Corpus Callosum
Wt
hHSP27(18)

FIGURE 19

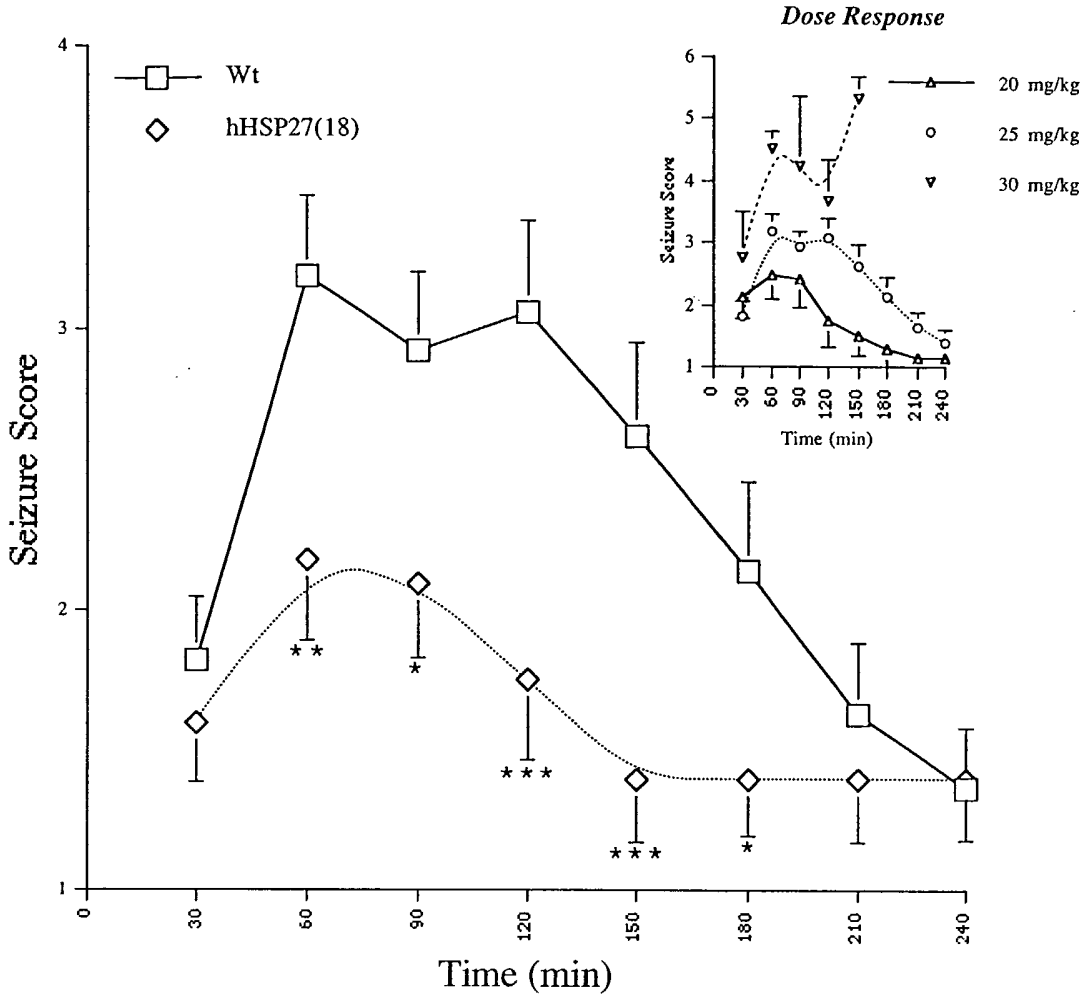

Dose Response
Wt
hHSP27(18)
20 mg/kg
25 mg/kg
30 mg/kg
Seizure Score
Time (min)
Seizure Score
Time (min)

FIGURE 20

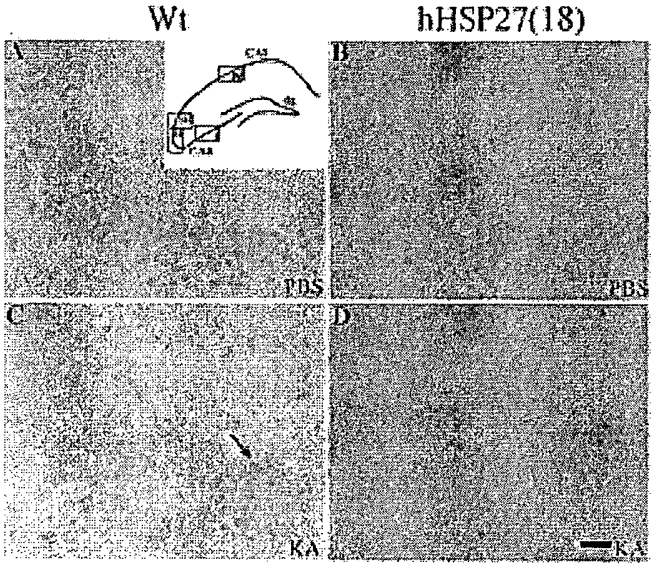


FIGURE 21

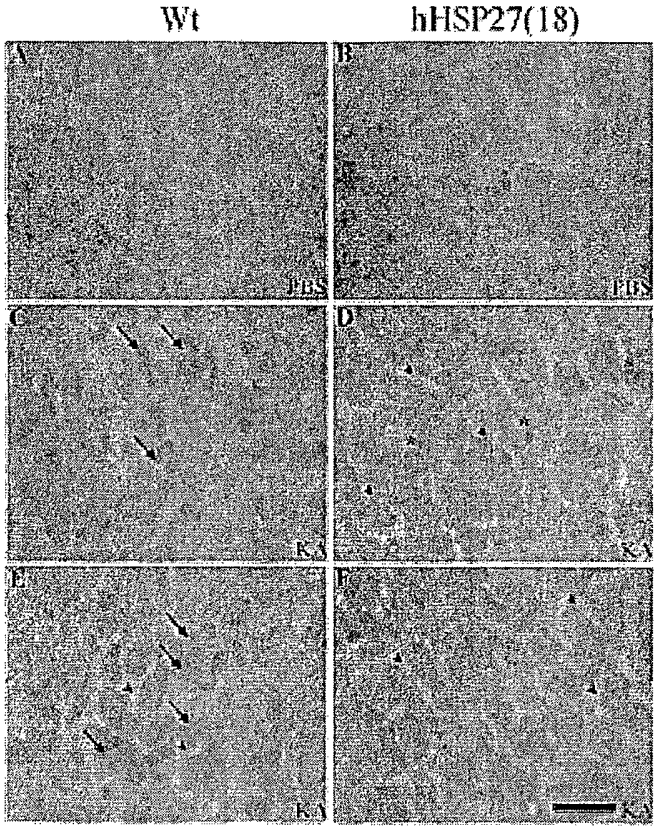


FIGURE 22

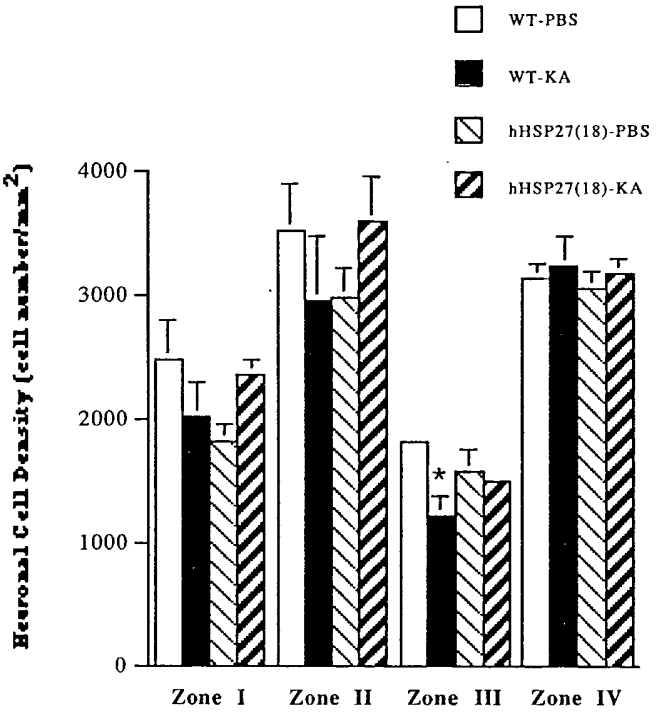
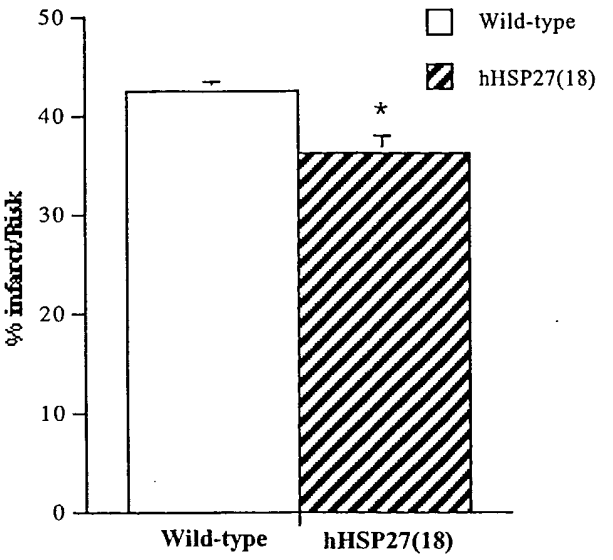

WT-PBS
WT-KA
hHSP27(18)-PBS
hHSP27(18)-KA
Neuronal Cell Density (cell number/mm2)
4000
3000
2000
1000
0
*
Zone I
Zone II
Zone III
Zone IV

FIGURE 23


Wild-type
hHSP27(18)
50
40
30
20
10
0
% infarct/Risk
*
Wild-type
hHSP27(18)