Characterisation of the Heat Shock Response in a Transgenic Mouse Model of ALS

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is an adult onset neurodegenerative disorder characterised by the selective degeneration of motor neurones in the motor cortex, brainstem and spinal cord. There is no cure and no effective treatment for this disease. Approximately 20% of familial ALS cases are due to a mutation in the gene encoding Cu/Zn superoxide dismutase (SOD1). Heat shock proteins (Hsps) are intracellular chaperones that normally aid protein handling and prevent the aggregation and misfolding of proteins. Failure of the ubiquitin proteasome system (UPS) has been implicated in this disease leading to intracellular protein aggregation.

It has previously been demonstrated that treatment of SOD1 mice with arimoclomol, a co-inducer of the heat shock response (HSR) has beneficial effects on disease progression. Much of the action of arimoclomol is not clear. In this study I examined possible alternative mechanisms of action of arimoclomol in SOD1 mice by investigating the effects of treatment on various markers of disease, including aggregation, inflammation, and ER stress. I also characterised components of the heat shock response (HSR) in disease progression of SOD1 mice and arimoclomol treated SOD1 mice. Western blot and immunohistochemical techniques were conducted. My results show that arimoclomol not only activates the heat shock response (HSR), causing increased expression of heat shock proteins (hsps) as well as important co-chaperones, but also considerably reduces aggregation, inflammation and ER stress. Arimoclomol could have therapeutic value for treatment of SOD1 mediated ALS in the future.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is an adult onset neurodegenerative disease, first described by the French neurologist Jean-Martin Charcot in 1869. ALS is characterised by the selective degeneration of motor neurones in the motor cortex, brainstem and spinal cord, resulting in progressive muscle paralysis and death, usually within 1-5 years of diagnosis. At present there is no cure for this debilitating disease and no effective treatment to alleviate symptoms. Fatality usually ensues as a result of respiratory failure or swallowing complications due to denervation of the muscles involved in these vital functions.

ALS is defined by the selective vulnerability of motor neurones to apoptosis, in particular those with larger cell bodies. Although the precise aetiology for this selective cell death is not clearly defined, motor neurones do possess specific characteristics that may increase their susceptibility to a variety of cellular insults such as excitotoxicity and oxidative stress (Shaw *et al.* 2001). For example, high energy demands make motor neurones specifically vulnerable to mitochondrial dysfunction. Since motor neurones possess some of the longest axons in the body, they are particularly susceptible to the effects of impaired axonal transport (Bruijn *et al.* 2004).

Pathological mechanisms of ALS

As with many neurodegenerative diseases, the majority of ALS cases are sporadic, of unknown aetiology. Of the 10-15% of familial cases which are known to have a genetic basis, around 20% are caused by mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1) (Rosen 1993). The SOD1 gene is localised to the long

arm of chromosome 21. The pathological characteristics of sporadic and familial disease show significant similarity (Rosen 1993), thus implying the possibility of parallel underlying mechanisms. Understanding the foundations of familial ALS may therefore also have significant implications for the understanding of sporadic disease (Bruijn *et al.* 2004). Transgenic mice modified to over-express human mutant SOD1 (mSOD1) with a G93A amino acid substitution display a phenotype and pathological features that mimic human ALS (Wood *et al.* 2003; Clement *et al.* 2003), thus presenting a good animal model for the study of the disease.

In excess of one hundred different mutations in the SOD1 gene have been identified to date. These are most commonly substitutions of single amino acids at different positions throughout the 153-residue sequence (Shaw & Valentine 2007). Mutant SOD1 mediated neurotoxicity is not well understood, however it is known to involve a toxic gain of function (Watanabe *et al.* 2001; Clement *et al.* 2003; Kabashi & Durham 2006). This hypothesis is supported by the absence of ALS pathology in SOD1 null mice and in transgenic mice modified to over-express wild type SOD1 (Bruijn *et al.* 2004). Altered functional properties of SOD1 therefore appear to be important in causing toxicity leading to motor neurone death. Bruijn et al, concluded that co-aggregation of important proteins with mSOD1 was an important factor in disease pathology.

Various cellular processes have been identified as possible contributing factors to ALS including excitotoxicity, axonal transport deficits, oxidative damage and inadequate protein handling within cells (Shaw *et al.* 2001). A typical pathological hallmark of

ALS is protein aggregation, involving both intracellular and extracellular deposition of a number of non-functional proteins (Watanabe *et al.* 2001). Numerous studies have shown that mutant SOD1 has a greater tendency to aggregate than wild type SOD1 and that initiation of disease corresponds with SOD1 aggregation in ALS mouse models (Shaw & Valentine 2007). It appears that a wide range of SOD1 mutations cause similar patterns of pathology such as aggregation of aberrant proteins. The precise cytotoxic effects of aggregated SOD1 remain elusive and the significance of aggregated proteins in ALS, and neurodeneneration more generally, is not clearly defined (Bruijn *et al.* 2004) It therefore remains unclear whether aggregates are toxic, are incidental by products of failed protein quality control or even represent a protective mechanism employed by the cell to sequester toxic species into inclusions, making them less harmful and less likely to trigger cell death. In any case, the presence of aggregates in ALS tissues of both patients and transgenic mice is indicative of proteasome dysfunction (Urushitani *et al.* 2002; Kabashi & Durham 2006).

Endoplasmic reticulum stress (ER stress) in ALS

Another pathological feature of ALS that suggests a disturbance in protein metabolism is the occurrence of ER stress. This condition is caused by the overload of ER with misfolded proteins which activate the ER's own stress response called the unfolded protein response (UPR). There are various markers of the UPR, one of which is the ER resident chaperone CHOP. CHOP is thought to be pro-apoptotic via activating the translocation of Bax to the mitochondria from the cytosol (Gotoh *et al.* 2004).

The non cell autonomous nature of ALS

In the healthy neuromuscular system, motor neurones in the spinal cord are supported by surrounding non-neuronal cells, for example astroglia. These astroglial cells release trophic factors and clear up the excess of neurotransmitters from the extracellular space (Barbeito *et al.* 2004). Astrocytes are the most abundant cell type present in the CNS. They provide structural support to neurones and have a direct influence upon the excitability of neurones via their modulation of ion and neurotransmitter concentration in the extracellular environment. The contribution of non-neuronal cells in the pathogenesis of ALS is becoming increasingly clear (Julien 2007). Astrogliosis is a process in which the characteristics and reactivity of astrocytes are changed in response to stress or injury. This is characterised by an increased expression of glial fibrillary acidic protein (GFAP), a class III intermediate filament. Neurones and reactive astroglia maintain a complex relationship with immune cells such as microglia which initiate the release of pro-inflammatory factors (Alexianu *et al.* 2001).

Evidence suggests that mutant SOD1 must be expressed in neuronal and non-neuronal cells for ALS pathology to manifest (Gong *et al.* 2000; Clement *et al.* 2003). Interaction between these cells is an important factor in causing the neurodegeneration that constitutes the disease. The manipulation of mSOD1 expression has helped to elucidate the non-cell autonomous nature of ALS. Evidence suggests that the isolated expression of mSOD1 in either motor neurones or glial cells does not instigate disease (Clement *et al.* 2003; Gong *et al.* 2000). Additionally, increasing the proportion of non mSOD1 expressing glial cells surrounding mSOD1 expressing motor neurones has a beneficial effect on disease progression (Beers *et al.* 2006). Evidence has suggested

that astrocytes expressing mSOD1 release soluble factors that have a selectively toxic impact on motor neurones (Nagai *et al.* 2007; Di Giorgio *et al.* 2007).

Inflammation in ALS

Neuroinflammation is evident in both sporadic and familial ALS including the $SOD1^{G93A}$ mouse model. Inflammation is not thought to be the underlying cause of the disease, however, evidence shows that inflammation does play a significant role in the progression of disease. Reactive microglia and astroglia have been found to accumulate in the ALS spinal cord (Consilvio *et al.* 2004). This is the fundamental cellular indication of neuroinflammation (McGeer & McGeer 2002). The activation of microglia results in the generation of reactive oxygen species (ROS) and nitric oxide (NO) which may be toxic to the cell. Microglial activation also initiates the release of pro-inflammatory cytokines (Barbeito *et al.* 2004; Lucas *et al.* 2006). It is apparent that activated glial cells are responsible for initiation of inflammatory pathways. Activation of microglia represent a primary inflammatory state. Immunohistochemical evidence of this can be observed using antibodies against any of the pro-inflammatory proteins such as nitric oxide synthase (NOS) and cyclooxygenase-2 (COX-2) (Barbeito *et al.* 2004). Inflammation causes oxidative stress, which is thought to be a contributing factor in ALS pathology. The source of reactive oxygen species is commonly related to leakage from the mitochondrial electron transport chain. However, the respiratory burst system of activated microglia is also a potent source of oxygen free radicals, thus inflammation may contribute to oxidative damage. Activated T cells present in ALS affected tissue may be a component of inflammatory pathways due to the release of factors which instigate a specific response. A strong stimulant of

microglia is the inflammatory cytokine γ-interferon, which is produced by T-cells. Activated glial cells and regions of ALS pathology have been associated with expression of phospholipase A2, which produces arachidonic acid (AA), a substrate to cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) activities (Kiaei *et al.* 2005). These are both important enzymes produced in neurones, astrocytes and endothelial cells that catalyse the conversion of AA into biological mediators including prostaglandins. It has been demonstrated that prostaglandins are signalling factors for degeneration in other neurological conditions such as Alzheimer's disease. COX activity in glial cells indicates the potential for localised inflammation in regions of ALS pathology. Elevated prostaglandin synthesis induced by increased levels of COX-2 results in glutamate excitotoxicity due to excess glutamate release by astrocytes, and the production of free radicals initiating oxidative stress (Consilvio *et al.* 2004; Minghetti 2004). It has also been reported that activated microglia produce COX-2. Although both COX-1 and COX-2 are involved in the same reaction, they are differentially synthesised. COX-1 is a constitutively expressed enzyme which plays a key role in the maintenance of normal tissue. COX-2 however, is a highly inducible enzyme which appears to play a significant role in inflammation.

Nuclear factor kappa B (NFκB) is a transcription factor which is a component of the cellular stress response linked to apoptosis, excitotoxicity induced neurodegeneration and inflammation. NFκB is expressed in regions of neurodegeneration and glial toxicity (Casciati *et al.* 2002). The protein phosphatase calcineurin (CaN) and oxidative stress regulate the activation of this ubiquitously expressed transcription factor. Evidence has suggested that NFκB is activated by mSOD1 via inactivation of

CaN, which in turn causes phosphorylation and degredation (via the UPS) of NFκB inhibitors (IκB). IκB acts as an inhibitor of NFκB suppressing its activity under normal conditions, retaining it in the cytoplasm by concealing domains which signify nuclear localisation. Casciati et al (2002) demonstrated increased activation of NFκB in a cell model of $SOD1^{G93A}$ mediated toxicity. Additionally, it is thought that NFκB is a transcription factor that mediates NO-synthase (Chen & Smith 1998) and COX-2 (Casciati *et al.* 2002). Post-mortem analyses of sporadic ALS patients and findings from fALS transgenic mouse models have justified these claims. Elevated production of spinal cord COX-2 has been shown to correspond with motor neurone loss in ALS.

Activation of inflammatory pathways may indicate that auto-toxicity may play a role in ALS possibly through the persistent stimulation of microglia. The initiation of the inflammatory response corresponds with disease onset and the severity is amplified with disease progression (Alexianu *et al.* 2001).

Intracellular role of heat shock proteins

Molecular chaperones or heat shock proteins (hsps) are ubiquitously present in all cells and are vital to the maintenance of cellular homeostasis (Muchowski 2002). They assist in protein synthesis, transport and degradation, thus promoting optimal protein handling and preventing the accumulation of aberrant proteins into aggregates (Nollen & Morimoto 2002). Heat shock cognates are constitutively expressed proteins which perform maintenance and organisational roles in cellular processes such as signal transduction and protein synthesis. Other hsps are expressed in response to elevated temperature or alternative forms of cellular stress to assist with increased protein

handling demands. There are five families of hsps categorised according to molecular weight: small hsps (16-36kDa), hsp40, hsp60, hsp70, hsp90 and hsp110. Hsp70 and hsp90 are important in maintaining proteins in a transitional state during folding to prevent aggregation. There are two main components of the hsp70 family; constitutively expressed hsp70 (Hsc73) and the highly inducible form Hsp72. These are synthesised via differential signalling pathways but possess very similar compositional and functional characteristics. Inducible Hsp70 is considered to be a fundamental component of the heat shock response. Thus, during stress, cellular survival is dependent upon the endogenous capacity of cells to synthesize hsps, especially Hsp70 (Chen and Brown, 2007).

The synthesis of hsps and the regulation of the HSR are dependent on the activation of the specific transcription factor, HSF-1. HSF1 is located in the cytoplasm, however, upon stress it gets activated by phosphorylation and trimerisation followed by translocation into to the nucleus where it binds to heat shock elements on heat shock promoter genes and initiates the transcription of hsp genes (Stuart *et al.* 1998; Muchowski & Wacker 2005). This accounts for the endogenous HSR and upregulation of molecular chaperones to allow restoration of cellular homeostasis following stress. The mechanism(s) by which Hsp70 exerts its cytoprotective effects have been the subject of scientific investigation for many years and today are still not clearly defined. It is thought that Hsp70 helps to maintain the structural integrity of proteins which may be lost in circumstances of cellular stress. Conformational change induced by stress may result in the exposure of hidden domains, leaving proteins in an unstable state and cells in a vulnerable condition. The heat shock response (HSR) can restore

structural integrity of damaged, misfolded proteins or promote ubiquitination and degradation by the proteasome, preventing accumulation of aberrant proteins (Nollen & Morimoto 2002).

Another important characteristic of hsps is that they also perform an anti-apoptotic function achieved through binding to pro-apoptotic factors and preventing the downstream activation of members of the cell death cascade. Thus, molecular chaperones provide essential support to cells in both health and disease and promote survival through interaction with signalling pathways involved both in death and survival.

Surprisingly, it has been shown that motor neurones have an inpaired ability to activate the heat shock response (HSR) in response to stress (Batulan *et al.* 2003). In ALS, motor neurones are exposed for prolonged periods to stress such as increased excitotoxicity. However, upregulation of the HSR in SOD1 mice by treatment with a novel co-inducer of the HSR called arimoclomol, has significant beneficial effects (Batulan *et al.* 2003; Kieran *et al.* 2004; Kalmar *et al.* 2005). Thus, treatment of $SOD1^{G93A}$ mice with Arimoclomol from a presymptomatic (35 days) and symptomatic (75 days) stage of disease results in a delay in disease onset, significantly improved motor neurone survival and an extension in lifespan compared to age matched saline treated animals. These effects have been attributed to the ability of arimoclomol to prolong the activation of the main hsp transcription factor called HSF1, resulting in an up-regulation in the expression of hsp70 and hsp90.

However, it is unlikely that the beneficial effects of arimoclomol in SOD1 mice is simply the result of increasing the expression of particular hsps such as hsp70 or 90. Indeed, despite the extensive evidence for the fundamental role of hsp70 in cellular protection and the demonstration of beneficial effects due to elevated expression in vitro, it would appear that up-regulation of hsp70 alone is not effective in combating stress induced conditions in vivo. For example, in an ischemia induced model of brain damage it has been demonstrated that transgenic mice over-expressing hsp70 showed no significant neuroprotective effects compared to control mice. Additionally, in SOD1 mice crossed to transgenic mice generated to express a 10-fold increase in inducible hsp70, no significant differences in the progression and severity of ALS was observed compared to normal SOD1 mice (Liu *et al.* 2005). Thus, it appears that hsp70 up-regulation alone is not sufficient to alter disease course.

Although hsp70 has been identified as a major component of cellular protection following stress, there is increasing evidence for its function as a modifier of inflammatory processes. Elevation of hsp70 levels has been demonstrated to alleviate both COX-2 expression and production of NO (Zheng et al, 2007). These anti-inflammatory activities are thought to be achieved via the direct interaction of hsp70 with the transcription factor NFkappaB. Hsp70 binds to NFkappaB and inhibits its pro-inflammatory actions. A reduced inflammatory response implies that pro-apoptotic signals will also be reduced, promoting cell survival. It is possible that the beneficial effects of arimoclomol in SOD1 mice may involve activation of such an anti-inflammatoty response.

hsp90 is an essential component of the HSR in conjunction with hsp70. Hsp90 is not a necessity for de novo protein synthesis but its importance in reducing aggregation has been indicated (Miyata & Yahara, 1992). Hsp90 binds to partially folded proteins and maintains their transitional state so that they can be effectively refolded by other molecular chaperones which have the functional capacity of folding such as Hsp70.

Assistant proteins in the HSR machinery: Co-chaperones

Co-chaperones are proteins that interact with chaperones in the maintenance of optimal protein handling and cellular homeostasis. It is likely that it is the protein-protein interactions between chaperones and co-chaperones that determines the fate of proteins, rather than the isolated actions of individual hsps alone. There are numerous co-chaperones which interact with hsps and modify their functions in different ways by binding to specific sites in order to influence the handling of their substrates.

Hsp70-Hsp90 organising protein (Hop) is a co-chaperone which forms a reversible link between the two inducible chaperones Hsp70 and Hsp90 (Chen & Smith, 1998) and is therefore important in forming and modulating a chaperone complex fundamental to the handling of aberrant proteins. Hsc70-interacting protein (hip) is present in the cytosol of eukaryotic cells in a homo-oligomeric form and functions to regulate activity of Hsc70 (Hohfeld *et al.* 1995; Irmer & Hohfeld 1997; Batulan *et al.* 2003). Hip exerts its modulatory effects on Hsc70 by binding to the ATPase domain in an Hsp40 dependent manner (Michels *et al.* 1997), through its tetratricopeptide repeats (TPR) and positively charged flanked α-helices. It has been suggested that hip is important in the early recognition and binding of substrates via the carboxyl-terminal

domain of Hsc70 (Irmer & Hohfeld, 1997). Once bound to Hsc70 hip increases its affinity for unfolded, damaged proteins, via stabilisation of the ADP bound state. Thus, hip promotes the refolding of damaged proteins. Additionally, there is evidence that it binds to unfolded proteins itself, suggesting a possible chaperone function. Hip competes with another co-chaperone for binding at the ATPase domain of Hsc70. This protein is called Bcl-e Associated Anthanogen-1 (Bag1) and it binds to B-cell lymphoma 2 (Bcl-2) promoting cell survival. Bag1 has numerous cellular functions including the regulation of growth via communication with the Raf1 signalling pathway (Stuart *et al.* 1998). Additionally, it also acts as a negative regulator of Hsc70 refolding activity through its inhibition of Hsp70 ATPase and ubiquitin lipase function by which it achieves the redirection of client proteins towards the proteasome for degradation. Another co-chaperone that has recently prevailed as a significant contributor to the degradation pathway is Carboxy terminus of Hsc70 Interacting Protein (CHIP), which also inhibits ATPase activity of Hsc70, instigating the premature release of substrates and subsequent signalling for degradation (McClellan & Frydman 2001; Choi *et al.* 2004; Dickey *et al.* 2007). The N terminal region of CHIP comprises 3 TPR units through which it interacts with other proteins. The U box domain of the C terminal of CHIP functions as an E3 ubiquitin ligase, (Petrucelli *et al.* 2004) which tags client proteins with ubiquitin as signals for degradation (Connell *et al.* 2001; Petrucelli *et al.* 2004; Dickey *et al.* 2007).

The essential role of co-chaperones in the assistance of Hsps functions is becoming increasingly evident, especially in managing an effective heat shock response. Hsps cannot perform successfully without support from co-chaperones which mediate their

function. Co-chaperones interact with hsps in different ways and thus influence the handling of their substrates. **The refolding pathway** involves recognition of damaged, misfolded proteins and requires the binding of co-chaperones including hip, Hop and Hsp40 to re-establish structure and function. Alternatively, a pathway leading to the **proteasomal degradation** of client proteins is pursued in situations when refolding is not appropriate or achievable. This degradation pathway requires interaction with other co-chaperones, including Bag1 and CHIP which inhibit ATPase activity of hsp70 resulting in the premature release and ubiquitination of client proteins and redirection towards the proteasome for degradation. The evidence discussed illustrates the complexity of the endogenous HSR employed by cells to combat stress.

The role of hsps in ALS

It has been suggested that an impaired ability to activate the heat shock response, characterised by a high threshold to activate HSF1, contributes to the specific vulnerability of motor neurones to death in ALS. Batulan *et al* (2003) investigated this possibility by examining the expression of Hsp70 and other components of the endogenous cellular defence system in primary motor neurone cultures and in spinal cords of $SOD1^{G93A}$ mice following exposure to ALS associated stress conditions. Thermal stress, glutamate excitotoxicity and $SOD1^{G93A}$ expression did not induce Hsp70 in motor neurones. Evidence was indicative of a high threshold for initiation of the heat shock response in motor neurones due to the lack of HSF-1 activation, and a consequent failure to up-regulate transcription of hsps in these cells (Batulan *et al.* 2003). In view of the complexity of hsp functions, it appears that strategies that target and amplify the HSR more effectively in motor neurones could be more successful in

alleviating disease pathogenesis. It is likely that the induction of multiple hsps will be a more successful strategy than the up-regulation of individual hsps (Batulan et al, 2006; Patel et al, 2004). Chaperones require their associated proteins to function effectively and therefore a stable balance is required for optimal protein handling.

Evidence suggests that arimoclomol augments the HSR by prolonging the activation of HSF1, allowing for amplified transcription of Hsps. Consequently, due to the complexity of the HSR the mechanisms underlying the beneficial effects of arimoclomol treatment identified in $SOD1^{G93A}$ mice (Kieran et al, 2004) may be more diverse than previously accounted for. In particular, co-chaperone expression and the mediation of inflammatory pathways by Hsp70 have not been investigated.

The current study aimed to investigate aggregation, inflammation and co-chaperone expression in progression of ALS in $SOD1^{G93A}$ mice and to establish the mechanism(s) by which arimoclomol may act on ALS pathology.

Experimental Procedures

In the experiments described in this thesis, the following procedures were performed by Dr Bernadett Kalmar: maintenance and genotyping of SOD1 mice; treatment with arimoclomol or saline; terminal anaesthesia and perfusion; removal of spinal cords, preparation of spinal cord sections and homogenates. Throughout this study I performed all immunohistochemical staining procedures, followed by microscopic analysis and image processing. I also performed all the analysis of the spinal cord homogenates including the determination of protein content, sample preparation, western blot analysis and data processing.

Genetic background and breeding of transgenic SOD1^{G93A} mice

Transgenic mice expressing the SOD1^{G93A} mutation were maintained in Biological Services Unit of the Institute of Neurology. The genotype of each mouse was established by PCR for human mutant SOD1 using tailsnips.

In this study, there were 3 experimental groups i) wild type mice ii) SOD1^{G93A} mice treated with arimoclomol and iii) SOD1^{G93A} mice treated with saline. Arimoclomol was administered interperitonially (ip) on a daily basis at 10mg/kg dissolved in saline from 35. Studies were carried out under license from the UK Home Office and following ethical review by the Institute of Neurology. Chaperone and co-chaperone expression levels were examined at various stages in these animals: at a presymptomatic age (30days) and near end stage (120 days).

Immunohistochemistry

Mice of each experimental group at 30 or 120 days of age were deeply anaesthetised (4% chloral hydrate, 1ml/100g body weight). The animals were perfused transcardially first with saline and then with a fixative containing paraformaldehyde (4% in phosphate buffered saline, PBS). The spinal cords were removed, postfixed in the same fixative for 4 hours then cryoprotected in sucrose (30% in PBS). The lumbar region of the spinal cord was then serially sectioned (10um) using a cryostat. The spinal cord cross-sections were subsequently processed for immunohistochemistry using a number of antibodies listed in Table 1.

Two different techniques were employed for immunohistochemical analysis of sections: some staining was visualised using diamino benzidine (DAB) and for others an immunofluorescence procedure was adopted using Avidin Alexa 488 and/or texas red. Sections for DAB analysis were initially washed for 5mins in PBS 0.1% Triton x-100 plus 3% H_2O_2 to block endogenous peroxides, whereas slides to be processed for immunofluorescence were washed in PBS 0.1% Triton x-100 only. All sections were incubated for 1hr in PBS containing 5% milk proteins, 0.1% Triton x-100 and 3% normal serum of the animal in which the secondary antibody was raised (blocking solution). Sections were incubated overnight at 4°c in the primary antibody. Negative control slides were also prepared in which the primary antibodies were omitted; these sections were incubated in blocking solution overnight. After washing in PBS 0.1% Triton x-100 the sections were incubated in the secondary conjugated antibodies for 2 hrs at room temperature. All sections were washed three times for 5 minutes in PBS 0.1% Triton x-100.

Table 1: *Primary antibodies used for immunohistochemistry*

Antibody	Clonality	Dilution	Source
GFAP	Mouse Monoclonal	1:500	Chemicon International
CHIP	Rabbit Polyclonal	1:400	Petrucelli, Leonard
CHOP	Mouse	1:200	Santa Cruz Biotechnology inc.
KDEL	Mouse	1:1000	Unknown
COX1	Rabbit	1:300	Unknown
COX2	Rabbit	1:400	Unknown
COX2	Rabbit Polyclonal	1:50	Unknown
HSP40	Rabbit Polyclonal	1:500, 1:1000, 1:2000	Stressgen
Ubiquitin	Rabbit Polyclonal	1:500	DAKO Cytomation
SOD1	Mouse	1:200	Stressgen Bioreagents Corp.
HDJ1	Mouse	1:200, 1:500	Mike Cheetham Lab
HSP110	Rabbit Polyclonal	1:100	Stressgen
HIP	Rabbit Polyclonal	1:1000	Stressgen Bioreagents Corp.
HOP	Mouse Monoclonal	1:100, 1:300, 1:500, 1:1000	Stressgen Bioreagents Corp.
NFκB	Rabbit Polyclonal	1:100	Santa Cruz Biotechnology inc.

Table 2: *Primary antibodies used for western blot*

Antibody	Clonality	Dilution	Source
CHIP	Rabbit Polyclonal	1:500	Petrocalli
NFκB	Rabbit Polyclonal	1:500	Santa Cruz Biotechnology inc.
HSP110	Rabbit Polyclonal	1:2000	Stressgen
Hip	Rabbit Polyclonal	1:1000	Stressgen Bioreagents Corp.
Hop	Mouse Monoclonal	1:1000	Stressgen Bioreagents Corp.
COX-2	Rabbit Polyclonal	1:1000	Unknown

Sections to be analysed using DAB were incubated for 1hr in avidin-biotin complex (Vector ABC kit) After washing slides for 5 minutes in PBS 0.1% Triton x-100, DAB solution (SIGMA, fast DAB) was applied until a sufficient level of staining was achieved. Sections were then washed three times in PBS 0.1% Triton x-100 and soaked in a Nissl stain (gallocyanin) for 25mins. Following three washes in PBS sections were dehydrated by soaking them in a series of solutions containing increasing volumes of ethanol. Each soaking step lasted for 2 minutes, beginning with 70% ethanol, then 90% ethanol, followed by two rinses in absolute ethanol. Coverslips were then mounted on the slides using Depex .

In the case of fluorescent slides, following the incubation in secondary antibody and washing, slides were incubated in Avidin Alexa 488 (dilution 1:400) for 1.5hrs, then washed and coverslipped.

Western Blot

Spinal cord tissue from a separate group of mice were processed for western blot analysis. Mice from each experimental group were terminally anaesthetised at 30 and 12 days of age, and transcardially perfused with saline only. The spinal cords were quickly removed and snap frozen in liquid nitrogen. Samples were stored at -80 degrees until further analysis. At the time of analysis samples were homogenised in homogenising buffer, centrifuged at 14000rpm for 10 minutes and the supernatant was collected.

Determination of protein concentration was achieved using a BIO-Rad assay system and a series of BSA standards. Protein levels were adjusted by diluting them with western sample buffer and then boiling them for 5-10 minutes to allow the denaturing of proteins. Polyacrylamide gel electrophoresis (PAGE) was performed using resolving gel with a 7.5% or 10% acrylamide concentration and 3% stacking gel. 5ul of the protein marker was loaded into the first well of each gel followed by equal quantities of the samples into subsequent wells. The gel was run at 160v for 1hr. Electrophoresis was used to transfer the proteins from the gel to a nitrocellulose membrane at 100V for 1hr. The membranes were then incubated for an hour at room temperature in a blocking solution consisting of PBS 0.1% tween containing 5% milk powder, whilst shaking. Blots were incubated overnight at 4°c in primary antibodies made up with the same blocking solution. The primary antibodies used in western blot procedures are listed in table 2. Following three washes of 10mins each in PBS containing 0.1% tween the membranes were incubated for 2hrs at room temp in horse radish peroxidise (HRP) conjugated secondary antibodies made up with PBS 0.1% tween containing 1% dried milk powder (dilution 1:1000) whilst shaking. Membranes were thoroughly washed 3 times for 10mins each, followed by treatment with a chemiluminescent detection reagent. Kodak film was used to visualise the blots followed by fixation.

Results

In this study, spinal cords of SOD1^{G93A} transgenic mice at 30 and 120 days of age were analysed using immunohistochemical and western blot techniques to assess protein aggregation, the presence of inflammatory markers and co-chaperone expression. In addition, spinal cord sections of arimoclomol treated SOD1 mice were also examined in order to investigate the mechanisms by which arimoclomol may influence pathogenesis in SOD1 mice. All negative control slides were assessed prior to analysis of experimental slides to ensure that the pattern of immunoreactivity observed in experimental slides was specific.

Protein aggregation in spinal cords of SOD1^{G93A} mice

Based on previous reports describing vacuolisation and aggregation, in this study I stained spinal cord sections with SOD1 and ubiquitin to label protein aggregates. Examples of SOD1 and ubiquitin stained spinal cord sections from wild type, SOD 120 day old and arimoclomol treated SOD 120 day old mice are shown in figure 1. I confirmed the presence of inclusions in motor neurones and glial cells of SOD1^{G93A} mice as indicated by the arrows. Similar patterns of immunoreactivity were observed for both proteins although the pattern differed between experimental groups. In wild type animals there was weak staining for SOD1 and ubiquitin in the cytoplasm of motor neurones localised to cell bodies and neither protein was expressed in axons or dendrites. The SOD1 and ubiquitin proteins were evenly distributed in the cytoplasm and no inclusions were observed. At 120 days of age spinal cord sections of SOD1 mice displayed a substantially greater level of immunoreactivity for the two proteins. Inclusions were evident in motor neurones and in glial cells and were strongly stained

Figure 1

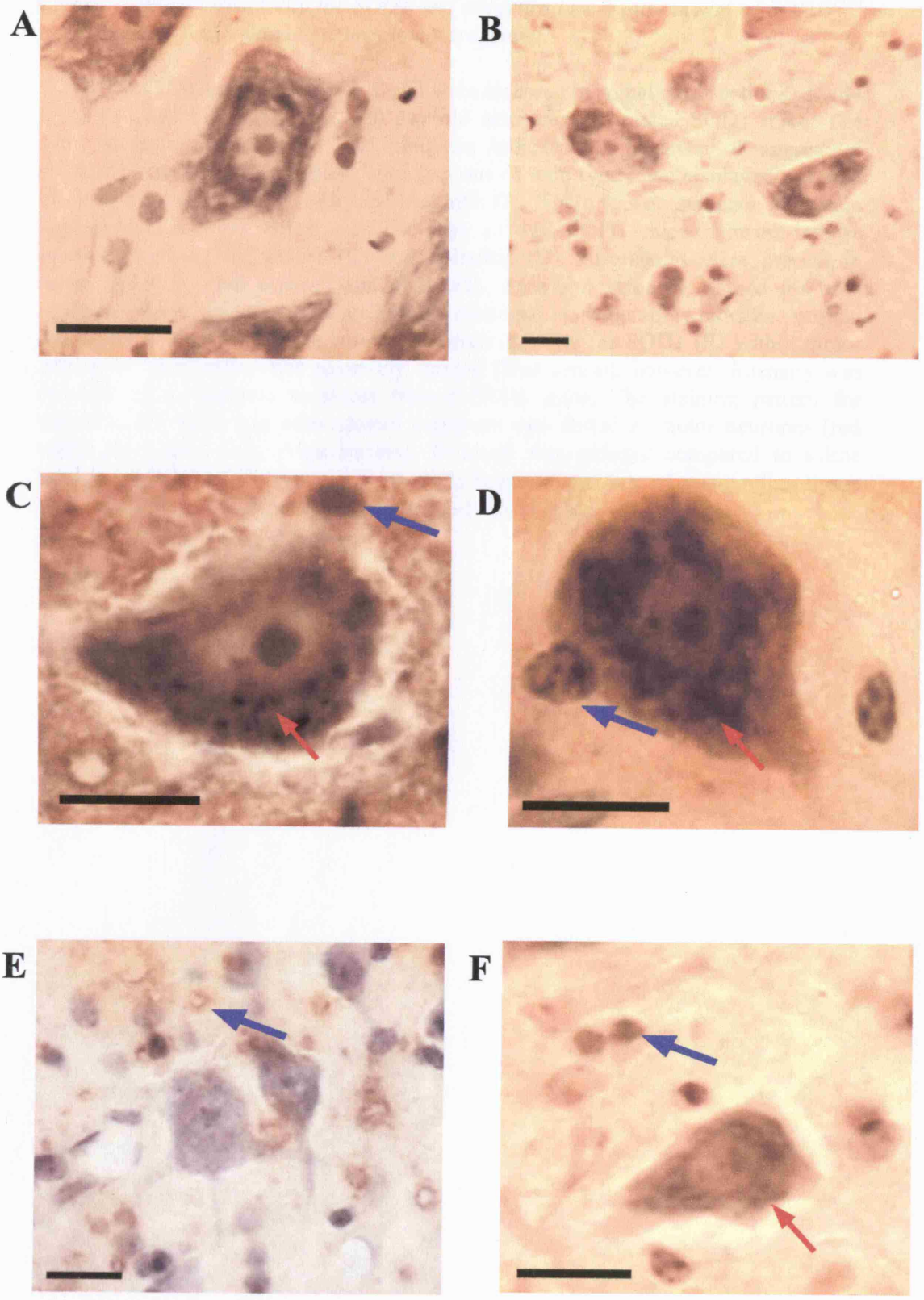


Figure 1: *Immunoreactivity for SOD1 and ubiquitin in a mouse model of SOD1^{G93A} mediated ALS and following arimoclomol treatment.*

SOD1 (A, C, E) and ubiquitin (B, D, E) were assessed in spinal cord sections of wild type, 120 day old SOD1 and 120 day old arimoclomol treated SOD1 mice. The staining patterns for SOD1 and ubiquitin indicate the presence of aggregates containing these proteins. Spinal cord sections of wild type mice displayed minimal immunoreactivity for SOD1 (A) and ubiquitin (B). There was no evidence of protein aggregation in these mice. At 120 days of age SOD1 mice showed intense immunoreactivity for SOD1 (C) and ubiquitin (D). Aggregates were present in motor neurones (red arrows) and glial cells were also strongly stained for both proteins (blue arrows). Arimoclomol treatment considerably reduced protein aggregation. Thus, there was minimal immunreactivity for SOD1 (E) within motor neurones. Glial cells were positively stained (blue arrow), however, intensity was reduced in comparison to saline treated SOD1 mice. The staining pattern for ubiquitin (F) following arimoclomol treatment was dotted in motor neurones (red arrow) and glial cells (blue arrows). Intensity was reduced compared to saline treated control mice. Vacuolisation and the immunoreactivity of surrounding tissue were also reduced compared to saline treated SOD1 mice.
Scale bar = 20um

for both SOD1 and ubiquitin (see Fig1 C and D). In contrast SOD1 mice treated with arimoclomol showed a similar pattern of immunoreactivity for SOD1 and ubiquitin, intensity was significantly reduced compared to saline treated SOD1 mice. Although some glial cells still showed immunoreactivity for both SOD1 and ubiquitin in arimoclomol treated tissue, the overall intensity of staining was reduced. The quantity and size of vacuoles evident in the spinal cord sections of arimoclomol treated SOD1 mice appeared to be smaller, with less SOD1 and ubiquitin immunoreactivity in the surrounding tissue.

Examination of inflammatory markers in spinal cords of $SOD1^{G93A}$ mice

In this study three specific inflammatory markers (GFAP, COX-2 and NFkB) were examined to investigate inflammation in progression of ALS and changes which occur in response to arimoclomol treatment in spinal cords of SOD1 transgenic mice. Astrogliosis was demonstrated using an antibody against GFAP, as can be seen in figure 2. Minimal immunoreactivity for GFAP was observed in spinal cords from wild type mice (Fig 2A). By 30 days of age, there was a slight increase in immunoreactivity in spinal cords of SOD1 mice (Fig 2B). The immunofluorescent staining demonstrated that astrocytic processes were long and thin. However, by age 120 days, intense astrogliosis was evident in the spinal cords of SOD1 mice. Astrocytes appeared to have altered morphology compared to those seen in wild type and 30 day old animals. The cell bodies were enlarged with shorter, fatter processes (Fig 2C). In contrast, as can be seen in Fig 2D, in SOD1 mice treated with arimoclomol, astrocytes maintained

Figure 2

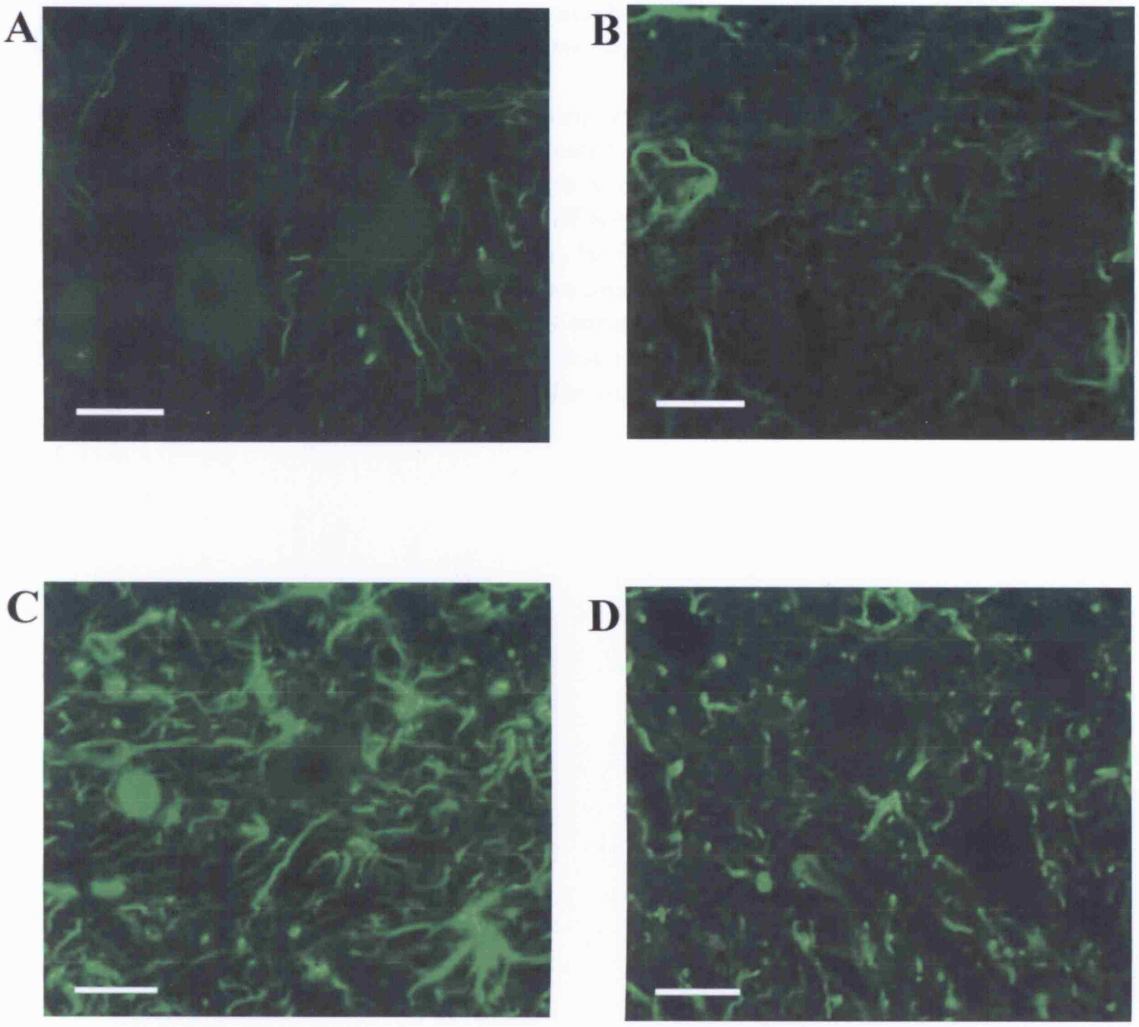


Figure 2: *Immunoreactivity for GFAP, as a marker of astrogliosis in spinal cord sections of a SOD1^{G93A} mice and following arimoclomol treatment.*

GFAP expression was assessed in spinal cord sections of wild type, 30 day old SOD1, 120 day old SOD1 and arimoclomol treated 120 day old SOD1 mice. Minimal immunoreactivity was observed in wild type mice (A). At 30 days of age SOD1 mice showed a slight increase in expression of GFAP (B) in comparison to wild type mice, processes of astroglia appeared long and thin. In SOD1 mice at 120 days of age there was intense immunoreactivity for GFAP, astroglia displayed altered morphology compared to 30 day old SOD1 mice; cell bodies were enlarged with shorter, fatter processes. In arimoclomol treated mice GFAP expression was reduced compared to SOD1 mice at 120 days of age. Astroglia had a morphology more similar to wild type and 30 day old SOD1 mice.
Scale bar = 20um

a more normal morphology and GFAP immunoreactivity was similar in intensity to that observed in wild type spinal cords.

The pattern of NFκB immunoreactivity was also established. As can be seen in Fig 3, there were significant differences in immunoreactivity for NFκB in spinal cords of wild type, SOD1 and arimoclomol treated SOD1 mice. In wild type mice, NFκB immunoreactivity in motor neurones and glia was detected at low levels (3A). Interestingly, no significant differences were detected at 30 days of age in the SOD1 mice (Fig 3B). By 120 days in SOD1 mice, positive staining for NFκB was observed in the nuclei and cytoplasm of motor neurones and in glial cells (Fig 3C). However, in SOD1 mice treated with arimoclomol, NFκB expression was dramatically reduced from that observed in saline treated SOD1 mice (Fig 3D).

In order to quantify the differences in expression levels of NFκB between the experimental groups, spinal cord homogenates were analysed by western blot. Initial attempts of visualisation did not reveal significant exposure of specific bands. Therefore, the blot was left for two days to develop, which resulted in the extensive staining of non-specific bands. This blot is not a reliable representation of NFκB activation as specificity could not be determined.

An attempt was made to analyse COX-2 expression in disease progression and in response to arimoclomol treatment. Unfortunately the antibody I used failed to recognise this enzyme in the experimental tissues.

Figure 3

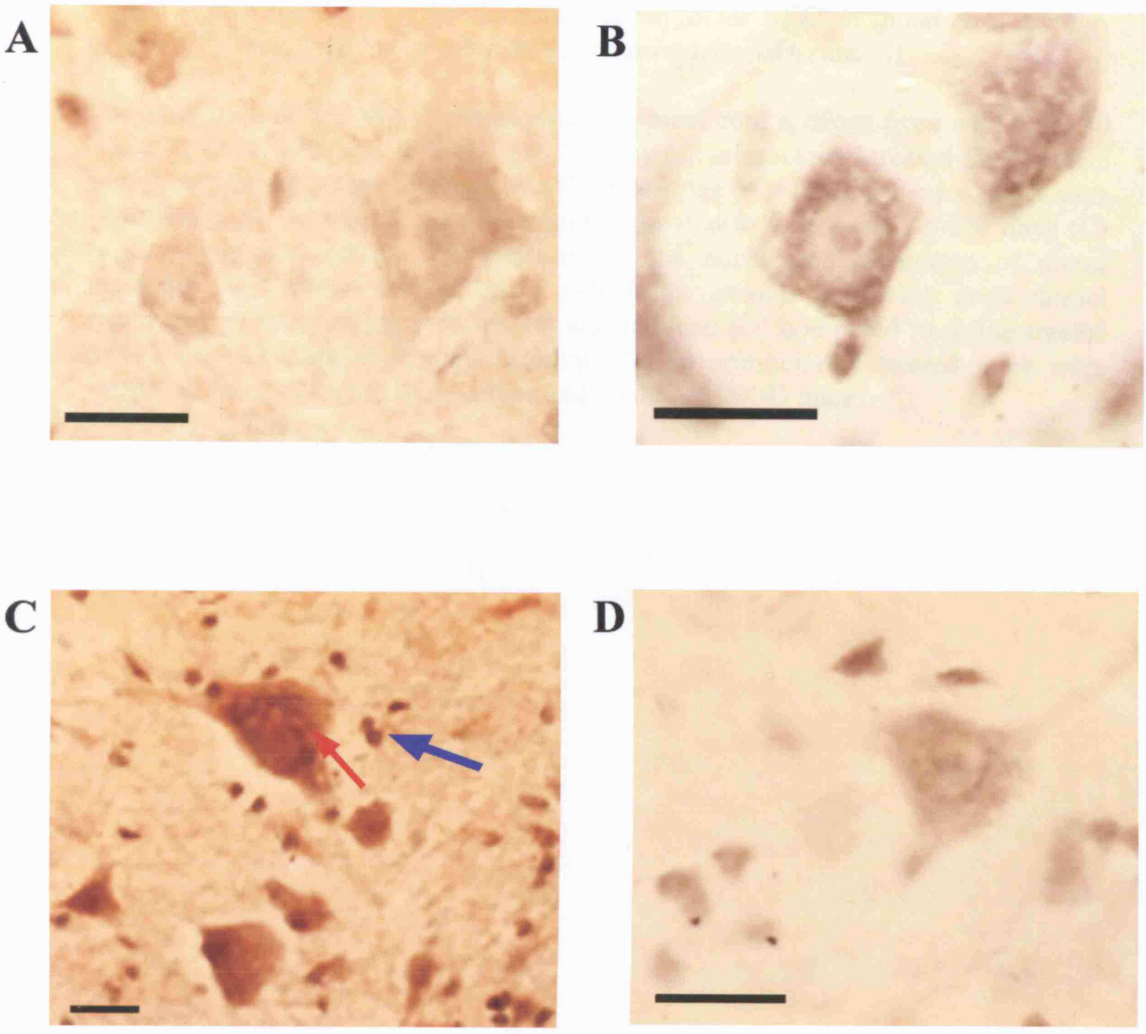


Figure 3: *Immunoreactivity for the transcription factor NFκB in spinal cord sections of SOD1^{G93A} mice and SOD1 mice following arimoclomol treatment.*

Immunoreactivity for NFκB was assessed in spinal cord sections from wild type, 30 day old SOD1, 120 day old SOD1 and 120 day old arimoclomol treated SOD1 mice. Low level staining was observed in wild type mice (A). In SOD1 mice at 30 days (B) there were no significant differences compared to wild type. At 120 days (C) intense immunoreactivity was observed in the nuclei and cytoplasm of motor neurones (red arrow) and in glial cells (blue arrow). Following arimoclomol treatment immunoreactivity for NFκB was reduced (D) compared to saline treated SOD1 mice. Levels of immunoreactivity for in arimoclomol treated mice were similar to those observed in wild type and 30 day old SOD mice.
Scale bar = 20um

Western blot analysis of COX-2 was conducted to quantify expression levels of this enzyme in the different experimental groups. Results did not comply with the well documented upregulation of COX-2 in SOD1 tissue, possibly due to problems with the antibody itself or with the spinal cord homogenates. Due to time restrictions the use of an additional antibody was not possible.

Markers of ER stress in spinal cords of SOD1^{G93A} mice

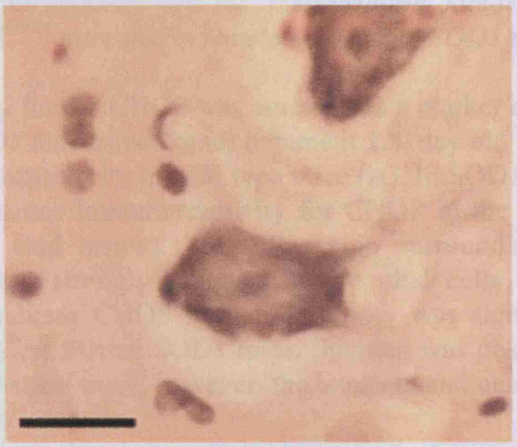
Spinal cord sections of SOD1 mice were examined for expression of CHOP as a marker of ER stress, as shown in Fig 4. Minimal immunoreactivity was observed in wild type mice (Fig 4A). However, by 120 days spinal cords of SOD1 mice showed intense immunoreactivity for CHOP in the cytoplasm and nuclei of motor neurones, and in glial cells (Fig 4B). The ER regions surrounding the nuclei were very strongly stained for CHOP. In contrast, the spinal cord sections of SOD1 mice treated with arimoclomol showed significantly reduced immunoreactivity for CHOP even at 120 days (Fig 4C).

Co-chaperone immunoreactivity in SOD1 mice

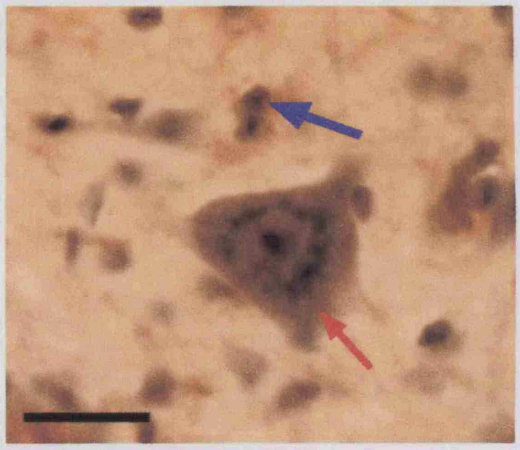
The current study examined the heat shock response and co-chaperone immunoreactivity in progression of ALS disease in SOD1 mice and tested the response to arimoclomol treatment. Spinal cord sections were processed for immunostaining of CHIP, hip, Hop, Hsp40 and HDJ1 in tissue of mice from all experimental groups. In addition, we also examined the expression of Hsp110, which has not previously been described in this ALS model.

Figure 4

A



B



C

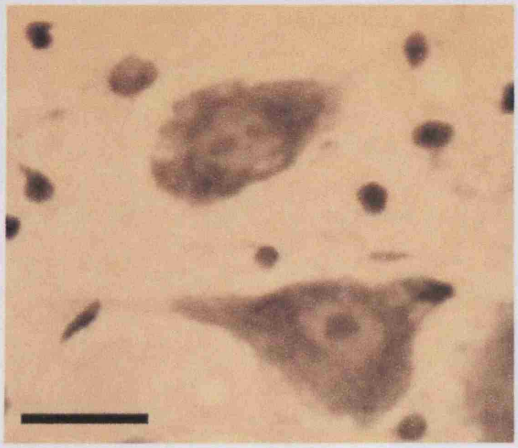


Figure 4: *Immunoreactivity for the transcription factor CHOP in spinal cord sections of $SOD1^{G93A}$ mice and arimoclomol treated SOD1 mice.*

Immunoreactivity for the CHOP was assessed as a marker of ER stress in wild type, 120 day old SOD1 and arimoclomol treatment 120 day old SOD1 mice. There was a low level immunoreactivity in wild type mice (A). In SOD1 mice at 120 days of age (B) there was intense immunoreactivity for CHOP in the nuclei and cytoplasm of motor neurones (red arrow). The ER region surrounding the nuclei in motor neurones was very strongly stained as were glial cells (blue arrow). Following arimoclomol treatment CHOP immunoreactivity was significantly reduced (C) in comparison to saline treated SOD1 mice. Staining was observed in some glial cells in arimoclomol treated mice; however, the intensity and quantity were reduced.
Scale bar = 20um

A. Chip immunoreactivity in spinal cord tissue.

Immunoreactivity for Chip was visualised using a DAB stain. Typical patterns of immunoreactivity in each experimental group can be seen in Fig 5. In wild type mice, Chip immunoreactivity in motor neurones and glial cells was very weak (Fig 5A). A similar pattern of staining was displayed in SOD1 mice at 30 days of age with slightly elevated reactivity in some glial cells, most likely microglia (Fig 5B). At 120 days of age in SOD1 mice there was a dramatic reduction in the expression of Chip, particularly in motor neurones (Fig 5C). In SOD1 mice treated with arimoclomol Chip immunoreactivity was observed in some astroglia and there was an intense up-regulation of Chip immunoreactivity within the nuclei and cytoplasm of motor neurones (Fig 5D).

Due to the apparent astroglial localisation of CHIP in spinal cords of SOD1 mice, I co-stained sections for CHIP and GFAP. Examples of the patterns of immunoreactivity in the different experimental groups are shown in Fig 6. As GFAP is a marker of astrocytosis this technique would clarify of the possible astrocytic localisation of CHIP. Astrocyte activation is not observed in wild type mice. Minimal immunoreactivity for both CHIP and GFAP were evident at 30 days in SOD1 mice. SOD1 mice at 120 days show intense immunoreactivity for CHIP (Fig 6A) and GFAP (Fig 6C) and these proteins co-localise within astroglia shown by the overlay image (Fig 6E). Increased expression levels of both proteins correspond to increased severity of disease. The redistribution of CHIP to motor neurones in arimoclomol treated animals is elucidated using this staining technique. The cytoplasm of motor neurones

Figure 5

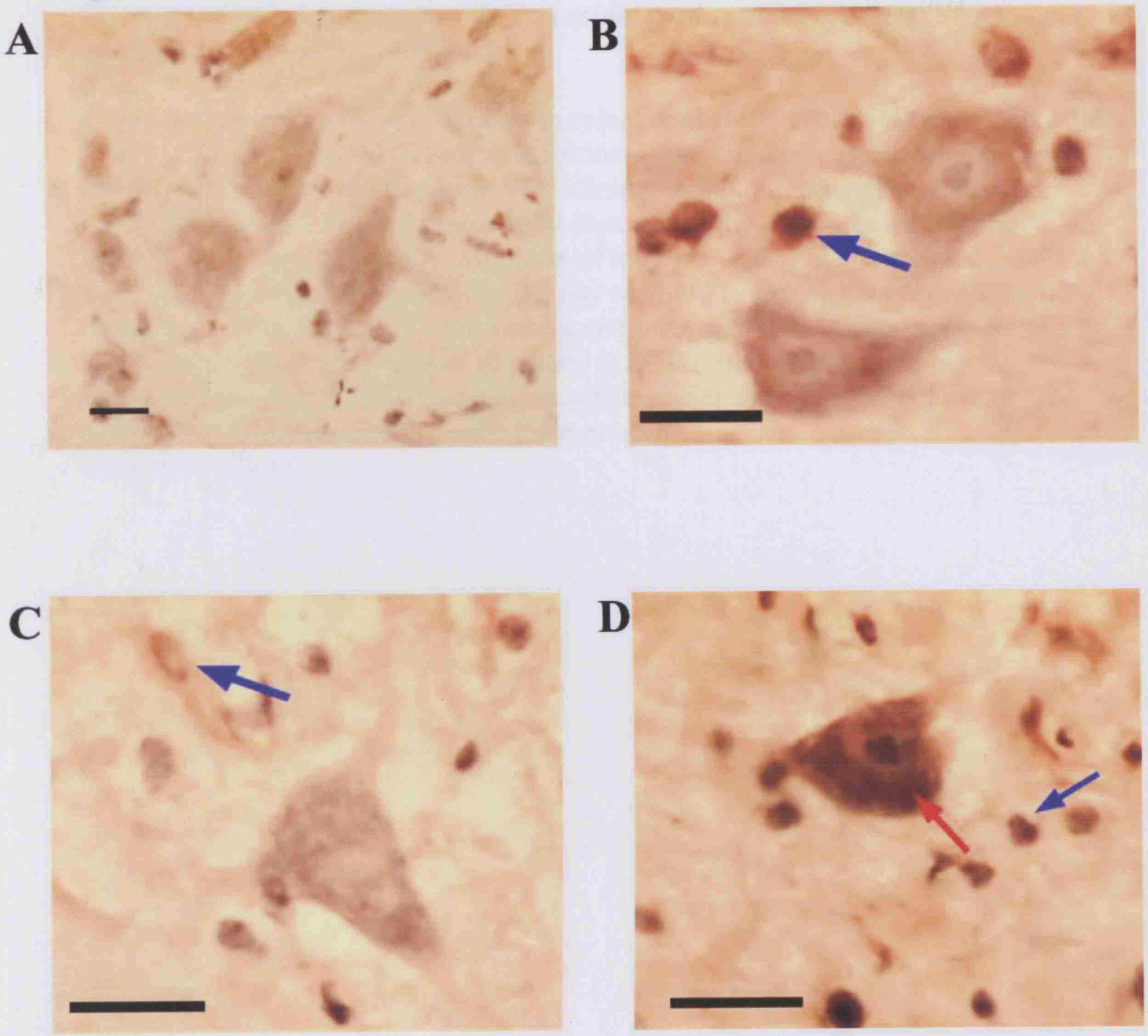


Figure 5: *Immunoreactivity for CHIP in spinal cord sections of SOD1^{G93A} mice and in arimoclomol treated SOD1 mice.*

CHIP immunoreactivity was assessed in spinal cord sections of wild type, 30 day old SOD1 and 120 day old SOD1 mice. Additionally staining patterns in arimoclomol treated SOD1 mice were also observed. Minimal immunoreactivity was shown in wild type mice (A). In SOD1 mice at 30 days of age (B) there was a moderate increase in staining within the cytoplasm of motor neurones and some glial cells were strongly stained (blue arrow). 120 day old SOD1 mice (C) displayed very little immunoreactivity for CHIP within motor neurones and weak staining in some glial cells (blue arrow). Arimoclomol treatment in SOD1 mice (D) resulted in a substantial increase in CHIP expression within nuclei and cytoplasm of motor neurones (red arrow) and glial cells (blue arrow).
Scale bar = 20um

Figure 6

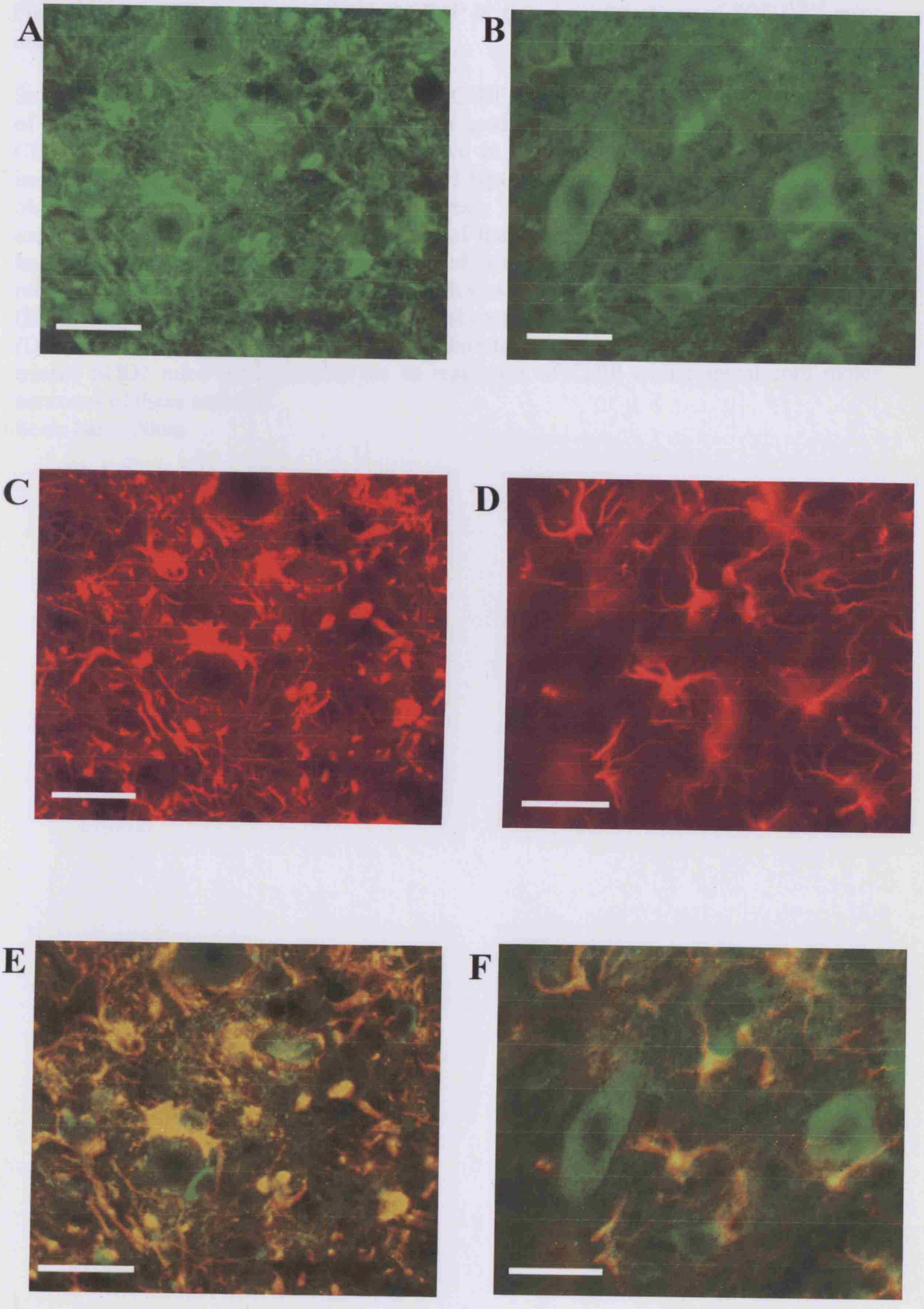


Figure 6: *A double stain for CHIP and GFAP in spinal cord sections of SOD1^{G93A} mice and in SOD1 mice treated with arimoclomol.*

Spinal cord sections were double stained for CHIP and GFAP. GFAP is a known marker of astrogliosis. Therefore, this technique was used to clarify the apparent localisation of CHIP to astroglia in SOD1 tissue. As shown in the DAB stains there was very little immunoreactivity for CHIP or GFAP in wild type mice and a slight increase in 30 day old SOD1 mice. At 120 days in SOD1 mice both CHIP (A) and GFAP (C) were expressed in glial cells. The co-localisation of these two proteins is demonstrated using an overlay image (E). In SOD1 mice treated with arimoclomol there appears to be a redistribution of CHIP to motor neurones with less intense immunoreactivity in glial cells (B). A GFAP stain of the corresponding spinal cord tissue as that shown in B is displayed (D). The overlay image of CHIP and GFAP immunoreactivity in SOD1 and arimoclomol treated SOD1 mice (F) highlights the up-regulation of CHIP within spinal cord motor neurones of these animals.
Scale bar = 20um

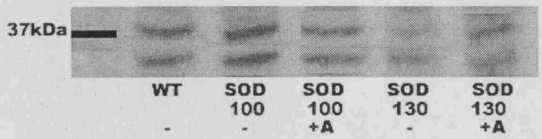
is strongly stained with CHIP (Fig 6B) and immunoreactivity is reduced in glial cells. A GFAP stain of the corresponding spinal cord tissue as shown in B is displayed (Fig 6D). The overlay image (Fig 6F) shows the redistribution of CHIP to motor neurones in arimoclomol treated animals.

To quantify these changes in CHIP expression, a western blot analysis was conducted to observe expression levels in the spinal cord homogenate of mice from each experimental group at different ages (Fig 7). These results support the immunohistological findings. However the data are non-quantifiable due to excessive non-specific labelling.

B. hip immunoreactivity in SOD1^{G93A} spinal cord tissue

Due to the documented interaction of hip with the main chaperone Hsc70 I investigated the immunoreactivity in mice from all experimental conditions using immunohistochemistry (Fig 8). Minimal immunoreactivity was observed within motor neurones and astroglia of wild type mice (Fig 8A). Hip expression appears to peak at 30 days with very strong immunoreactivity in the cytoplasm of motor neurones and in glial cells. In SOD1 mice at 120 days of age the immunoreactivity for hip was evident within the nuclei and cytoplasm of motor neurones and in glial cells (Fig 8C). The intensity is however reduced in comparison to SOD1 30 day old mice. Arimoclomol treated SOD1 mice at 120 days of age display some immunoreactivity for hip in motor neurones and glial cells (Fig 8D). However, there is no considerable differences between these animals and saline treated controls.

Figure 7: *CHIP expression in SOD1 mouse spinal cords during progression of ALS and following arimoclomol treatment.*



CHIP expression in spinal cord tissue is shown for wild type, SOD 100 day old and SOD 120 day old mice as well as SOD mice at 100 and 120 days that had been treated with arimoclomol. There does not appear to be any dramatic differences between wild type, SOD 100 day old and arimoclomol treated SOD 100 day old mice. At 120 days CHIP expression is reduced in SOD1 mice. Arimoclomol treatment results in elevated expression of CHIP in end stage SOD1 mice (120 days).

Figure 8

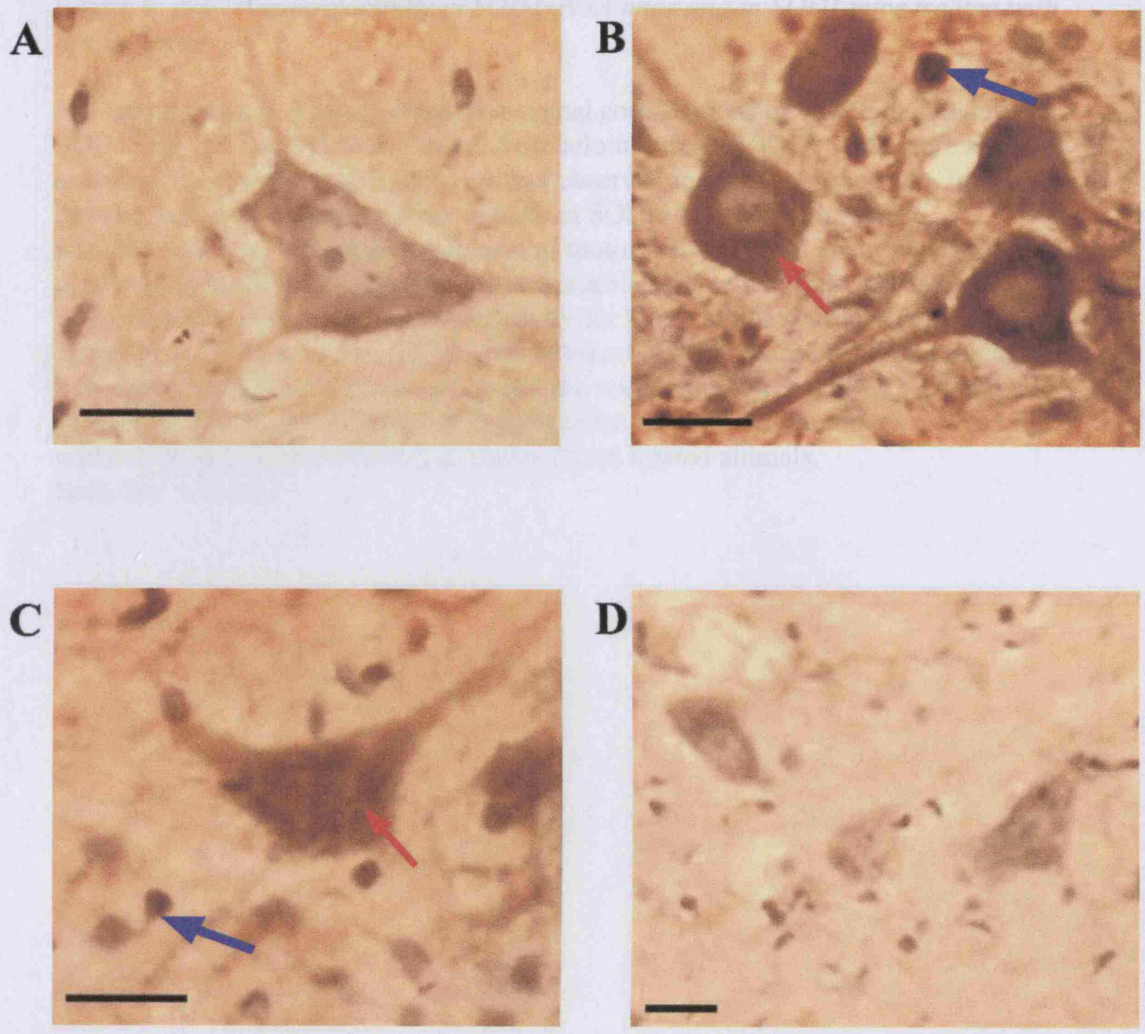


Figure 8: *Hip immunoreactivity in SOD1G93A mice and in SOD1 mice treated with arimoclomol.*

Hip immunoreactivity was assessed in spinal cord sections of wild type, 30 day old SOD1 and 120 day old SOD1 mice. Arimoclomol treated SOD1 mice were also assessed. Minimal immunoreactivity was observed in wild type mice (A). In age 30 day the expression of hip appears to peak in SOD tissue (B) with intense immunoreactivity within the cytoplasm of motor neurones (red arrow) and within glial cells (blue arrow). Neuronal processes are also labelled. In SOD1 mice at 120 days (C) there is reduced immunoreactivity for hip in motor neurones (red arrow) and glial cells (blue arrow). There is no substantial differences in staining intensity between the SOD 120 day old group and the arimoclomol treated SOD 120 day old group (D). There is low level immunoreactivity within motor neurones and some positive glial immunoreactivity in arimoclomol treated animals.
Scale bar = 20um

Western blot data cannot be quantified due to an insufficient number of repeats, however, specific bands were observed and confirmed the results obtained in immunohistochemical analysis (see Fig 9).

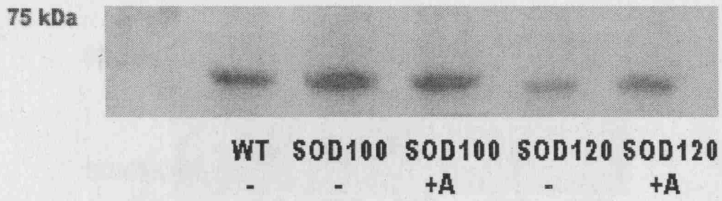
C. Hop immunoreactivity in SOD1^{G93A} spinal cord tissue

Immunohistological analysis was not possible using the antibody against Hop and therefore cellular distribution could not be determined. However, western blot data (figure 10) demonstrated that there is a marked reduction in Hop expression in SOD1 mice at 120 days of age compared to wild type and younger SOD1 mice. SOD1 mice treated with arimoclomol showed substantially less motor neurone loss and consequently higher expression levels of Hop.

D. Hsp110 immunoreactivity in SOD1^{G93A} spinal cord tissue

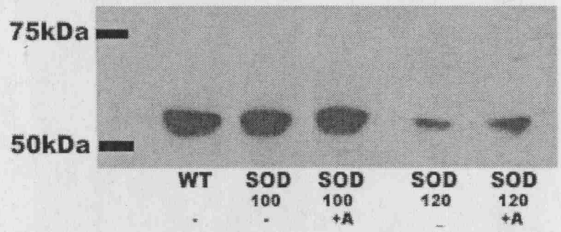
Low level immunoreactivity was observed in motor neurones and glial cells of wild type mice (figure 11A). Hsp110 did not appear to be expressed in motor neurones. At 120 days in SOD1 mice (B) there was strong immunoreactivity in some glial cells. In arimoclomol treated SOD1 mice the intensity of hsp110 staining was similar to that observed in SOD1 saline treated mice; however, the number of activated glial cells was elevated.

Figure 9: *Hip expression in SOD1 mouse spinal cords during progression of ALS and following treatment with arimoclomol.*



A) Immunohistochemical analysis in wild type, end-stage SOD1 and end-stage SOD1 mice treated with arimoclomol demonstrates that Hip expressing is induced in motor neurones by ALS and is substantially reduced in end-stage SOD1 mice following treatment with arimoclomol. B) Western blot data also confirm these findings, showing that Hip expression is amplified as disease progresses. The reduced levels at end stage in SOD1 mice can be explained by the localisation of expression to motor neurones and the substantial loss of these cells in SOD1 mice.

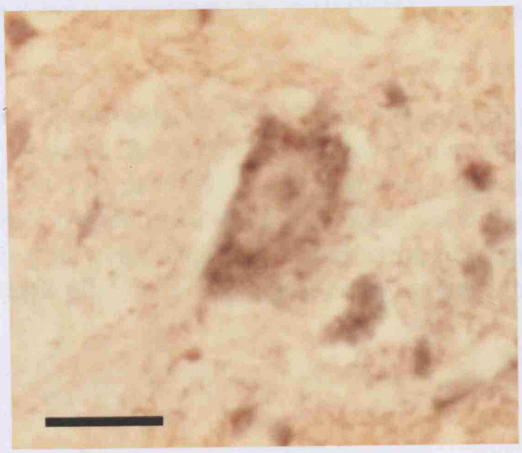
Figure 10: *Western blot analysis displaying expression levels of hop during progression of ALS and following arimoclomol treatment.*



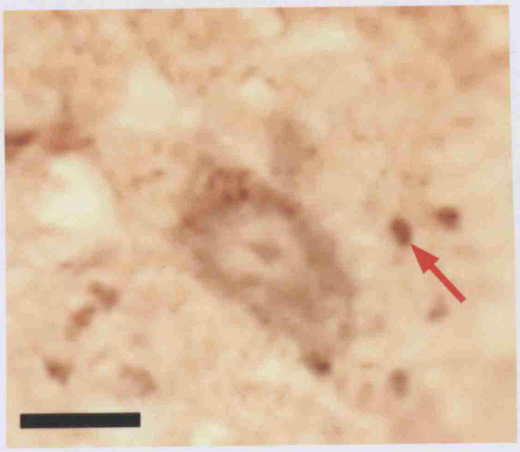
Expression levels of hop are displayed in wild type, SOD 100 and SOD 120 day old mice and in arimoclomol treated SOD 100 and SOD 120 day old mice. Qualitative analysis indicates that there are no obvious differences in expression of hop between wild type and SOD 100 day old mice or following arimoclomol treatment at 100 days. At 120 days expression of hop is substantially reduced. Following arimoclomol treatment hop expression levels are elevated in end
stage mice.

Figure 11

A



B



C

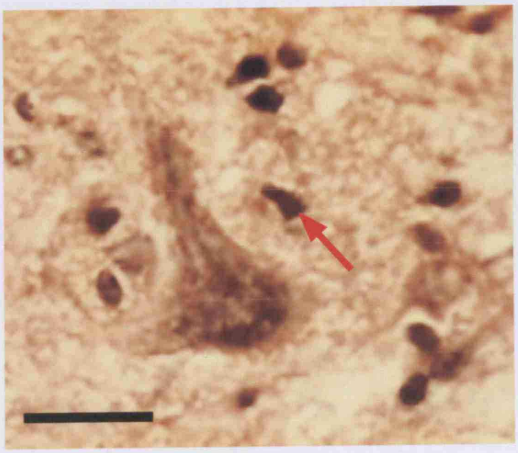


Figure 11: *Hsp110 immunoreactivity in SOD1^{G93A} mice and in arimoclomol treated SOD1 mice.*

Spinal cord sections of wild type, SOD 120 day old and arimoclomol treated SOD 120 day old mice were assessed for hsp110. Low level immunoreactivity was observed in wild type mice (A). At 120 days (B) positive staining was observed in glial cells (red arrow). In arimoclomol treated mice (C) motor neurones showed weak immunoreactivity for hsp110, however glial cells displayed a very strong staining pattern (red arrow) and the number of activated glial cells was considerably greater than in saline treated SOD1 mice.
Scale bar = 20um

The results obtained from the western blot are inconclusive due to a problem with the antibody which produced a dotted staining pattern on the blot and a high level of non-specific labelling.

E. Hsp40/HDJ1 immunoreactivity in SOD1^{G93A} spinal cord tissue

Attempts were also made to analyse expression of Hsp40 and HDJ1 (another member of the Hsp40 family), however these antibodies were not successful at recognising the appropriate proteins in our tissues.

Discussion

Mutations to the SOD1 gene account for 20% of familial ALS cases. The precise mechanisms by which mSOD1 initiates this disease are unknown. This study has shown that in a transgenic SOD1^{G93A} mouse model of ALS: aggregation, inflammation and co-chaperone expression are all induced during disease progression. Arimoclomol treatment has been previously shown to reduce the typical pathology exhibited in these mice by up-regulating expression of hsp70 and hsp90 (Kieran et al, 2004). In this study I have shown that arimoclomol also has other effects on ALS pathology. Thus, arimoclomol treatment reduced aggregation in motor neurones and markers of glial activation as well as inflammation. Moreover, treatment with arimoclomol increased the expression of co-chaperones in motor neurones and glial cells compared to vehicle treated SOD1 mice. These results therefore demonstrate that the mechanisms by which

arimoclomol acts on the pathogenesis of mSOD1 mediated ALS are more diverse than previously described.

Protein Aggregation

Previous evidence has suggested that mSOD1 has a higher propensity to aggregate than wild type SOD1, possibly via mechanisms involving alterations in: conformation, net charge or metal binding properties (Shaw and Valentine, 2007). Although the mechanisms by which mSOD1 induces ALS remain elusive, a toxic gain of function has been proposed (Bruijn et al, 2004). One mechanism by which mSOD1 may be toxic for motor neurones involves the formation of protein aggregates. In this study I observed the presence of SOD1 positive aggregates in late stage disease in SOD1 mice. Protein aggregation is a typical pathological hallmark of ALS. This study also shows that arimoclomol treatment ameliorates this pathological feature, and in arimoclomol treated SOD1 mice there were fewer protein aggregates present in motor neurones and the intensity of staining for SOD1 and ubiquitin appeared to be reduced compared to the pattern of staining observed in saline treated SOD1 mice. The mechanism by which arimoclomol achieves these beneficial effects may be via the altered solubility of mSOD1, making mSOD1 less likely to form insoluble inclusions and improving its degradability by the UPS.

Substantial evidence documents the presence of protein aggregates in spinal cord tissue of ALS patients and mSOD1 transgenic mice (Watanabe *et al.* 2001). Aggregates containing SOD1 and ubiquitin were observed not only in motor neurones but also in glial cells in this study. This observation demonstrates the non-cell

autonomous nature of this disease. Additionally, intense vacuolisation was observed in end-stage SOD1 mice and surrounding tissue was positively stained for both SOD1 and ubiquitin. This finding suggests that there may be a causal relationship between aggregation and vacuolisation. Indeed, the size and quantity of vacuoles is reduced in arimoclomol treated SOD1 mice which corresponds with the reduction in aggregation observed in these animals.

A documented feature of mSOD1 induced ALS is the sequestration of other important proteins as well as SOD1 into aggregates. This element of disease pathogenesis has also been demonstrated in the current study through positive staining of aggregates in motor neurones and glial cells for the protein ubiquitin. Ubiquitin plays a crucial role in intracellular protein degradation. Multiple ubiquitin chains are tagged to client proteins to mark them for recognition by the proteasome and thus for subsequent degradation. The sequestration of ubiquitinated proteins into aggregates observed in this study therefore indicates that aberrant proteins marked for degradation could not be cleared from the cell by the UPS. This indicates a diminished efficiency of the UPS and lays foundations for aggregate formation.

It has been suggested that inhibition of the UPS promotes increased refolding activity (Marques et al, 2006). However, in circumstances when the HSR is insufficient to meet protein handling demands then intracellular protein accumulation will occur. Previous studies have shown that components of the HSR, particularly hsp70 are also sequestered into aggregates in SOD1 mice. An investigation of hsp70 containing aggregates was not in the scope of the current study. However, future studies should

explore other components of protein aggregates in SOD1 mediated ALS. This could be achieved using a double staining technique for SOD1 and different HSR constituents.

Co-chaperone expression

In this study, the expression pattern and levels of co-chaperone that are important in protein handling was investigated at different stages of disease progression and following treatment with arimoclomol. Western blot analyses in this study have shown that expression levels of hsp110, hip, chip and hop are elevated during disease progression. Increased expression is evident at 100 days of age compared to wild type animals. However, this approach does not distinguish between functional and non-functional proteins. Some of these proteins may actually be constituents of intracellular inclusions and are therefore non–functional. The sequestration of co-chaperones into aggregates represents a means by which efficiency of the HSR may be diminished. Hsps require their assistant proteins (co-chaperones) to function optimally. Future analysis of this phenomenon may help to clarify the mechanisms by which mSOD1-mediated ALS results in disease pathology. Once activated, the accumulation of proteins seems to evolve into a detrimental cascade of events, which without intervention will result in cell death.

ER Stress

This study also demonstrates the presence of ER stress as indicated by the altered expression pattern of CHOP in spinal cord sections of mSOD1 mice. ER stress is initiated by protein overload and mitochondrial dysfunction (Boyce et al, 2006; Vlug et al, 2005). Previous studies have suggested that accumulation of misfolded proteins

within cells corresponds with downstream activation of pro-apoptotic factors. Up-regulation of CHOP, a marker of ER stress, was observed within the nuclei and cytoplasm of motor neurones with intense immunoreactivity in the ER region surrounding the nuclei in end-stage SOD1 tissue and represents early signs indicating the activation of apoptotic pathways. Strong immunoreactivity was also observed in glial cells. This indicates a role for non-neuronal cells in the initiation of apoptotic cascades leading to neuronal death. Arimoclomol treated SOD1 mice sowed a reduced expression of CHOP compared to saline treated SOD1 mice, suggesting a reduction in apoptotic activities in these animals. Motor neurone counts were not within the scope of this study but it was clear that significantly more motor neurones survived in the end-stage $SOD1^{G93A}$ mice that had been treated with arimoclomol compared to saline treated SOD1 mice. This has also been previously established (Watanabe *et al.* 2001; Kieran *et al.* 2004). Thus, the results of this study support an association between aggregation and apoptosis in this SOD1 model of ALS. Additionally, hsp70 acts in an anti-apoptotic manner through binding to Bax (a pro-apoptotic factor from the Bcl-2 family of proteins) and preventing it from translocating to the mitochondria where it initiates apoptotic cascades. Therefore, the improved motor neurone survival observed in SOD1 transgenic mice may be assisted by anti-apoptotic functions of hsp70.

Inflammation

Previous studies have revealed the non-cell autonomous nature of ALS and the importance of inflammatory processes including astrogliosis in ALS pathogenesis. This study examined the process of astroglial reactions in disease progression, using GFAP as a marker of astrogliosis. GFAP is an intermediate filament protein expressed

by astrocytes and its level of expression is elevated during stress. In SOD1 mice and ALS patients, as disease progresses, GFAP expression is increased. The increased expression of this protein is identifiable at 30 days of age in SOD1 mice, a pre-symptomatic disease stage, indicating that astroglial changes occur early on in the disease process. Arimoclomol treated SOD1 mice show considerably less astrocytosis at endstage compared to age matched saline treated animals. Inflammation is postulated to augment pathogenesis in ALS. Reduced reactivity of astrocytes in arimoclomol treated animals therefore suggests that arimoclomol has reduced activation of inflammatory pathways. This may be a mechanism via which arimoclomol promotes motor neurone survival in SOD1 mice, resulting in reduced disease pathology.

COX-2 is an enzyme produced by neurones, activated microglia and astroglia and is involved in inflammation. Substantially increased expression of COX-2 in the spinal cords of ALS patients and SOD1 transgenic mice is a recognised feature of the disease. COX-2 inhibition may therefore represent the most obvious target for reducing ALS associated inflammation to alleviate toxicity and reduce activation of downstream events that lead to apoptosis and motor neurone loss. Although I attempted to examine COX-2 expression, due to unforeseen circumstances I was unable to do so in this study and therefore could not measure the influence of arimoclomol on expression levels of this protein. Frank et al (2001) have provided evidence to support the beneficial effects of COX-2 inhibition in a $SOD1^{G93A}$ transgenic mouse model. Animals were treated with the SC236 COX-2 inhibitor and demonstrated a 20% prolongation in survival. Inhibition of COX-2 synthesis may also produce additional beneficial effects through a

reduction in excitotoxicity, which is another established contributor to ALS pathology. Mice treated with COX-2 inhibitors display reduced susceptibility to excitotoxic insults.

The transcription factor NFκB is involved in mediation of inflammatory responses. In this study, minimal immunoreactivity for NFκB was observed in wild type mice and in $SOD1^{G93A}$ mice at 30 days of age. This implies that the inflammatory pathway associated with NFκB activation is not activated in wild type mice and in presympomatic SOD1 mice, which is consistent with claims that activation of inflammatory pathways corresponds with disease onset (Alexianu *et al.* 2001), which occurs around 75 days of age in this ALS model. Both inactivation of the protein phosphatase CaN and oxidative damage are responsible for the activation of NFκB through phosphorylation and proteasomal degredation of IκB. This exposes nuclear localisation domains resulting in translocation from the cytoplasm (Lee *et al.* 2006). This study provides immunohistochemical evidence illustrating an augmented inflammatory response during progression of disease in SOD1 mice. NFκB is expressed in motor neurones and glia cells in $SOD1^{G93A}$ end-stage mice, thus indicating the existence of inflammatory processes in numerous cell types. Once activated NFκB is involved in transcription of NOS and COX-2, both of which are established contributors to pathology in ALS. Therefore, reducing the activation of NFκB in ALS may represent a mechanism via which disease progression can be delayed. Elevated manganese superoxide dismutase levels have been found in 30% of sporadic ALS cases (McEchern et al, 2000) and NFκB is responsible for the transcription of this enzyme (Mattson et al, 1997). The results of this study

demonstrate altered levels of NFκB as disease progresses in SOD1 mice. Inflammation associated with activation of this transcription factor may therefore represent a common mechanism involved in both familial and sporadic ALS (which also present remarkably similar disease pathology). The results of this study also show that arimoclomol reduces the activation of NFκB in end stage SOD1 mice which corresponds with reduced signs of pathology.

Our results therefore show that arimoclomol significantly reduces inflammation, as demonstrated by the reduced expression of both GFAP and NFκB (and possibly COX-2) in arimoclomol treated SOD1 mice compared to saline treated SOD1 mice. Inflammation does not however represent a causal factor in ALS and therefore, therapeutic intervention aimed at reducing inflammatory processes will only be successful in delaying disease progression and not in treating the underlying cause. Indeed, a number of clinical trials of anti-inflammatory drug therapies have failed to produce outcomes for its patients.

The HSR in SOD1 mice

The interaction of arimoclomol with the HSR has previously been documented. Arimoclomol activates the HSR via prolonged activation of HSF-1 allowing amplified transcription of hsps. Kieran et al (2004) identified the up-regulation of both hsp70 and hsp90 in arimoclomol treated $SOD1^{G93A}$ mice at end-stage compared to age matched saline treated controls. Although the chaperone role of hsp70 is well established it is thought that it may posses other capabilities which are also beneficial to the cell, including the direct interaction with apoptotic cascades and inflammatory pathways.

HSP70 downregulates the transcription factor NFκB which is activated in response to stress. Hsp70 binds to NFκB preventing its activation and therefore preventing the induction of inflammatory processes. In this study, the up-regulation of NFκB in SOD1 tissue has been demonstrated. A plausible explanation for this could be the sequestration of hsp70 proteins into inclusions rendering them non-functional. Arimoclomol has been shown to augment transcription of hsp70 and as it is shown here, this corresponds with reduced activation of inflammatory pathways. These factors may be directly linked as the up-regulation of hsp70 may result in increased binding of NFκB by hsp70 therefore explaining the reduced activation of this inflammatory marker.

The current study shows that arimoclomol also influences other components of the HSR: co-chaperones, the proteins that assist hsps in the handling of substrates. CHIP is a co-chaperone specifically involved in the degradation pathway. CHIP interaction with hsp70 results in the ubiquitination and subsequent degradation of client proteins. Findings from this study demonstrate the intriguing redistribution of CHIP to motor neurones in arimoclomol treated mice. The high threshold for induction of the HSR in motor neurones is well documented. These results may therefore represent an improved intrinsic capability of motor neurones to synthesise hsps, specifically CHIP. CHIP may be particularly important in protecting motor neurones from apoptosis in mSOD1 mediated ALS due to its important co-chaperone activity of assisting hsps in the degradation of aberrant proteins. The mSOD1 protein is recognised by the cell as atypical due to alterations in the amino acid sequence and it will therefore be targeted for degradation. The mSOD1 mice used in this investigation possess a high number of

mSOD1 repeats. Therefore elevated transcription of the genes encoding CHIP and hsp70 within motor neurones represents improved degradation machinery and an improved capability of the cell to deal with the high level of aberrant proteins. Enhanced protein handling will result in a reduction of aggregate formation as demonstrated in this study, as well as in reduced activation of downstream events that lead to apoptosis. This results in improved motor neurone survival as has been shown in arimoclomol treated $SOD1^{G93A}$ mice (Kieran et al, 2004).

In immunohistochemical analyses of the co-chaperone hip, expression seems to peak at 30 days of age in SOD1 mice. hip may therefore represent one of the initial proteins to be up-regulated upon activation of the HSR during stress. This co-chaperone forms a reversible link between hsp70 and hsp90 and therefore is important in forming chaperone complexes which promote refolding. Expression of hip is still evident at 120 days of age in SOD1 mice, however, immunoreactivity is reduced. It appears to be expressed in glial cells and in the cytoplasm and nuclei of motor neurones in late stage disease. The nuclei of motor neurones located in spinal cord sections however are not evident from my experimental series. Therefore positive immunoreactivity may be non-specific and infact be staining of dead motor neurones that have not yet been fully degraded. These results demonstrate that early efforts of the cell in the combat of stress are directed towards the refolding of aberrant proteins.

Hsp110 has been recently recognised as a co-chaperone which assists hsp70 chaperone activity (Dragovic *et al.* 2006). This co-chaperone is the only protein recognised to date that acts directly on protein aggregation inducing disaggregation to resolubilise

denatured proteins (Shaner & Morano 2007). In this study expression levels of hsp110 assessed by western blot are greater at 120 days in arimoclomol treated SOD1 mice compared to saline treated SOD1. Therefore, increased hsp110 expression may also explain the reduced aggregation evident in arimoclomol treated mice. However, immunohistochemical analysis showed weak immunoreactivity for hsp110 in wild type and SOD1 mice at 120 days. Arimoclomol treatment did not induce motor neurone expression of this co-chaperone. Strong immunoreactivity was evident in glial cells at 120 days of age. Following arimoclomol treatment, the intensity of glial staining for hsp110 remained unchanged, the number of reactive cells was elevated. Aggregation is dramatically reduced in SOD1 mice treated with arimoclomol compared to saline treated controls. Therefore, these findings support the proposal that glial cells provide support to motor neurones neurone.

In addition to hsp110, CHIP is also important in the intracellular degradation of proteins. A therapeutic strategy aimed at targeting these two co-chaperones may prioritise mechanisms acting directly on alleviating pathology observed in ALS.

All data presented in the current study is qualitative and so future studies should look at quantifying expression levels of different co-chaperones and markers of disease pathology in arimoclomol treated and saline treated mice. This could be achieved through repeated western blot analysis. Western blots conducted in this study, generally produced qualitative data on differences between wild type, SOD1 and arimoclomol treated SOD1 spinal cord samples. Due to time constraints and the high number of antibodies used in this study, the exact quantitative determination of

western blot line densities for each antibody was not possible. Additionally, excessive labelling of non-specific bands made the data presented in some blots questionable (hsp110, NFκB and CHIP). In addition to non-specific labelling the antibody against hsp110 also created a strange dotted pattern of staining on the blot, seemingly unrelated to electrophoretic protein bands. This even occurred when the procedure was repeated and retested using BSA as a blocking agent instead of milk powder. Thus in the future, other antibodies against COX-2, NFκB, and hsp110 should be employed if quantitative western blot analyses are to be carried out.

It could be argued that the beneficial results of arimoclomol treatment observed in this study are only present due to the treatment of SOD1 mice with arimoclomol from 35 days of age, when symptom onset does not occur until around 75 days of age. However, a novel property of arimoclomol is that it is a co-inducer of the HSR, and therefore only amplifies this response when it has already been activated by cellular stress. It is likely that arimoclomol is only effective after motor neurones and glia are stressed, so likely after 75 days. Therefore, the treatment between 35 and 75 days of age is unlikely to have any effect on outcome. All beneficial effects of this drug are likely to occur after disease onset and therefore findings of this study are relevant to the treatment of patients. Kieran et al (2004) demonstrated that there were no significant differences in the beneficial effects of arimoclomol treatment between mice treated from 35 days and 75 days of age. Future research should examine co-chaperone expression following treatment of SOD1 mice from pre-symptomatic and post-symptomatic disease stages as this could have significant implications for therapy. Additionally, the SOD1 mice used in this study suffer a very vigorous form of disease

due to the high number of mutant SOD1 proteins compared to fALS patients (10 fold greater). Therefore, the beneficial effects seen in these mice highlight the potential for treatment of patients.

Conclusion

Arimoclomol exerts beneficial effects upon several components of ALS pathology. Amplification of the HSR induces increased transcription of hsps and their assisting co-chaperones. Redistribution of assisting proteins, specifically Chip, to motor neurones improves the intrinsic capability of motor neurones to deal with stress induced conditions. Arimoclomol also reduces the activation of inflammatory pathways either by reducing the toxicity in mSOD1 mediated ALS tissue and therefore promoting better cellular state and reduced activation of pro-inflammatory factors, or by acting directly as an anti-inflammatory drug. The influence of arimoclomol on several disease processes in ALS results in reduced pathology, in particular aggregation and motor neurone loss. This beneficial effect is achieved via interactions with both neuronal and non-neuronal cells. Thus, as I confirmed in this study, enhancement of the heat shock response has widespread effects on ALS pathology in SOD-1 mice. These changes result in better survival of motor neurones, making them more stress resistant to SOD1 induced toxicity. Therefore, an hsp based therapy can be a very promising one in the treatment of ALS. Indeed, an ALS clinical trial using arimoclomol is ongoing in the United States.

Finally, protein aggregation and selective neuronal loss are pathological hallmarks of numerous neurodegenerative diseases including Parkinson's disease and Alzheimer's disease. Therefore, augmentation of the heat shock response may also be

effective in alleviating cellular stress and thus reducing activation of apoptotic pathways in other neurological conditions.

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