ROLE OF SONIC HEDGEHOG IN AMYOTROPHIC LATERAL SCLEROSIS

INVESTIGATION OF THE CYTOPROTECTIVE EFFECTS OF SONIC HEDGEHOG IN CELLULAR AND ANIMAL MODELS OF AMYOTROPHIC LATERAL SCLEROSIS

BY

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A Thesis

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a fatal progressive neurodegenerative disease with no known cause. Despite the efforts of investigators over the past 150 years, there remains no effective cure which substantially prolongs life. Therapeutic strategies have explored all of the proposed underlying pathological pathways of the disease from increased oxidative damage to impaired axonal transport, with little to no success. In the following pages, a novel perspective will be presented outlining the preliminary investigations of a new line of research demonstrating that Sonic hedgehog (Shh) protein and its agonists have cytoprotective effects on motor neurons. To begin these investigations, initial experiments were conducted *in vitro* utilizing a mouse hippocampal cell-line (HT-22) which served as a model for transient transfection and oxidative challenge assays. The results are reported in Chapter 2. Building upon these introductory findings, further investigations were conducted exploiting the $SOD1^{G93A}$ mouse model of ALS. Chapter 3 summarizes key observations pertaining to the abundance of a key cellular organelle in the sensing of Shh signalling, the primary cilium, in the spinal cord of $SOD1^{G93A}$ mice. In Chapter 4, a semi-quantitative analysis of the effects of Shh and Shh agonists pre-treatment *in vitro* on primary mixed spinal cord cultures are described. Subsequent challenge with an excitotoxic NMDA treatment was also conducted, as well as an *in vivo* survival study exploring the potential therapeutic effects of chronic Shh administration on $SOD1^{G93A}$ mice. The cumulative research presented here represents the very first investigation into the unique application of Shh and its agonists as potential therapeutic agents for the treatment of ALS, and our findings indicate that Shh has the potential of becoming a novel therapeutic agent for the treatment of ALS.

For Abagail & Grace……..

"What would you attempt to do if you knew you could not fail?"

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To my entire family for always being there through good and bad, especially my mother Catherine Peterson who has shown me what true strength, character, and perseverance are and to whom I owe everything.

"Heroism consists in ... ...Hanging on one minute longer"

Aims of the Thesis

The foundation for the research being presented here deals with the heretofore unexplored role of the developmental morphogen Sonic hedgehog in neuronal protection against cellular stress in the context of Amyotrophic Lateral Sclerosis.

Our investigations had three major objectives:

(i)   Establish proof-of-concept in a defined *in vitro* cell culture system;

(ii)   Identify and survey for the abundance of primary cilia in primary mixed spinal cord cultures of wild-type and transgenic mice;

(iii)   Elucidate if exogenous administration of Shh could prolong survival of transgenic animals and could also confer resistance to an applied excitotoxic challenge in primary mixed spinal cord culture.

Preface

The research presented herein includes observations which have been previously published/prepared for submission for publication. As such chapters 2-4 represent three original scientific manuscripts which, as of January 2012, are in print or have been submitted. The work presented in each manuscript was a collaborative effort that involved several colleagues, and thus has resulted in multiple authors.

Chapter 2:   Peterson R, Turnbull J. Sonic Hedgehog is Cytoprotective against Oxidative Challenge in a Cellular Model of Amyotrophic Lateral Sclerosis. J mol.Neurosci. Oct.7, 2011.

This work was conducted over the period of 2009-2010. I designed and conducted the experiments, analyzed and interpreted the data, and contributed to the writing of the manuscript.

Chapter 3:   Ma, X, Peterson R, Turnbull J. Adenylyl Cyclase type 3, a marker of primary cilia, is reduced in primary cell culture and in lumbar spinal cord in situ in G93A SOD1 mice. BMC Neuroscience 2011, 12:71

These investigations were conducted from 2010-2011. All *in vitro* experiments were conducted and analyzed by me along with the establishment of the primary culture system. All *in vivo* experiments were designed by Dr. John Turnbull and conducted by Dr. Xioxing Ma. Data collection for these studies was conducted by Dr. X. Ma and analysis was completed

collaboratively by Dr. Ma and Dr. Turnbull. Manuscript preparation was collaboratively done among the three researchers.

Chapter 4:   Jiang F*, Peterson R*, Turnbull J. Sonic hedgehog reduces excitotoxicity in cultured mouse motor neurons and improves survival in the G93A SOD1 mouse model of ALS. Submitted PLOS neuroscience. *(*These authors contributed equally to this work)*

This study was performed over the period of 2010-2011. I am responsible for all *in vitro* experimental design, data collection, analysis, and interpretation. I also contributed to the design of the *in vivo* experiments. *In vivo* experiments were conducted by Dr. Fan Jiang, along with data collection. Data analysis and interpretation was completed by Dr. Turnbull. Manuscript preparation was collaboratively done between me and Dr. Turnbull.

List of Papers

Chapters 2-4 represent the findings of the published manuscripts below, and have been reprinted in their entirety.

I.   Sonic Hedgehog is Cytoprotective against Oxidative Challenge in a Cellular Model of Amyotrophic Lateral Sclerosis. **Peterson R**, Turnbull J. J. mol Neurosci 2011 Oct 7.

II.   Adenylyl Cyclase type 3, a marker of primary cilia,is reduced in primary cell culture and in lumbar spinal cord in situ in G93A SOD1 mice. Ma X, **Peterson R**, Turnbull J. BMC Neurosci. 2011 Jul 18;12:71.

III. Sonic hedgehog reduces excitotoxicity in cultured mouse motor neurons and improves survival in the G93A SOD1 mouse model of ALS. Jiang F*, **Peterson R***, Turnbull J.

List of Abbreviations

A   Activator

ALMS   Alström syndrome

ALS   Amyotrophic Lateral sclerosis

ALS-PDC   ALS-Parkinsonian dementia complex

AMPA   α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ATP   Adenosine triphosphate

Bax   Bcl-2 associated X protein

BBS   Bardet-Biedl syndrome

Bcl-2   B-cell lymphoma 2 protein

BDNF   Brain derived neurotrophic factor

Bim1   Bismoloylmalimide inhibitor 1

BMAA   β-methylaminoalanine

CASP   Caspases

Cg   Chromogranin

CI   Cubitus interruptus

CK1   Casein kinase 1

CNS   Central nervous system

COX-2   Cyclooxygenase 2

CPe   Choroid plexus epithelial cells

CSF   Cerebrospinal fluid

DNA   Deoxyribonucleic acid

DS   Down Syndrome

DV   Dorsal Ventral

EAAT2	Excitatory amino acid transporter 2
ECM	Extracellular Matrix
ERK	Extracellular signal regulated kinase
EVC	Ellis von Creveld syndrome
FALS	Familial ALS
FGF	Fibroblast growth factor
FL	Full-length
FOXE1	Forkhead box protein E1 (thyroid transcription factor)
FOXF1	Forkhead box protein F1
FOXM1	Forkhead box protein M1
FP	Floor plate
FTLD	Frontotemporal Lobar degeneration
FUS/TLS	Fused in sarcoma/translated in liposarcoma oncogene
Gli	Glioma associated oncogene
GPCR	G-protein coupled receptor
GSH-PX	Glutathione peroxidase
GSK3	Glycogen Synthase Kinase 3
Hh	Hedgehog protein
HH	Huntington's gene
hnRNPs	Heterogeneous nucleoproteins
HPE	Holoprosencephaly
HSPs	Heat shock Proteins
IFT	Intraflagellar transport
IGF-2	Insulin-like growth factor-2

IL	Interleukin
IM	Intramuscular
iNOS	inducible nitric oxide synthase
IP	Intraperitoneal
ITT	Intent-to-treat
JATD	Jeune asphyxiating thoracic dystrophy
JBTS	Joubert syndrome
JNK	C-jun N-terminal kinase
LC_{50}	Concentration which causes 50% lethality
LCA	Leber congential amcurosis
LMN	Lower motor neuron
LPS	Lipopolysaccharide
LV	Lateral ventricle
MHC	Major histocompatibility complex
miRNA	micro RNA
MKS	Meckel syndrome
MMP9	Matrix metallopeptidase 9
MN	Motor neuron
MND	Motor neuron disease
mRNA	Messenger RNA
MS	Multiple Sclerosis
MT	Microtubule
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-L	Neurofilament light chain

NGF	Nerve growth factor
NMDA	N-methyl-D-aspartic Acid
N-Myc	Neuroblastoma derived myelocytomatosis related oncogene
NO	Nitric oxide
NPHP	Nephronophthisis
NPHP1	Nephrocystin
NR2F2	COUP transcription factor 2
NTD	Neural tube defects
OFP-1	Oral-facial-digital type I
PD	Parkinson's disease
PKA	Protein Kinase A
PKCε	Protein kinase C epsilon
PKD	Polycystic kidney disease
PNS	Peripheral nervous system
PP	Per protocol
Ptch 1	Patched 1
R	Repressor
RA	Retinoic acid
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
S1009a	S100 calcium binding protein 9a
S100A7	S100 calcium binding protein A7
SALS	Sporadic ALS

SETX	Senataxin
Shh	Sonic hedgehog
SMARD	Spinal muscular atrophy with prominent respiratory dysfunction
SMO	Smoothened
SNLS	Senior-Löken syndrome
SOD1	Superoxide dismutase 1 (Cu/Zn Superoxide dismutase)
SuFu	Suppressor of fused
SVZ	Subventricular zone
TDP-43	Trans-activation response DNA-binding protein weighing 43 kDa
TFs	Transcription factors
TIMP3	Metalloproteinase inhibitor 3
TNFα	Tumour necrosis factor alpha
UMN	Upper motor neuron
VAPB	VAMP-associated protein B
VEGF	Vascular endothelial growth factor

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------CHAPTER 1------

INTRODUCTION

1) Neurobiology of Amyotrophic Lateral Sclerosis (ALS)

1.1 Introduction

ALS is a fatal progressive neurodegenerative disease, and is the most common form of adult-onset motor neuron dysfunction. First described over 150 years ago by Charcot [1], it is known in North America as Lou Gehrig's disease and in Europe as Motor Neuron Disease (MND). Its hallmark is the death of specific populations of motor neurons located in the brain and spinal cord, which eventually leads to the paralysis of voluntary muscle [2].

The reported incidence of ALS in North America is approximately 1-2 per 100,000; the prevalence is slightly higher at 4-6 per 100,000 [3]. Due to the relatively low number of reported cases in the general population, ALS has status as an 'orphan' disease. The risk of developing ALS during a person's lifetime demonstrates a gender bias, with men seemingly being more susceptible (1:350) compared to women (1:400). The average age of onset for the disease is between 58-63 years (sporadic form) and 47-52 years (heritable or familial version) [4].

The clinical manifestations of the disease are not uniform and show great heterogeneity. Therefore clinical definition of ALS is based upon the site of weakness and also depends on whether the upper or lower motor neurons are involved. The main clinical presentations of motor neuron disease include: a) disease onset in limbs, involving both upper and lower motor neurons (UMN and LMN, respectively); b) bulbar disease onset which presents with speech and swallowing impairments; c) disease onset with a purely UMN involvement; and d) pure LMN with progressive muscular atrophy [5]. Patients who present with bulbar-onset disease comprise 25% of all cases, while 70% of cases are due to limb-onset ALS; the remaining 5% involve initial trunk or respiratory involvement [6].

UMN involvement leads to spasticity, weakness, and exaggerated deep tendon reflexes. By contrast, LMN involvement leads to muscular fasciculation, wasting, and weakness. Bulbar UMN degeneration results in spastic dysarthria and defects in speech. LMN bulbar dysfunction is characterized by tongue wasting, weakness, and fasciculations, and late onset dysphagia [7]. Fully 50% of all ALS patients succumb to the disease within 30 months of symptom onset, with 20% of patients surviving between 5-10 years [8]. The prognosis for survival for patients with ALS is dependent upon the demonstrated symptoms, and age at the time of disease onset. Generally younger age is associated with longer survival. In all patients early respiratory muscle dysfunction and bulbar-onset disease are associated with shorter survival periods [8].

Currently, the only drug licensed for the treatment of ALS by the FDA is Riluzole. The effectiveness of the drug was determined in a randomized trial of approximately 900 patients, who took 50 mg twice daily [9]. Results from this study showed that the overall probability of survival after 1 year of treatment with the drug was 9% greater than placebo, which functionally translates to a life-span extension of 2-3 months. Given the relative ineffective nature of available treatment options, the best care for patients with ALS focuses on a multidisciplinary integrated approach involving a team of several health care professionals including neurologist, physiotherapist, occupational therapist, psychologist, dieticians, speech language therapist, and a respirologist [8].

1.2 Pathobiology of ALS

1.2.1 Environmental Factors and ALS

ALS has been widely accepted as a complex disease with a complicated aetiology likely involving both genetic and environmental factors. So far, epidemiologic studies have been able to conclusively link increased age, male sex, and smoking as risk factors for ALS [10]. Less certain are findings that suggest that agricultural work, along with exposures to such chemicals as pesticides, lead, mercury, and those used in the textile and plastics industries, are linked to increased incidence of ALS [11]. Recently, more controversial associations between ALS and military service and playing professional soccer have also been made [12].

Rare geographical clusters of ALS have been linked on the island of Guam to the neurotoxin beta-methylaminoalanine (BMAA), which is derived from the cycad nut. This feature of the disease is part of the ALS Parkinsonism dementia complex (ALS-PDC). However, the pathology suggests that it is more closely related to a tauopathy rather than traditional ALS [13]. In addition, viral infection has also been investigated as an environmental risk factor. More recently, a number of cases of HIV infected patients have developed ALS-like syndromes [14]. Other viruses such as poliovirus (which selectively infects motor neurons) along with the discovery of both enteroviral and retroviral sequences in ALS patient tissues have contributed to the evidence for the possibility of viral involvement in ALS [15].

1.2.2 Genetic Mutations and ALS

Studies of family pedigrees where ALS, and other motor neuron disease phenotypes, have been segregating have led to the identification of a number of disease associated genes. Approximately 5-10% of all reported ALS cases have a heritable genetic component with an autosomal dominant pattern of inheritance. This subsection of cases has thus been called Familial

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ALS or FALS. By contrast, the majority of all reported cases of ALS have no clear cut heritable component and are called Sporadic ALS or SALS. Although genetically distinct, both FALS and SALS share common cellular pathological features, which suggest that there is a similar underlying disease-causing mechanism.

To date, a number of different genetic mutations have been identified in ALS including deletions, insertions, and polymorphisms. Among these genetic defects the five most prominent are mutations in the Cu/Zn superoxide dismutase (SOD1) [16], alsin [17], senataxin [18], synaptobrevin/VAMP (vesicle-associated membrane protein)-associated protein B (VAPB) [19], dynactin [20], and most recently a newly discovered hexanucleotide repeat expansion in chromosome 9 (C9ORF72) which has been linked to ALS-FTD and has been identified in 46% of FALS cases and 21.1% of SALS cases reviewed in this study [21]. Alsin (coded by the ALS2 gene) is comprised of 34 exons which encodes a protein which is 184kDa in size. The protein is found universally, and is particularly abundant in neurons where it locates to the cytosolic segment of the endosomal membrane [22]. The intracellular mechanisms in which alsin takes part, have not been fully uncovered, but it is known that one of its roles is to act as an exchange factor during endosomal trafficking [23]. A number of different mutations have been discovered in ALS2, the most recent being a homozygous missense mutation [24]. The majority of the mutations found within ALS2 have been predicted to produce a truncated form of the protein [17]. Protein truncation was found to be indicative of the resultant disease phenotype, with less truncated forms being correlative with milder disease presentations [17]. All mutations within ALS2 render the protein unstable, which would indicate that this form of ALS is due to a loss of native functionality [25].

Defects in the Senataxin (SETX) gene result in a juvenile form of motor neuron disease which is characterized by muscle weakness, and atrophy [26]. Patients struck with this form all display missense mutations in SETX, which codes for a 330kDa DNA/RNA helicase. Mutations in SETX have implicated changes in DNA/RNA processing as a possible pathogenic mechanism, and these have also been associated in two other heritable diseases; namely, spinal muscular atrophy and severe infantile distal spinal muscular atrophy with prominent respiratory dysfunction (SMARD).

Mutations in the VAMP-associated protein B gene cause adult onset, autosomal dominant ALS and also an atypical ALS variant. The latter is slow to progress and involves tremors, but does not involve frontotemporal dementia [27]. VAPB has six exons and codes for a ubiquitously expressed 27.2 kDa protein, which is involved in vesicle transport within the cell. Another gene which has recently been associated with the atypical form of ALS is Dynactin. Mutations within this gene have been found to affect the p150 subunit, an integral component of the dynein complex that is responsible for the majority of axonal retrograde transport [20].

1.2.2.1 Superoxide dismutase 1 (SOD1)

Most of the knowledge on ALS pathogenesis has been derived from studies based on animal and cellular models centering on mutant SOD1 protein expression. In its native wild type form, SOD1 is a ubiquitous cytosolic protein consisting of 153 amino-acids. It functions as a homodimer with each subunit binding to a single zinc and copper atom [28]. SOD1 converts the superoxide anion, which is a by-product of normal oxidative phosphorylation by the mitochondrion, to hydrogen peroxide via cyclical reduction/oxidation mechanism termed dismutation. Mutations in SOD1 (located on the long arm of chromosome 21) account for

approximately 20-25% of all reported FALS cases [16], with over 150 mutations having been identified along the entire length of the gene [29]. These mutations span all 5 coding exons and include 114 disease-related mutations, 6 silent mutations and 5 intronic variants.

Of the reported mutations, the vast majority are classified as missense, but 12 mutations are non-sense or deletions which produce a truncated form of SOD1. It has been found that most mutations do indeed reduce the dismutation action of SOD1. However there does exist a small minority of mutations that do not, and in those cases SOD1 retains its full enzymatic capacity [30]. There is still debate on whether there is a correlation between enzyme activity, clinical progression and disease characteristics [31].

The most widely accepted view of how mutations in SOD1 cause disease is related to the fact that the mutant protein has a predilection for aggregation [32]. Mutations cause conformational change in the resultant protein, which increase instability leading to defects in proper folding and assembly of abnormal SOD1 dimers [33]. Aggregation of mutant SOD1 within the cytosol, and more specifically the endoplasmic reticulum (ER), of neurons represents a toxic gain-of-function of the protein. ER bound aggregates of mutant SOD1 have been found to inhibit proteosomal degradation [34].

A consequence of impaired proteosome function is the aberrant association of mutant SOD1 protein with other binding partners, such as mitochondrial membranes and other essential proteins, leading to disruption of normal cellular processing [35]. Although the pathogenic role of these mutant protein aggregates still remains to be fully understood, it is generally accepted that the beginning stages of oligomerisation constitutes a toxic event, while the aggregates themselves may be a latent by-product which are by and large inert [36].

1.2.3 Animal Models of ALS

1.2.3.1 SOD1 knockout mice

The discovery of mutations within SOD1, in a subset of reported ALS cases, led to the development of various genetically engineered mice models. The initial approach was based on two observations: 1) the global distribution of SOD1 protein; and 2) early observations that patients heterozygous for SOD1 had erythrocytes with diminished reductase capacity [37]. This led researchers to hypothesize that the pathogenesis of ALS was mainly due to a loss-of-function of the native wild-type enzyme. Therefore SOD1 knockout mice were created. These knockout mice developed normally and did not display any motor abnormalities or disease characteristics resembling ALS [38]. Fully developed adult mice did however display a hypersensitivity to axotomy [38], ischemia [39, 40], and paraquat-induced toxicity [41]. Characterization of the knockout model proved that disruption of SOD1 was in itself insufficient to cause spontaneous motor neuron degeneration. However the SOD1 knockout mouse model may be relevant to age-related peripheral axonopathy, involving denervation of muscle [42], muscle atrophy [43], and sarcopenia resulting in major locomotor deficiencies [44]. More recently, research has gone on to demonstrate a 50% drop in innervation of hindlimb muscle in 4-month old SOD1 knockout mice, which proceeds to complete denervation by 18-months [45]. These age-related neurodegenerative defects in the SOD1 knockout model suggest that complete loss of SOD1 functionality can contribute to the pathogenic development of ALS in mutant mice.

1.2.3.2 SOD1 Wild-type Transgenic Mice

Prior to mutation in SOD1 being linked to FALS, the gene was implicated in Down syndrome (DS). This disease is usually linked to the duplication of a number of genes on chromosome 21 where SOD1 is located. Transgenic mice overexpressing wild-type SOD1 (SOD1^{wt}) were first

reported in 1987 [46]. Although phenotypically normal initially, later in development they exhibited clinical features of DS [47]. Age-related neural degeneration in the spinal cord of SOD1[wt] mice was observed. These aged mice also displayed axonal loss and motor neuron degeneration [48], along with a 40% loss in anterior horn cells in heterozygous mice at 600 days of age [49] and impairment of nerve regeneration after peripheral injury [50]. No lines of SOD1[wt] mice however have died due to ALS-like symptoms, although animals do appear to undergo a sub-clinical loss of motor neurons [51]. Taken together, findings from studies of this transgenic model do indicate out that overexpression of SOD1 can cause some motor neurodegeneration.

Studies using the aforementioned knockout technology and SOD1[wt] models hinted at a biphasic model of SOD1 expression in relation to neurodegeneration, whereby SOD1 levels at both low and high extremes are potentially pro-oxidative and toxic [52]. Therefore intermediate SOD1 expression levels are essential for the proper and long-term preservation of motor neurons and their functioning.

1.2.3.3 SOD1 Transgenic Mice

Once mutation linkage to FALS had been established, a giant leap in ALS research was taken with the creation of transgenic mice bearing the various reported human mutations in SOD1. These various transgenic mouse lines constitutively express mutant SOD1, typically involving 12-15kb human genomic fragments encoding SOD1 driven by promoters and other endogenous regulatory elements [53]. To date, there are 12 different human SOD1 mutations expressed in mice, with 9 missense and 3 C-terminally truncated variants (reviewed in [47]). Although each separate transgenic line has its own unique biochemical features, the one common characteristic

among them all is fatality due to progressive paralysis, as in human disease. The demonstrated disease phenotype of SOD1 mutant transgenic mice points to a dominant gain-of-function pathway of pathogenesis for ALS [53].

The most popular transgenic animal model in use today is the SOD1^{G93A} transgenic mouse line, followed by SOD1^{G37R}, SOD1^{G85R}, and SOD1^{G86R}. These four models can be categorized as abundant and stable (SOD1^{G93A}, SOD1^{G37R}), or marginally expressed and unstable with little to no activity in case of CNS (SOD1^{G85R}). SOD1^{G93A} mice develop hindlimb weakness and tremor at approximately 3 months of age which progresses to hyper-reflexia, paralysis and eventual death at 4 months of age [53]. Pathological features of the SOD1^{G93A} mouse include: 1) degeneration of the neuromuscular junction at 47 days of age; and 2), proximal axonal loss by 80 days of age, accompanied by motor impairment and a 50% loss in lower motor neurons by 100 days of age [54]. Based on these features, ALS can be loosely described as a distal axonopathy of the lumbar motor neurons [54]. The key pathological features of diseased spinal motor neurons include mitochondrial vacuolisation, Golgi apparatus fragmentation, neurofilament-positve inclusion, and cytoplasmic SOD1 aggregates (reviewed in [47]). In addition, extensive microgliosis and astrocytosis is evident at disease onset [55], along with degeneration of cranial nerves such as the trigeminal, facial and hypoglossal [56].

Initially several SOD1^{G93A} mouse lines were created and the first published line was labelled G1 had 18 predicted transgene copies. This initial line was further divided into 2 subcategories, a high-copy variant termed G1H with 25 transgene copies and a low copy variety with only 8 transgene copies called G1L [57]. The G1H line was imported into the Jackson Laboratory as the strain B6SJL-TgN(SOD1-G93A)1Gur [58]; the low copy variant is designated B6SJL-

TgN(SOD1-G93A)1Gur[dl].The clinical phenotype and disease progression of G1H and G1L are dependent on transgene copy number, with the high-copy variant developing early-onset symptoms associated with rapidly progressing disease and the G1L inducing a delayed disease onset and longer duration [47].

Transgenic overexpression of the various human SOD1 mutations in mouse models, at 0.2 to 24 fold protein and 0 to 14.5-fold enzyme activity levels, induces fatal motor neuron degeneration [59]. This indicates that the disease-causing pathway involves a toxic gain-of-function of mutant SOD1 protein which may be related to the level of protein over-expression but not to its native functioning as an antioxidant.

1.3 Proposed pathogenic mechanisms in ALS

1.3.1 Oxidative Stress

One of the initial areas of intense research was in the field of oxidative damage to motor neurons. Cumulative oxidative stress occurs over the normal life-span progression, and elevated oxidative damage in motor neurons of ALS patients is a key feature of the disease. Indeed several studies using post-mortem samples from ALS patients [60] have confirmed elevated biomarkers of increased oxidative metabolism.

Mutations in SOD1 may alter the native enzymatic activity through either aberrant copper catalysis or improper metal binding. Speculation on defective enzymatic SOD1 functioning have been linked to alterations in the configuration of the active pocket that allows atypical substrates to interact with copper [61]. These atypical substrates may include peroxynitrite (which is a spontaneous product of superoxide and nitric oxide combining), or hydrogen peroxide (which is the native product released from the first step of the dismutase reaction catalyzed by SOD1).

These substrates may bind to mutant SOD1 and catalyze the nitration of tyrosine residues [62]. Alternatively, mutant SOD1 may lead to the improper binding of zinc which allows the rapid reduction of bound copper. When SOD1-bound copper is in its reduced state, it catalyzes the formation of the superoxide anion. This reaction is called 'backward catalysis' [63]. Reduced metal binding ability of mutant SOD1 may result also in an increase in free copper and zinc which can lead to neurotoxicity.

Currently there is still ongoing debate on whether mutant SOD1-induced oxidative stress represents a pathogenic mechanism in ALS. The argument for oxidative stress damage is mainly derived from studies in the SOD1^{G93A} mouse model and from SALS patient samples [64]. On the other hand, studies using the SOD1^{G37R} mouse model yielded contradictory findings, with results showing no increase in oxidative biomarkers in spinal cord motor neurons [65]. Additional conflicting evidence was provided from a study where SOD1^{G85R} was co-expressed with SOD1^{wt}. Here, there was no impact on age of onset, rate of progression, or survival [51]. Paradoxically, overexpression of SOD1^{wt} in various other transgenic models (i.e., SOD1^{G93A}, SOD1^{L126Z}, and SOD1^{A4V}) resulted in accelerated onset of disease, which was attributed to increased formation of mitochondrial aggregates consisting of both mutant and wild type SOD1 [66]. Collectively, these findings would not strongly suggest a role for increased oxygen-mediated toxicity in the pathogenesis of motor neuron disease [67].

1.3.2 Aberrant Protein Aggregation

Mutation in SOD1 produces conformational instability in the protein that results in the formation of intracellular aggregates. These aggregates have been found in the various transgenic ALS mouse models, as well as in a small number of human cases. These protein aggregates have been

found to be ubiquitinated and immunoreactive to SOD1; they have been located in the neuropil of motor neurons and also in astrocytes [36]. Aggregates become evident by time of disease onset, and increase with its progression [35, 36].

Unlike wild-type SOD1, which forms a stable dimeric complex, mutant SOD1 oligomerizes forming a small cytosolic pore-like structure very much akin to some forms of β-amyloid proteins [68]. The key question with respect to protein aggregation is whether these complexes are indeed toxic or pathogenic. Preliminary studies in the transgenic rodent model revealed that there was indeed a link between the clinical phenotype of the disease and protein aggregation, which was proportional to the severity of the mutation [69].

The underlying mechanism of toxic protein aggregation has been highly speculative. Many hypotheses have been put forth, including mediation of oxyradical formation, dysfunctioning of the proteosome, and protein sequestration [28]. Mutant SOD1 has been found to directly bind the chaperone family of heat-shock proteins (HSPs), specifically to HSP70, HSP40, HSP27 and αβ-crystallin [70]. This interaction may result in impaired chaperone activity, thereby exacerbating dysfunctional protein folding. Overexpression of HSP70 in several mutant SOD1 models failed to affect either disease onset or survival. Surprisingly different HSP70 & HSP90 expression was observed between the $SOD1^{G85R}$ and $SOD1^{G93A}$ models [71], suggesting that chaperone function was mutation dependent. Studies addressing protein aggregation and proper functioning of the proteosome revealed that modulation of ubiquitination was largely ineffective in the $SOD1^{G93A}$ model [72]. To date, these and other studies have failed to make a causal link between aberrant mutant SOD1 protein aggregation and motor neuron degeneration *in vivo*.

More recently, transactivation response DNA-binding protein with a molecular weight of 43kDa (TDP-43) was identified as a major component of intraneuronal inclusions in SALS, mutant SOD1 FALS and in frontotemporal lobar degeneration (FTLD) [73]. TDP-43 is a 424 amino-acid protein encoded by the *TARDBP* gene located on chromosome 1 [74]. TDP-43 possesses 2 RNA-binding domains, and a glycine-rich region located at the C-terminal end which binds to a host of heterogeneous nucleoproteins (hnRNPs); it is more abundantly found in the nucleus than in the cytoplasm. The exact molecular function of TDP-43 is incompletely understood, but it is implicated in RNA processing, stabilization and transport [75]. There have been over 25 mutations found in *TARDBP*, which are principally missense mutations primarily located in the C-terminal portion of the protein [76]. *TARDBP* mutations in ALS are rare and only account for 5% of all FALS cases and only 1% of all ALS [77].

In the past three years, major interest in TDP-43 has been related to the fact that the wild-type TDP-43 protein has been found within inclusions in the majority of SALS patients [73]. Mutant TDP-43 (TDP-43^{mutant}) are mislocated, ubiquitinated, abnormally processed, or hyperphosphorylated [75]. In contrast to wild-type TDP-43, mutant varieties of TDP-43 are mainly localized to the cytoplasm, and also appear to be depleted within the nucleus [78]. These observations suggest that nuclear depletion results in failure of normal RNA metabolism possibly resulting in abnormal RNA splicing. One study did find that wild-type TDP-43 does interact with neurofilament-light (NF-L) mRNA, which has been implicated in the pathogenicity of ALS [79]. Abnormal processing of TDP-43 has been found in a study of FTLD/ALS [80] and results in the accumulation of the small molecular weight species of 25kDa consisting of the C-terminal fragment of the protein. Expression of the C-terminal fragment *in vitro* leads to aggregate formation, but the exact mechanism and its implication for pathogenicity are yet to be

determined [81]. Overexpression of TDP-43^{mutant} in mice resulted in degeneration of both spinal and cortical (layer V) neurons, which contained the aforementioned ubiquitinated inclusions and leads to a loss of upper and lower motor neurons [78].

While research into TDP-43, its mutant forms and their role in ALS and FTLD is still in its infancy, it marks a significant gain in our understanding of the disease. Their discovery has introduced impaired RNA processing as a possible pathogenic cause of these diseases; it also represents a key finding in SALS pathology. Interestingly TDP-43 was not found in the vast majority of mutant SOD1 FALS cases [82]. Finally it appears that TDP-43 may play a similar role as α-synuclein in Parkinson's disease and amyloid precursor protein in Alzheimer's disease. Mutations in these diseases are also rare, and mutant protein inclusions are evident in each. More recently still, mutations in another protein, fused in sarcoma/translated in liposarcoma (FUS/TLS) with TDP-43 like DNA/RNA binding functions has been linked to FALS [83].

1.3.3 Excitotoxicity

Glutamate, which is released from the presynaptic neuron, is the main excitatory neurotransmitter in the CNS. Glutamate binds to receptors on the post synaptic neuron which, in turn, causes the influx of sodium and calcium. Under usual conditions the influx of these ions is very tightly regulated; in a diseased state however, an increase in synaptic glutamate levels or elevated sensitivity of the postsynaptic neuron to glutmatergic stimulation can lead to neuronal death. This process is called excitotoxicity. The key receptors involved in this pathological pathway are the N-methyl-D-aspartic (NMDA) and the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) types [84].

The excessive glutamate induced stimulation of post-synaptic NMDA and AMPA glutamate receptors results in the massive influx of calcium into motor neurons [84]. Motor neurons are especially sensitive to excitotoxicity for two main reasons: 1) the abundance of calcium-permeable AMPA receptors; 2) the relatively low quantity of calcium buffering proteins such as parvalbumin and calbindin [85]. This diminished capacity to buffer calcium can trigger calcium-activated processes and downstream release of effector molecules such as proteases, nuclease and lipases [86].

AMPA receptors have been crystallized and are composed of a tetrameric complex of 4 subunits (GluR1-4), with calcium permeability being dictated by the GluR2 subunit. The presence of the GluR2 subunit renders the AMPA channel impermeable to calcium. Motor neurons express the GluR2 subunit at a relatively low level, which functionally translates to higher calcium permeability and increased sensitivity to excitotoxicity [87]. The GluR2 subunit mRNA is heavily edited, and there is some suggestion that normal mRNA processing is altered in ALS. The importance of the GluR2 subunit was demonstrated in SALS patients, for whom the GluR2 mRNA was aberrantly spliced [88]. This resulted in increased calcium permeability of the AMPA receptor, and ultimately increased susceptibility to excitotoxic cell death. Indeed, further studies into GluR2 subunit modulation revealed that deletion of the GluR2 encoding gene in mutant SOD1 mice accelerated motor neuron degeneration resulting from increased calcium influx [89], while its overexpression provided protection for motor neurons which translated to increased life span [90].

Increased calcium buffering, *via* overexpression of calcium binding proteins such as parvalbumin and calbindin, leads to higher resistance of cultured motor neurons to excitotoxicity [91, 92].

Spinal motor neurons express these proteins in low amounts and are particularly vulnerable to excitotoxicity, while motor neurons which are spared during ALS progression have been found to express both [85]. The functional outcome for calcium-binding protein-deficient motor neurons is increased stress on the mitochondria. This leads to depolarization and the increased production of reactive oxygen species [93].

In mutant SOD1 animal models, as well as in patient samples, elevated levels of extracellular glutamate have been detected [94, 95]. The main receptor responsible for clearance of the synaptic cleft from excess glutamate is the EAAT2 glial transporter (also called GLT-1). In both patients and rodent models of ALS, levels of this glutamate transporter have been reported to be diminished [96, 97]. Interestingly, mutant SOD1 was found to damage the C-terminal portion of EAAT2 indirectly by triggering its cleavage by caspase-3 [98]. Axonal-loss, a hallmark of ALS, also resulted in decreases in EAAT2 expression in astroglia [99]. Pharmacological upregulation of the EAAT2 transporter, via the β-lactam antibiotic ceftriaxone, resulted in an increased life span of mutant SOD1 mice. This indicated that increased clearance of the synaptic cleft has a beneficial effect and can protect motor neurons during disease progression in the mouse model [100]. Thus in addition to GluR2, dysfunctional EAAT2 activity can contribute to the pathogenic mechanism of excitotoxicity.

1.3.4 Mitochondrial Dysfunction and Apoptosis in ALS

Evidence for the role of mitochondria in ALS pathogenesis was reported from a subset of SALS patients who had abnormal mitochondrial morphology in both skeletal muscles, and in intramuscular nerves [101, 102]. In these cases, further biochemical characterizations revealed defects in the respiratory chain complexes I & IV in muscle [103]. Increased intra-mitochondrial

calcium in both the muscle and spinal cord also occurred and aberrant mitochondrial morphology was evident in the various mutant SOD1 mouse models. These mitochondria were vacuolated, which was a consequence of a detachment between the inner and outer mitochondrial membrane [104]. Misshapen mitochondria were found to increase in number and the severity of vacuolization also worsened with disease progression [105].

Support linking mitochondrial dysfunction and mutant SOD1 has come from *in vitro* cell culture of neuronal cell-lines and primary motor neurons, where there is mitochondrial depolarization, impaired calcium homeostasis, and impaired ATP production (reviewed in [106]). There is still much debate about the exact role mutant SOD1 plays in mitochondrial dysfunction. Certain reports suggest that mutant SOD1 can block mitochondrial protein import channels [107], and that overexpression of stable mutant SOD1 ($SOD1^{D90A}$, $SOD1^{G93A}$) disrupts normal intracellular radical chemistry that leads to aberrant mitochondrial uptake of mutant SOD1 [108]. Interestingly, unstable mutant variants ($SOD1^{G85R}$ and $SOD1^{G127X}$) were not taken up by the mitochondria in this study.

One key pathological pathway which is triggered by mitochondrial stress is apoptosis. In both human and SOD1 mutant mice, biomarkers of apoptosis are discovered during the late stages of the disease (reviewed in [109]). Mutations in SOD1 were thought to convert it from an anti- to a pro-apoptotic protein. Indeed cultured neurons which ectopically expressed mutant SOD1 succumbed to apoptotic death [110, 111]. Mutant SOD1 association with mitochondrial membranes that leads to pore formation may also potentiate the release of cytochrome C, which in turn triggers the intrinsic apoptotic cascade [112]. Mutant SOD1 appears to sequentially trigger activation of apoptotic effector caspases (CASP). In the $SOD1^{G85R}$ mouse, there is early

activation of CASP1, which appears months prior to both CASP3 activation and clinical disease onset [113]. CASP3 is naturally found in motor neurons and in astrocytes, where it directs cleavage of astroglial EAAT2 [114]. Mutant SOD1 interaction in this pathway provides evidence for a link between two pathological pathways in ALS, namely excitotoxicity and apoptosis.

SOD1^{G93A} mice exhibit activation of CASP7, which has also been found to coincide with onset of disease [115]. Indeed, there seems to exist a hierarchical cascade of caspase activation in this mouse model beginning with cytochrome C translocation into the cytosol. The latter triggers CASP9, followed by CASP1; they precede triggering of CASP3 and CASP7 [115]. Further studies seeking to achieve total caspase inactivation revealed that its inhibition *via* ectopic p35 expression had little effect on survival [116].

In addition to indirect triggering of apoptosis via activation of caspases, mutant SOD1 was found to bind and sequester the anti-apoptotic protein BCL-2 [117]. Binding of BCL-2 by mutant SOD1 results in a detergent insoluble inclusion which renders BCL-2 non-functional. These BCL-2/mutant SOD1 complexes are toxic to the cell and can trigger apoptosis. In contrast, neuronal overexpression of BCL-2 in SOD1^{G93A} extended lifespan, and also resulted in partial motor neuron rescue [118]. Further, genetic deletion of pro-apoptotic proteins Bax/Bim resulted in increased survival in mutant mice; it did not rescue lost muscle innervation, suggesting that distal synaptic dysfunction is sufficient for lethal mortality in mice [119].

1.3.5 Neuroinflammation

Once the apoptotic cascade has been initiated, secondary cellular events in the spinal cord come into play which serves to intensify disease progression. These include activation of the resident immune cells such as microglia and T cells, which produce pro-inflammatory factors and

cytokines such as interleukin 1β, cyclooxygenase 2 (COX2) and tumour necrosis factor-α (TNFα). These discoveries have revealed that motor-neuron death is not cell autonomous, with other cells contributing to disease progression. The first evidence of non-neuronal involvement came from mice studies in which mutant SOD1 expression was limited to motor neurons. These animals failed to develop disease-like symptoms or mortality [120]. Subsequently, other studies seeking to selectively express mutant SOD1 in a cell-restricted manner resulted in similar findings (i.e., no classical ALS phenotype being exhibited) [121, 122]. Taken together, these investigations clearly indicate that the effect of mutant SOD1 expression is not limited to motor neurons and that motor neuron degeneration involves multiple other cell types.

Mutant SOD1 expressing motor neurons surrounded by wild-type non-diseased cells had increased survival in chimeric mice [123]. Similarly, decreasing the expression of mutant protein in selective cell types slowed disease progression (reviewed in [124]). Although these findings demonstrated that restricting mutant SOD1 expression to just one cell type was favourable, they were not capable of delineating the role each cell type had in disease progression.

Astrocytes are the main macroglial cell-type within the CNS. Their main function deals with uptake of glutamate from the synaptic cleft, and also as the major cytotrophic provider to motor neurons [125]. Astrocyte participation in ALS was first shown in both patients and animal models which involved a decreased expression of EAAT2 [126]. Primary culture experiments showed that mutant SOD1 expressing astrocytes were neurotoxic to motor neurons [127]. Grafting wild-type lineage-restricted astrocyte precursors into the cervical spinal cord of SOD1^{G93A} rats prolonged survival [128]. Along these lines, an astrocyte-centric therapeutic strategy focusing on boosting antioxidant defences was implemented and it showed that

overexpression of the transcription factor Nrf2 was able to extend survival in the SOD1^{G93A} mouse model.

Microglial cells are the resident CNS immune cells. Under normal physiological conditions, they actively survey the environment for foreign antigens. Once these cells have been activated, their response depends on the nature and length of the stimulus. Microglial activation results in either cellular proliferation or phagocytosis, along with the release of such factors as neurotransmitters, pro-inflammatory cytokines, reactive oxygen species, anti-inflammatory cytokines, and neurotrophic factors [129]. Microglial activation and proliferation is a hallmark of post-mortem ALS tissues, and coincides with increased expression of cell surface markers such as major histocompatibility complex (MHC) antigens I & II, integrins and reactivity to immunoglobulins [130]. These findings represented end-stage microglial involvement of pathology and did not elucidate the role of microglia in the progression of the disease. Evidence for a role of these cells in disease progression was subsequently explored in various studies in mutant animal models (reviewed in [124]). Others used radiolabelled ligands and PET imaging to demonstrate that activated microglia are widespread in both the motor (cortex, and pons) and the extra-motor (dorsolateral prefrontal cortex and thalamus) regions of live ALS patients [131].

Activated microglia and astrocytes produce reactive oxygen species (ROS), which have been shown to upregulate the expression of inducible nitric oxide synthase (iNOS). This occurs early in the pre-symptomatic stage of ALS [132] and constitutes a marker of increased oxidative stress. Primary culture studies revealed that mutant microglia produce more nitric oxide (NO) than wild-type microglia, and are also more toxic to motor neurons. This toxicity was abrogated with the addition of an iNOS inhibitor [121, 133]. Microglial cells also produce superoxide ions

via the NADPH oxidase enzyme [134]. During disease progression, this enzyme is upregulated in the spinal cord resulting in increased ROS production. The latter contributes to the pathology of neurodegeneration through oxidative damage of proteins [135]. Deletion of NADPH oxidase activity in a SOD1^{G93A} model resulted in significantly increased survival [135]. Apocynin, a NADPH oxidase blocker, was also shown to prolong survival by 100 days when given in the drinking water of low-copy number SOD1^{G93A} mice [136]. These results proved exciting, but further investigation revealed that when apocynin was administered after disease onset the outcome was not as striking.

As mentioned previously, pro-inflammatory factor production is coupled to microglial activation. One such signal is TNFα, which is a potent cytokine with direct toxic effects on motor neurons, astrocytes, and microglia. Levels of TNFα were shown to be elevated in both the blood and plasma of ALS patients [137, 138]. Elevated TNFα mRNA along with protein levels were found in pre-symptomatic SOD1^{G93A} mice, which correlated directly with disease progression [139]. Primary microglial cultures isolated from SOD1^{G93A} mice showed increased TNFα production when activated by lipopolysaccharide (LPS) [140]. A closer inspection of the role TNFα has in ALS was undertaken by researchers who crossed mutant SOD1 models and TNFα-deleted mice [141]. The results of this particular study showed that neither axonal degeneration nor activation of astrocyte and microglial cells were attenuated due to the lack of TNFα. This suggested that TNFα is not a key factor in motor neuron degeneration caused by FALS, but perhaps more of a modulator of disease. In accordance with these results clinical trials of the TNFα inhibitor drug thalidomide were conducted, but they failed to improve symptoms or mortality of ALS patients [142].

In addition to the pro-inflammatory response exhibited by diseased microglia, there is a concurrent decrease in the expression of anti-inflammatory and trophic factors. One of these key trophic factors is insulin-like growth factor 1 (IGF-1). SOD1^{G93A} primary microglia in culture have been shown to produce less of this factor when compared to wild-type microglia even after activation via LPS [133]. It was also shown that IGF-1 has anti-inflammatory properties by inhibiting the release of TNFα and nitrous oxide (NO), along with neuroprotective action on motor neurons and astrocytes which express mutant SOD1 [143].

Cellular communication between motor neurons and microglia is vital in the normally functioning CNS. Therefore, diseased motor neurons can trigger activation of microglia, initiating the inflammatory cascade. Damaged neurons release ATP which activate microglia *via* binding to the P2 purinergic receptor, which in turn activates the inflammatory response [144]. Activation of these inflammatory receptors translates into increased production of TNFα and COX-2 by mutant SOD1-expressing microglia [145]. These receptors were also found in great abundance on the surface of microglia and macrophages in spinal cords from ALS patients [146]. Mutant SOD1 itself activates microglia once it has been secreted from diseased motor neurons. This egress from motor neurons is facilitated by its interaction with the chaperone-like factor chromogranin A (CgA) [147]. Both CgA and CgB were found to co-localize with mutant SOD1 within motor neurons, but not wild-type SOD1 in ALS mice. Secreted mutant SOD1 activates microglial cells by binding to the CD14 receptor [148].

1.3.6 Impaired Axonal Transport and ALS

Reduced axonal transport is yet another pathological feature of ALS. First demonstrated in patients with the disease [149], and it was subsequently observed in the various transgenic rodent models [150]. Axonal transport is vital to the normal functioning of neurons and is responsible for the movement of molecules and organelles within the axon. Many axonal proteins are synthesized within the cell body (or soma), and then transported in an anterograde manner along the axons towards the synapse. On the other hand, peripheral trophic factors and neurotransmitters must be transported in a retrograde manner from the synapse to the soma. The protein complexes responsible for the movement of cargo within the axons are kinesin and the dynein-dynactin complexes.

Prior to disease onset in the SOD1^{G93A} model anterograde transport was found to be slowed, and this amplified disease progression. These defects were not only restricted to anterograde transport as retrograde movement was also disrupted [151, 152]. The molecular basis for this impairment is not yet fully understood, but researchers have suggested that aggregations of neurofilaments (spheroids) might hinder the normal transport [149]. Diminished retrograde transport in ALS has also been linked to the disruption of dynein function [153]. Anecdotal evidence from other motor neuronal disorders in which perturbations in the structure and function of the proteins responsible for transport are causative suggest that this may also be the case in ALS. Induced mutations within the dynein gene in the SOD1^{G93A} model paradoxically improved symptoms; they also relieved the slowing of axonal transport [154, 155]. This surprising finding led researchers to suggest that some cytotoxic feature of mutant SOD1 requires dynein-based transport along the axon.

Although evidence for impaired axonal transport as a key pathological pathway is somewhat contradictory, support from other neurodegenerative diseases (e.g., Huntington's disease) indicate that impaired trafficking along the axon can lead to motor- neuron degeneration. Cell-culture studies of the effects of the Huntington's relevant proteins huntingtin and presenilin 1 showed that mutations in each resulted in impaired axonal transport [156, 157]. Additional evidence for the importance of proper axonal transport came from the *wobbler* mouse model. Mutations in a vesicular sorting protein leads to nerve degeneration [158], a finding also implicated in a human pedigree study of frontotemporal dementia [27].

1.4 Emerging Therapeutic Treatment in ALS

Over the course of the 150 years since ALS was first described, there have been many pathological pathways implicated or suggested as causing disease. The diversity of these etiologies suggests that, as with FALS, ALS might best be considered a syndrome rather than a unique disease. The one common feature is the overall disease phenotype (with exceptions in disease progression and prominence of affected motor neurons). Over 150 different compounds and diverse therapeutic strategies have been tested in the various mouse models of ALS. These include: 108 pharmacotherapies, 14 gene therapies, 9 cell transplantations, 3 immunisations and 7 dietary regimens [47]. Unfortunately however, treatments which had shown promise in prolonging the lifespan in animal models failed to recapitulate the same effect in humans [159]. The reasons for this apparent failure are multiple, but may in part be due to critical differences between the mouse models and human disease. For example, SOD1 overexpression resulting from multi-transgene copies does not closely resemble the genetic state in humans. The obvious differences in neuroanatomy, pharmacokinetics, and pharmacodynamics also need to be considered [160].

In addition to these intrinsic differences, problems with how drug trials have been designed and structured also contributed to the failure in finding a cure. Meta-analysis of a number of ALS drug trials in mice revealed that most were underpowered (small sample size) and confined to the presymptomatic stage of the disease for treatment administration which is the least relevant to the real-life clinical setting [160]. Future animal trials of promising therapeutics will need to take into considerations such factors as gender, genetic background, transgene copy number, disease onset and endpoint, and litter matched wild-type controls [161]. The following section summarizes some of the advances made in therapeutic development, and is organized by the primary action of the drugs.

1.4.1 Anti-oxidants

Generally speaking, the administration of various anti-oxidants (such as catalase, putrescine-modified catalase, ginseng, and vitamin E) in mice all delayed onset of ALS signs, but failed to have a significant effect on survival [162-164]. Other compounds such as NOS inhibitors (e.g., creatine, red wine, and SOD1/catalase mimetics) were able to extend survival but not age of onset. A number of other anti-oxidants (celastrol, green tea, and Neu2000) were able to both delay age of onset and extend survival. One very promising compound is Mn-porphyrin. When given at symptomatic onset of disease this agent was able to very significantly increase survival [165]. This class of compounds have underlined the hypothesis that oxidative stress may be an important initiating factor in ALS and that they demonstrate some utility as a treatment in the SOD1 animal models. By and large, when studied this group of drugs has failed to exhibit significant efficacy in human trials.

1.4.2 Anti-glutamatergics

This class of compounds focuses mainly on reducing the toxic effect of increased glutamate in the synaptic cleft, and has consistently resulted in extension of survival in animal models without a concurrent increase in age of onset. To date, the only US FDA approved drug for treatment of ALS is the anti-glutamatergic, riluzole. The effects of riluzole are moderate, increasing survival of patients in a phase 3 trial by only 2-3 months [166]. However, other compounds similar in action to riluzole (such as talampanel, memantine, topiramate, lamotrigine, gabapentin, and ONO-2506) all have resulted in no beneficial effects in human trials (reviewed in [167]). Increased expression of the glutamate transporter EAAT2 by the β-lactam antibiotic Ceftriaxone was associated with increased survival and elevated EAAT2 mRNA levels [100]. This compound is in clinical trials in the USA and Canada [168].

1.4.3 Anti-aggregates

Strategies which target the toxic gain-of-function of mutant SOD1 aggregates or other putative aggregates focus on upregulating the intrinsic molecular chaperone family of proteins, and in particular the heat shock proteins. Arimoclomol is a compound which has proved to be able to increase the expression of both HSP70 and HSP90 proteins. This upregulation resulted in a cytoprotective effect which could prevent motor neuron degeneration [169]. In animal studies, arimoclomol delayed disease progression and was also able to extend survival by 22% [170]. Initial phase-I human trials proved the drug was safe, and that it crossed the blood-brain barrier readily [171]; and further trials are underway [172].

1.4.4 Anti-apoptotics

Therapies designed to interfere with the intrinsic apoptotic cascade have shown their primary efficacy on disease endpoint, with little effect on onset or progression. Several animal model studies demonstrated that anti-apoptotic compounds were able to decrease mortality and in certain cases, were also neuroprotective. On the other hand, some compounds (valproate) have shown no beneficial effect on neuropathology (reviewed in [47]). Taken together, the overall consensus on the utility of anti-apoptotics for the treatment of ALS is that they would not provide a major therapeutic benefit.

1.4.5 Mitochondrial enhancers

Compounds such as creatine and minocycline, which have been reported to improve mitochondrial functioning in the SOD1 model, have been disappointing in human trials [173]. This reported failure may have been attributable to poor bioavailability and improper dosing. A second trial of creatine utilizing several doses discovered that administration of 30 mg daily resulted in increased brain creatine concentrations, and lower glutamate concentrations [174]. A phase-II clinical trial of creatine at this dose is planned for 2011. A mitochondrial pore modulator named olesoxime, which was initially discovered in a large screen of 40,000 compounds, also showed efficacy in animal models [175]. It is currently being tested in a phase-II/III study in Europe [176]. The oxidative stress reducer dexapramipexole is a dopamine agonist, which has been used for the treatment of Parkinson's disease. This drug was also shown to extend survival of SOD1 transgenic mice [177]. Results of a phase-II study showed that dexapramipexole was well tolerated, safe, and those higher doses were able to slow motor decline [178].

1.4.6 Neurotrophic factors

One of the most surprising and disappointing developments in ALS therapeutics has been the failure of neurotrophic factors to provide benefit in humans. Several different growth factors, which initially showed promise in animals failed to show the same results when tested in clinical trials (reviewed in [47]). Researchers have postulated that the failure of these factors may be based on a number of physiological properties (such as bioactivity, bioavailability, therapeutic dose, and immunodynamics). Administered systemically these factors become foreign antigens and elicit neutralising-antibody production, which in turn limits their functionality.

1.4.7 Gene therapies

These DNA approaches for the development of treatments for ALS are described below based on their method of delivery, and encompass 4 non-viral and 11 viral mediated platforms.

1.4.7.1 Non-viral mediated delivery

A number of studies have been conducted in which DNA plasmids, small interfering RNA (RNAi), or short peptide chains were delivered locally *via* electroporation or systemically by injection into transgenic SOD1 mice (reviewed in [47]). None were able to rescue animals from disease [179]. These non-viral approaches also have to deal with the same immunoreactive processes that systemically administered neurotrophins elicited and are generally viewed as being less efficacious than viral strategies.

1.4.7.2 Viral mediated delivery

The recent use of viral platforms has utilized retrograde delivery of viral vectors to deliver their therapeutic factors directly to their target tissues from peripheral sites of injection. These viruses have been genetically engineered to evade the immune response, and specifically transduce

target cells in the CNS with great efficiency. A number of different neurotrophic factors, along with various gene-silencing motifs, have been delivered by viruses and these studies have produced some exciting results. Intramuscular injection of viral-encoded neurotrophic factors such as cardiotrophin-1, GDNF, IGF-1, and VEGF all had significant beneficial effects on survival and onset of disease (reviewed in [47]). These studies demonstrated that sustained and prolonged delivery of these factors locally to the disease affected area significantly rescued ALS neurodegeneration. Lentiviral-mediated delivery of RNAi targeting mutant SOD1 dramatically slowed disease symptoms [180, 181].

These initial studies have highlighted the utility of viral-mediated delivery in therapeutics and offer great promise for future clinical trials. However, there are still many technical issues concerning safety and site of administration which have to be addressed. Also, many of these approaches targeting genetic defects only deal with SOD1-FALS, and may not necessarily be applicable to the majority of ALS cases.

1.4.8 Stem-cell therapy

Stem-cell-based approaches focus on the replacement of dying cells with stem-cell progenitors. In theory these cells then repopulate the target tissue and recapitulate the action of the diseased cell(s), or protect surviving ones. Recently, with production of pluripotent stem cells from skin fibroblasts, the troubling ethical issues related to the use of embryonic stem cells has been resolved. This new field of therapeutics has offered new hope to many patients afflicted with various degenerative diseases; indeed stem-cell therapy has been heralded publicly as a cure-all approach to sickness. Although many studies have demonstrated that pluripotent stem cells from

various sources can differentiate into motor neurons, their ability to replace dead motor neurons and reinnervate their target muscle is yet to be determined [182].

One study demonstrated that after direct spinal cord injection of stem-cell derived motor neurons the introduced cells were able to extend axons towards target muscle tissues. This resulted in improved limb function [183]. This preliminary study highlighted the efficacy of this strategy, but the functional relevance to patients with ALS is questionable given the rate of progression of the disease. Further, the length of the motor axon in the human would complicate reinnervation in humans compared to rodents. Other studies have gone on to inject stem-cells *via* different routes (intrathecal, intravenous, intrathoracic, intracervical, and cortical) directly into ALS patients, with some positive therapeutic effects (reviewed in [167]).

These initial pilot studies involved a relatively small number of subjects, who displayed heterogeneity of disease severity; they also lacked proper control groups. Assessment of post-mortem tissue from these patients would also have indicated how successful cell-implantation and engraftment were. Therefore the results of these investigations must be viewed with caution. While promising, several issues still need to be addressed in terms of the application of stem-cell therapy in ALS treatment. Clarification of parameters such as optimum cell type and number, dosing frequency, and location of administration still need to be rigorously tested before stem-cell therapy can be considered a viable treatment option.

1.4.9 Immunomodulatory therapies

Pharmacological approaches which focus on attenuation of the neuroimmune response in ALS have been partially successful to date; they increase survival, and delay onset in rodents. A number of different pharmacological compounds (COX2 inhibitors, cyclosporin, lenalidomide,

thalidomide, sulindac) which suppress neuroinflammation were able to prolong life (reviewed in [47]). In addition, other compounds such as GSK3 and MMP9 inhibitors were shown to be beneficial to mice when administered prior to symptom onset [184, 185]. These various studies have been the basis of the consensus that the immune system participates in motor-neuron degeneration in ALS. As with other agents why these anti-inflammatory drugs fail in human clinical trials is still not well understood (reviewed in [124]).

1.4.10 Future clinical trials

ALS presents certain inherent problems for the development of future clinical trials involving promising drugs and therapies. Complications include, but are not limited to: the rarity of the disease in the general population; the relatively unknown but possibly lengthy period between disease onset and symptom onset, and delays in diagnosis; the demonstrated heterogeneity of the disease; and the inherent high dropout rate in clinical trials [167].

The most valuable tool which will aid ALS researchers in the future is the identification of a reliable biomarker(s) of ALS. A well-characterized biomarker would allow for comparison between the animal and human models of a specific treatment, and facilitate more detailed pharmacokinetic studies. This would permit closer scrutiny of drug trials, and also would aid in trial design and implementation. A biomarker would also aid in the discovery of homogenous cohorts of patient cohorts, along with classification of the earliest phases of the disease when therapeutic intervention may be the most beneficial.

Phase III clinical trials often rely upon survival as the primary outcome when testing novel treatments for ALS. This paradigm is expensive and requires large numbers of patients being monitored over a long duration. These trials may experience low enrolment due to the

possibility that patients might receive the placebo and not the active compound. There has been some interest in the use of historical controls for comparison purposes. Increasingly, the primary outcome would be to use the revised ALS functional rating scale (ALSFRS-R) [186]. This clinical tool is easily administered, clinically relevant, and is a reliable indicator of progression and survival [187]. In fact this scale has already been used in a study of lithium and riluzole by the Northeast and Canadian ALS Research consortia [188].

2) Role of Sonic Hedgehog (Shh) during neural development

2.1 Introduction

Hedgehog (Hh) was first discovered in mutant *Drosophila melanogaster* (the common fruit fly) and was so named due to the appearance of spiky hairs on the cuticle of the larvae. The gene responsible for this phenotype coded for a protein which was implicated in segment polarity [189]. Subsequently, mammalian homologs were discovered, and were named Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh). Although they bear highly similar sequences and can be substituted for one another, their potencies differ and can be ranked by Shh>Ihh>Dhh [190].

Shh has been found to be expressed in many tissues throughout the body including the peripheral and central nervous systems, skeleton, skin, somites, and limbs. Shh has been found to play important roles in segmentation and cell proliferation, but most importantly in cell fate determination. Shh expression is very tightly regulated both spatially and developmentally and exerts its action in a paracrine and autocrine fashion. The numerous demonstrated responses to Shh have been found to be due to tight control of the production, concentration and modification of the Shh signal itself [191].

The Hedgehog family of signalling proteins are synthesized as 45kDa pre-proteins which undergo autocleavage to yield two fragments. The N-terminal, or 'Hedge domain' portion, contains the signal sequence, and is highly conserved. The C-terminal end is also highly conserved and is covalently coupled to cholesterol during cleavage; it contains the 'Hog' domain which promotes the autocleavage reaction [192]. In addition to the C-terminal cholesterol modification, the N-terminal portion of the protein also undergoes palmitoylation. The observed lipid-modification of both segments of the mature Shh protein enhances its membrane binding properties along with increasing its secretion and scope of action [191].

Intense study into the role of Shh during embryonic development has revealed that aberrant Shh signalling has been linked to birth defects such as cyclopia (development of only one eye at the midline), or holoprosencephaly (failure of the brain to develop into two hemispheres) [193]. More recently, deregulation of the Shh signalling cascade has been intensely scrutinized as a key contributor governing certain forms of neurological cancers such as medulloblastoma and gliomas [194].

Given the demonstrated role of Shh signalling in both development and certain forms of cancer, it was hypothesized that it may also play a role in disease repair and recovery. Shh may trigger repair and/or replacement of diseased or dying cells from a resident pool of *in situ* stem cells. This regenerative potential makes Shh especially attractive as a possible treatment for neurological disorders.

Although the field of Shh therapy for the treatment of neurological disease is still in its infancy, the available research shows that modulation of Shh and its signalling cascade have proven to be beneficial, and supports further studies [195].

2.2 Shh and neural fate determination

During vertebrate development, the central nervous system arises from a number of coordinated growth steps that culminate with the formation of distinctive structures and tissue types; it also specifies cellular fates [196]. One such structure is the notochord. It is located at the ventral midline under the floor plate, and is a major source of diffusible factors which are responsible for ventral neural patterning [197]. One of these factors is Shh [198], which is first expressed in the developing mouse embryo at embryonic day (E) 7.5 in the ventral node. By E9.5, Shh is expressed by cells in the ventral forebrain, notochord, floor plate of the neural tube, and limb buds [198].

Notochord-derived Shh is responsible for the establishment of molecular markers of six ventral neural progenitor niches (floor plate (FP), p3, pMN, p2, p1, and p0) [199]). Once these progenitors have been induced, the major source of Shh becomes the cells of the floor plate and not the notochord [200]. The mechanism by which Shh dictates neuronal fate is by activation of characteristic transcription factors (TFs) within each progenitor population. The ordered appearance of these TFs is dependent upon their requirement for increasing concentrations and/or duration of Shh exposure [201]. This graded response to Shh results in the establishment of the dorsal ventral (DV) axis such that cells which are exposed to lower concentrations, or shorter durations, develop into cell types observed more distally to the notochord. Cells which undergo a longer exposure, or are exposed to higher concentrations of Shh (such as motor neurons), develop into cells with a more ventral phenotype.

The molecular characteristics of Shh are consistent with those used to describe a morphogen in the classical sense. Hallmarks of the morphogenic action of Shh include: the ability to affect

cellular fate distal from the point of origin; the differential phenotypic response of cells based upon length of exposure and concentration; and the ability to modulate cellular proliferation. It is worth noting that motor neurons are not the only neuronal-cell type which responds to Shh signalling. Other more ventral neuronal types--nigral dopaminergic neurons, striatal projection neurons, basal forebrain cholinergic neurons, and oligodendrocyte progenitor cells--are also induced by Shh [202].

Shh distribution throughout the neural tube is highly regulated by a host of proteins which act to alter Shh secretion, diffusion, retention, and degradation. As stated previously, the lipid modification of Shh (palmitoylation at the N-terminus, and cholesterol modification at the C-terminus) prior to secretion into the extracellular matrix (ECM) affects the diffusivity of Shh. Expression of transmembrane receptors on target cells and extracellular proteins that bind Shh directly can also alter Shh molecular dynamics [203].

Deficiencies in Shh signalling have been linked to developmental defects both in humans and other animals most notably in the late 1960's, when the first appearance of cyclopia occurred in offspring of goats which had ingested the Lily plant *Veratrum californicum* [204]. The causal agent was a steroidal alkaloid called cyclopamine. In animal models of development, cyclopamine treatment resulted in cranio-facial abnormalities which were similar to those seen in Shh knockout mice, and to holoprosencephaly (HPE) in humans [205]. The antagonistic action of cyclopamine on Shh signalling occurred irrespective of Shh biogenesis, or of Shh binding to its cognate transmembrane receptor [206]. Cyclopamine exerts its action *via* direct binding to and inhibiting the action of the Shh downstream effector smoothened (SMO) [207]. The attenuating action of cyclopamine and other synthetic small-molecule Shh antagonists, have led

to preliminary clinical trials to test their efficacy as anti-cancer therapeutic agents. In cancer models where tumourigenic growth is associated with upregulation of the Shh signalling pathway, such as basal-cell carcinoma and medulloblastoma, Shh pathway inhibitors have been able to reduce tumour size and slow cancer progression [208].

In addition Shh pathway small-molecule agonists have also been discovered utilizing a high-throughput cell-based screen. These novel compounds trigger Shh signalling activation downstream of Shh binding at the level of SMO [209]. This new class of synthetic compounds has the potential to become therapeutic agents for the treatment of traumatic or chronic degenerative diseases.

2.3 The Shh signalling cascade

The Shh signal is initiated when Shh binds to its receptor Patched1 (Ptch1) [210]. Ptch1 is a 12-pass transmembrane receptor-like protein which in the absence of Shh ligand binding inhibits the heptahelical transmembrane protein SMO. SMO is a member of the G-protein coupled receptor (GPCR) superfamily and is mainly intracellular, unlike Ptch1 which is found at the membrane surface. Upon Shh binding to Ptch1, the receptor is internalized and degraded relieving the inhibition of SMO [211]. SMO translocates to the membrane surface after being phosphorylated by an as yet poorly understood mechanism. It goes on to switch the Glioma-associated (GLI) proteins from a repressed form to active forms. The GLI family of zinc-finger proteins is comprised of three members (namely Gli1, Gli2, and Gli3) and constitute vertebrate homologs to *Drosophila Cubitus interruptus* or CI.

 In the absence of Shh signal and thus SMO, GLI is cleaved and this cleavage is directed by motifs contained within the C-terminal end of the protein. These sites are phosphorylated, first

by protein kinase A (PKA), which is then followed by further phosphorylation by other serine/threonine kinases, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) [191]. Once the concurrent phosphorylation steps have taken place, secondary processing of the phosphorylated C-terminal GLI by other proteins results in ubiquitylation and targeting of GLI for degradation by the proteosome. This yields a truncated N-terminal form, which acts as a repressor of transcriptional activation.

It is believed that SMO inhibits proteolytic degradation of GLI by an as yet undefined mechanism, and causes accumulation of the full length, (or active form) of the protein. This then goes on to direct transcriptional activation by binding to GLI-responsive elements upstream of target genes.

The importance of GLI-mediated transcriptional activation during neural development has been demonstrated, and a mechanism by which differentiation of the various neuronal populations is beginning to come into focus. [212]. The interplay between the various GLI protein family members is complex and not yet fully understood [213]. Recent genetic studies have revealed that neuronal identity along the dorsal-ventral axis is dependent upon action of the various GLI proteins.

In the absence of Shh, ventral interneurons V0, V1, V2 and MN are specified if GLI in its repressive form is removed; whereas FP and V3 neurons (which reside in the most ventral neuronal niches) not only require Shh, but also GLI to be in its activator form [213]. Studies indicate that Gli1 functions mainly as a transcriptional activator, whereas Gli2 mediates the immediate-early response to Shh activation. A general model of how Shh would affect GLI

functionality is to establish a gradient of GLI activity along the DV axis with neuronal fate being dictated by either the repression or activation of transcription by Gli2 & Gli3.

Both Gli2 and Gli3 contain N-terminal repressor and C-terminal activator regions. Gli2 appears to direct the development of the more ventral regions of the neural tube, such as the floor plate and adjacent V3 neurons along with MN. Studies into the action of Gli3 during development have indicated that, in the absence of Shh, it functions primarily as an inhibitor of transcription. However, Gli3 can act as a transcriptional activator in the context of Shh stimulation and directs the development of more dorsal cellular phenotypes such as the FP and MN [214].

It is also noteworthy that the action of the individual GLI proteins can sometimes be substituted for one another within the same species, indicating a substantial amount of functional equivalency.

2.4 Shh signalling and the Primary Cilium

2.4.1 Cilial structure and functioning

All cells possess a cadre of receptors which transmit signals from the extracellular environment. These include a wide variety of receptors, ion channels, kinases, phosphatases and a host of secondary signalling molecules which are located at or near the cell's plasma membrane. On the cell surface of most types of eukaryotic cells there is an organelle which is uniquely suited to the task of signal transduction, namely the non-motile primary cilium.

Although cilia were discovered over 100 years ago [215], recent evidence indicates that these small organelles are critical cellular signalling hubs. A review of the scientific literature from the past few years reveals various metaphors used to describe cilia as: "cellular antennae", "global positioning systems", or "watchtowers" [216]. Cilia can be described as long and thin

microtubule (MT) structures which protrude from the cell surface; they continuously sample the external environment for signalling cues. These organelles are classified according to their MT makeup as 9+2 (motile), or 9+0 (primary) cilia [217]. This arrangement of the 9 doublet MT functions as the internal scaffolding this core structure is known as the axoneme. Along with the 9 doublet MT, motile cilia also possess an additional 2 MT doublet in the center (i.e., 9+2), while primary cilia do not (i.e., 9+0). Motile cilia are long and thin protrusions from the cell surface extending up to 20µM and tend to be concentrated on the apical surface of epithelial tissues such as the lining of the respiratory pathway, the intestinal tract; they can also be found in the fallopian tubes and in the ventricles of the brain. The rhythmic beating of the motile cilia found in these compartments, create waves to clear mucus, drive sperm, and move cerebrospinal fluid. Primary cilia however, once thought of as a vestigial organelle, are increasingly becoming recognized as signalling nexuses for cellular sampling of the extracellular environment.

All cilia are generated during interphase of mitosis and originate from a structure known as the basal body, which includes the centriole. At the interface of the axoneme and basal body are the terminal plate and transition fibres. Their main function is to regulate the trafficking of proteins into and out of the cilia [218]. The cilia are not static during the life cycle of the cell. On the contrary, given that the centriole is part of the basal body, the axoneme is resorbed during cell cycle progression. Thus cilia are hallmarks of fully differentiated or quiescent cells.

Since no actual protein synthesis occurs at the cilia during ciliogenesis, a transport system termed intraflagellar transport (IFT) is responsible for delivery of the various axonemal precursors. Construction of the axoneme proceeds from the base to the tip using IFT as a highway [219]. The IFT system consists of two types of MT motors, along with 17 different IFT

proteins. These elements combine together to form large 'IFT particles' which are actually visible under light microscopy [220]. Movement from ciliary base to tip (also called anterograde) requires type 2 kinesins IFT motors; for tip to base transport (retrograde), the IFT motor responsible is dynein or cytoplasmic dynein 1b/2 [220]. Given the highly specialized nature of the cilium, there must be a barrier at or near its entrance. Electron microscopic investigations revealed that at the site of the transitional fibres there exists a region known as the 'ciliary necklace'. It has been found to be a universal feature of both motile and primary cilia [221]. The function of the ciliary necklace is to ensure that only cilia-targeted proteins pass from the cytoplasm into the axoneme.

IFT may play a role in regulation, function and turnover of cilia-specific proteins. The mechanism of protein targeting to the cilium is not yet fully known, but drawing on studies in other animal models suggest that a number of post-translational protein modifications can determine ciliary specificity. Key among those appears to be myristylation. This modification of proteins at the amino terminus may enhance protein- lipid association [222]. Phosphorylation may also specify ciliary targeting. Recently, the protein nephrocystin (NPHP1) was shown to be specific for the base of the cilia of epithelial cells after phosphorylation by casein kinase 2 [223]. In addition to phosphorylation and myristylation, targeting may also involve specific peptide sequences. In mammals the receptor SMO contains the cilia-specific sequence motif consisting of a hydrophobic and basic amino acid residue immediately next to the carboxyl terminal of the seventh transmembrane segment [224].

2.4.2 Primary Cilia in the CNS

Within the CNS, the flow of cerebrospinal fluid (CSF) is coordinated by motile cilia on the surface of radial glia and choroid plexus epithelial (CPe) cells in conjunction with ependymal cells lining the brain ventricles [225]. CSF originates from the CPe cells which can regulate both the volume and contents of the CSF; they also possess both motile and non-motile cilia [226]. The primary cilia of the CPe cells regulate the transcytosis of CSF into the ventricles and have most recently been shown to be able to sense the presence of certain neuropeptides [226]. Irregular CPe ciliary structure or functioning can result in increased CSF volume and hydrocephalus during brain development [227], which disrupts CSF flow. The role of the CSF in normal CNS homeostasis is becoming increasingly important as a source of signalling factors that exert their action on both the developing and adult brain [228]. The directional movement of the CSF, which is synchronized by the ependymal cilia, establishes a gradient of these signalling factors. They can exert their action distally from the point of origin in a paracrine fashion, or locally on the surface of the cells which produce them in an autocrine manner.

2.4.3 Primary Cilia and disease

Diseases which affect primary cilial structure or functioning are collectively termed 'ciliopathies'. Although relatively rare, this group comprises afflictions which are associated with a wide range of multi-organ, multisystem pathologies. Ultimately they result in early death and decrease overall life quality. Ciliopathies can be categorized into two groups: motile and nonmotile. Although both involve the same cellular organelle and share many signalling processes, their distinction lies in differing clinical involvements. However, both types of cilia are involved in cystic kidney and liver disease [229]. Accompanying these diseases are increased prevalence of

situs inversus (lateral organ reversal), polydactyly, abnormalities in the corpus callosum, and mental retardation.

The list of ciliopathies seems to grow each year and at present includes: Bardet-Biedl syndrome (BBS), nephronophthisis (NPHP), Senior-Löken syndrome (SNLS), Alström syndrome (ALMS), Meckel syndrome (MKS), Joubert syndrome (JBTS), oral-facial-digital Type I (OFD 1), Jeune asphyxiating thoracic dystropy (JATD), Ellis van Creveld (EVC) syndrome, and Leber congenital amaurosis (LCA). In addition both forms of polycystic kidney disease (PKD) dominant and recessive, are regarded as ciliopathies [229]. Ciliopathies can be further sub-categorized into groups based upon their skeletal involvement. JATD, OFD1 and EVC have a significant skeletal contribution, while BBS, NPHP, MKS, JBTS, ALMS and LCA do not.

The prime example of a non-skeletal ciliopathy is Bardet-Biedl syndrome (BBS). BBS is the most widely studied ciliopathy because its primary aetiology involves defects in the primary cilia [230]. Patients afflicted with BBS present with all the common characteristics of ciliopathic disease namely: polydactyly, cystic kidneys, retinitis pigmentosa (RP), situs inversus, and nearly all are obese. BBS-affected individuals are born with postaxial polydactyly, and are night blind by the age of 8 that precedes total blindness by the age of 15. Patients commonly succumb to end-stage renal failure (ESRF) involving kidney cyst formation, subsequent to having undergone dialysis or transplantation. Fully 30% of BBS patients develop chronic kidney disease [231]. Other ciliopathies are less severe than BBS. NPHP primarily involves the kidneys and constitutes a causal agent in kidney failure in children [232]. NPHP is considered to be phenotypically milder than BBS or MKS and when presented with retinal degeneration is called SNLS. Two of the most severe ciliopathies are MKS and JBTS--both are perinatally lethal. MKS is associated

with neural tube defects (NTD) and is characterized by encephalocoele, polydactyly, and renal cystic disease. Similar to MKS, JBTS presents with ataxia and cerebellar vermis hypoaplasia [229]. Skeletal associated ciliopathies such as OFD1 and JATD present with shortened long bones and ribs, among other defects.

The genetic lesions which are associated with the mentioned ciliopathies all seem to involve either ciliary function or ciliogenesis. The first gene associated with ciliopathy was MKKS (or BBS6) and was identified in 2000 [233]. Since its discovery, over 30 other genes have been linked to various ciliopathic diseases. The majority of ciliopathies have an autosomal recessive mode of inheritance with the exception of OFD1; it is X-linked dominant. However the inheritance and penetrance of ciliopathic mutations can be highly complex and may involve more than one locus as suggested for BBS [234]. Furthermore, other studies have gone on to provide evidence suggesting that the phenotypic outcome and severity of any specific ciliopathy are due to the type, number, and position of the underlying genetic mutation.

In addition to developmental ciliopathies, certain types of cancers have been shown to be regulated by the various signalling pathways centering on the primary cilium. The pathogenic involvement of primary cilia in tumorigenesis is predicated on deregulation of the Shh pathway. Medulloblastoma is the most common form of brain cancer in children, and has been found to be caused by aberrant Shh signalling [235]. Medulloblastomas caused by constitutively active SMO rely on the primary cilium for tumour formation and progression. In contrast the primary cilium can also serve an inhibitory role in tumorigenesis, or cancers which need constitutively active Gli2 (GLI2CA) [236]. The duality of primary cilia in medulloblastoma biology is not surprising given the fact that SMO acts at the ciliary membrane.

2.4.4 Primary Cilia and Shh signalling

Although research into Shh and its signalling cascade has been well established and ongoing for the past 20 years, it was a surprising revelation that most if not all Shh signalling occurs at the site of the primary cilium. Initially, evidence linking Shh signalling to primary cilia emerged from classical forward genetic screens in mice involving surveys of neural tube defects and dorsoventral pattering abnormalities. These studies produced surprising evidence that linked the resultant mutant phenotypes to genes encoding IFT proteins and other ciliary machinery, and not the Shh signalling cascade [237]. Subsequently, researchers succeeded in localizing the key upstream signalling factors of the Shh pathway to the surface of the cilial membrane [238].

The functional outcome of Shh signalling is the regulation of the Gli transcriptional activators and repressors to control tissue patterning and development. Furthermore, this control is dependent upon the ability of Shh signalling components to associate with and travel within the primary cilium. Indeed, studies have shown that both Gli activator and repressor functions depend on primary cilia [239]. The central question concerning Gli processing from its full-length (FL) form into either repressor (R) or activator (A) versions is the exact cellular location where this occurs. Currently two models have been put forth. The first deals with the conversion of Gli3-FL to GliR in the absence of Shh. In this case, a complex including Kif7 [(suppressor of fused (Sufu)] protein, and protein kinases forms at the base of the primary cilium, while Ptch1 near the cilial base prevents entry and accumulation of Smo in the cilium [240]. At the ciliary base, protein kinases direct the conversion of Gli-FL into GliR. Upon Shh binding to Ptch1 and thus Smo activation, the Kif7/Sufu/Gli complex travels to the tip (anterograde) of the cilium (thus Gli-FL is transported distally from the protein kinases). This promotes the conversion of

Gli-FL to GliA within the ciliary tip [241]. In the second model proposed, Gli-FL transformation into GliA is presumed to occur within the nucleus [242].

3. Shh and Neurodegenerative Disease

3.1 Introduction

The role of Shh and its associated signalling partners and organelles have been intensely studied over the past 30 years for their role during development. Recent evidence has indicated that they continue to exert their action in fully developed quiescent adult tissues (both normal and diseased) by regulating the production of key growth and angiogenic factors, along with controlling cellular proliferation. The distribution of Shh ligand and its receptors has been found to be present throughout the adult rodent nervous system, but the degree of Shh signalling has been found to be somewhat attenuated and more restricted spatially [243].

In 2003, viral delivery of Shh was shown to be efficacious in stimulating the proliferation of neural stem cells in the hippocampus [244]. It had also been demonstrated that Shh was upregulated as part of the tissue repair mechanism [245]. These initial studies sparked interest into the exploration of the therapeutic potential of Shh and its signalling cascade for the use in neurodegenerative disease studies. A brief survey of the available research yields convincing evidence that Shh has neuroprotective and neuroregenerative capabilities in neural disorders, varying from Parkinson's disease (PD) to ischemic stroke and other peripheral neuropathies.

3.2 Shh and Parkinson's disease

PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra. During normal brain development Shh is known to direct growth and differentiation of this very class of neurons [246]. Shh has also been shown to have a neuroprotective effect on

differentiated fetal dopaminergic neurons when they are challenged with N-methyl-4-phenylpyridinium *in vitro* [247]. Neural progenitor cells isolated from the subventricular zone (SVZ) of fetal mice have also been proven to secrete Shh ligand that promotes neuroprotection of dopaminergic neurons during development [248]. Injection of 6-hydroxydopamine (6-OHDA) unilaterally into the striatum causes a decrease in dopaminergic innervation of the striatum, as well as a loss of dopaminergic neurons in the substantia nigra [195]. 6-OHDA injection into rat brains serves as a model of PD. When Shh protein was injected directly into the striatum of these animals a significant improvement was shown in this model [249]. In a separate study in which researchers transplanted neural progenitor cells from the SVZ into the striatum of PD rats, motor function was improved [248]. In these preliminary studies, Shh demonstrated its utility as a neuroprotective agent in the context of PD.

3.3 Shh and peripheral neuropathy

In addition to CNS diseases, hedgehog proteins have shown to be protective in rodent models of PNS injury. Shh mRNA was shown to be significantly upregulated in a mouse model of nerve-crush injury. Shh was also prognostic of recovery time after insult, as administration of anti-hedgehog antibody resulted in prolonged recovery time and dosing with recombinant Hh protein accelerated the recovery period [250].

Diabetes mellitus is often accompanied by peripheral neuropathy (manifested as pain and loss of sensation in the extremities) and the degeneration of nerve fibres. In the streptozotocin-induced diabetes rodent model the beta cells of the pancreas are killed, resulting in hyperglycemia [251]. Exogenous administration of Hh proteins 5 weeks after onset of diabetes in the rodent model, and continued for an additional 5 weeks, resulted in a complete restoration of nerve fibre

conductivity. It also relieved the diabetes-induced decrease in production of nerve growth factor [251]. Evidence from the nerve-crush model and the diabetes studies amply demonstrated the beneficial effect Shh can have on nerve recovery from both injury and disease, and that this effect is based at least in part on the ability of Hh to stimulate production of neurotrophic and angiogenic factors.

3.4 Shh agonist and treatment of CNS diseases

Given the therapeutic effect of recombinant Shh protein, it was a natural extension to study the efficacy of available small molecule agonists of the Shh signalling cascade. These agonists are active at low nanomolar concentrations, have good bioavailability, and cross the blood brain barrier easily following systematic administration [252]. Acute agonistic treatment of the 6-OHDA PD mouse model with the Shh agonist purmorphamine resulted in beneficial effects which lasted up to 6 weeks post lesion induction [253]. In an excitotoxic model of Huntington's disease (HD) using a malonate challenge, exogenous treatment with recombinant Hh protein was effective in limiting neuronal death [254]. Shh agonists were also tested in rodent models of peripheral neuropathy and were found to be advantageous in attenuating the effects of the applied insult to the nervous system [195].

Currently, the mechanism by which either Shh or its agonists induce neuroprotection remains unclear. Proposed hypotheses include Hh directly activating neuronal survival pathways, or directly regulating synaptic activity. However, there is some evidence indicating that Hh/agonist mediate neuroprotection by exerting their control over gene expression in Hh reactive cell lineages such as the astrocytes. The latter are known to respond to injury by secreting various neurotrophic factors. Interestingly, evidence for this theory comes from *in vitro* studies of

cultured fibroblasts that showed that Shh can induce production of brain-derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (IGF-1) [255]. Shh was also shown to regulate cytokines involved in angiogenesis, such as vascular endothelial growth factor (VEGF) and other angiopoietins from mesenchymal cells [245]. In the case of diabetes, rats showed an increase in nerve growth factor (NGF) mRNA levels in the sciatic nerves after recombinant Shh protein treatment [251]. All of these studies indicate that Hh/agonist can affect neuronal resistance to injury, by upregulating key cellular survival factors.

4) References

[1]   Charcot M. Demonstration of Arthropathic Affections of Locomotor Ataxy. British medical journal. 1881 Aug 13;2(1076):285.

[2]   Mulder DW. Clinical limits of amyotrophic lateral sclerosis. Advances in neurology. 1982;36:15-22.

[3]   McGuire V, Longstreth WT, Jr., Koepsell TD, van Belle G. Incidence of amyotrophic lateral sclerosis in three counties in western Washington state. Neurology. 1996 Aug;47(2):571-3.

[4]   Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, et al. Amyotrophic lateral sclerosis. Lancet. Mar 12;377(9769):942-55.

[5]   Gordon PH, Cheng B, Katz IB, Pinto M, Hays AP, Mitsumoto H, et al. The natural history of primary lateral sclerosis. Neurology. 2006 Mar 14;66(5):647-53.

[6]   Vucic S BD, Kiernan MC. Diagnosis of motor neuron disease. Sydney. Sydney Australasian Medical Publishing Company 2007.

[7]   Duffy JR, Peach RK, Strand EA. Progressive apraxia of speech as a sign of motor neuron disease. American journal of speech-language pathology / American Speech-Language-Hearing Association. 2007 Aug;16(3):198-208.

[8]   Talbot K. Motor neuron disease: the bare essentials. Practical neurology. 2009 Oct;9(5):303-9.

[9]   Riviere M, Meininger V, Zeisser P, Munsat T. An analysis of extended survival in patients with amyotrophic lateral sclerosis treated with riluzole. Archives of neurology. 1998 Apr;55(4):526-8.

[10]   Armon C. An evidence-based medicine approach to the evaluation of the role of exogenous risk factors in sporadic amyotrophic lateral sclerosis. Neuroepidemiology. 2003 Jul-Aug;22(4):217-28.

[11]   Strong M. Exogenous neurotoxins. In: Brown RHJ MV, Swash M, ed. *Amyotrophic Lateral Scerlosis*. London: Martin Duntiz 2000:279-87.

[12]   Armon C. Excess incidence of ALS in young Gulf War veterans. Neurology. 2004 Nov 23;63(10):1986-7; author reply -7.

[13]   Schmidt ML, Zhukareva V, Perl DP, Sheridan SK, Schuck T, Lee VM, et al. Spinal cord neurofibrillary pathology in Alzheimer disease and Guam Parkinsonism-dementia complex. Journal of neuropathology and experimental neurology. 2001 Nov;60(11):1075-86.

[14]   Moulignier A, Moulonguet A, Pialoux G, Rozenbaum W. Reversible ALS-like disorder in HIV infection. Neurology. 2001 Sep 25;57(6):995-1001.

[15]   Berger MM, Kopp N, Vital C, Redl B, Aymard M, Lina B. Detection and cellular localization of enterovirus RNA sequences in spinal cord of patients with ALS. Neurology. 2000 Jan 11;54(1):20-5.

[16]   Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature. 1993 Mar 4;362(6415):59-62.

[17]   Yang Y, Hentati A, Deng HX, Dabbagh O, Sasaki T, Hirano M, et al. The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. Nature genetics. 2001 Oct;29(2):160-5.

[18]   Chance PF, Rabin BA, Ryan SG, Ding Y, Scavina M, Crain B, et al. Linkage of the gene for an autosomal dominant form of juvenile amyotrophic lateral sclerosis to chromosome 9q34. American journal of human genetics. 1998 Mar;62(3):633-40.

[19]   Nishimura AL, Mitne-Neto M, Silva HC, Richieri-Costa A, Middleton S, Cascio D, et al. A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. American journal of human genetics. 2004 Nov;75(5):822-31.

[20]   Puls I, Jonnakuty C, LaMonte BH, Holzbaur EL, Tokito M, Mann E, et al. Mutant dynactin in motor neuron disease. Nature genetics. 2003 Apr;33(4):455-6.

[21]   Renton AE, Majounie E, Waite A, Simon-Sanchez J, Rollinson S, Gibbs JR, et al. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron. Oct 20;72(2):257-68.

[22]   Topp JD, Gray NW, Gerard RD, Horazdovsky BF. Alsin is a Rab5 and Rac1 guanine nucleotide exchange factor. The Journal of biological chemistry. 2004 Jun 4;279(23):24612-23.

[23]   Otomo A, Hadano S, Okada T, Mizumura H, Kunita R, Nishijima H, et al. ALS2, a novel guanine nucleotide exchange factor for the small GTPase Rab5, is implicated in endosomal dynamics. Human molecular genetics. 2003 Jul 15;12(14):1671-87.

[24]   Panzeri C, De Palma C, Martinuzzi A, Daga A, De Polo G, Bresolin N, et al. The first ALS2 missense mutation associated with JPLS reveals new aspects of alsin biological function. Brain. 2006 Jul;129(Pt 7):1710-9.

[25]   Yamanaka K, Vande Velde C, Eymard-Pierre E, Bertini E, Boespflug-Tanguy O, Cleveland DW. Unstable mutants in the peripheral endosomal membrane component ALS2 cause early-onset motor neuron disease. Proceedings of the National Academy of Sciences of the United States of America. 2003 Dec 23;100(26):16041-6.

[26]   Chen YZ, Bennett CL, Huynh HM, Blair IP, Puls I, Irobi J, et al. DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). American journal of human genetics. 2004 Jun;74(6):1128-35.

[27]   Skibinski G, Parkinson NJ, Brown JM, Chakrabarti L, Lloyd SL, Hummerich H, et al. Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. Nature genetics. 2005 Aug;37(8):806-8.

[28]   Pasinelli P, Brown RH. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat Rev Neurosci. 2006 Sep;7(9):710-23.

[29]   http://alsod.iop.kcl.ac.uk/.

[30]   Radunovic A, Delves HT, Robberecht W, Tilkin P, Enayat ZE, Shaw CE, et al. Copper and zinc levels in familial amyotrophic lateral sclerosis patients with CuZnSOD gene mutations. Annals of neurology. 1997 Jul;42(1):130-1.

[31]   Yamanaka K, Cleveland DW. Determinants of rapid disease progression in ALS. Neurology. 2005 Dec 27;65(12):1859-60.

[32]   Shaw BF, Valentine JS. How do ALS-associated mutations in superoxide dismutase 1 promote aggregation of the protein? Trends in biochemical sciences. 2007 Feb;32(2):78-85.

[33]   Nordlund A, Oliveberg M. SOD1-associated ALS: a promising system for elucidating the origin of protein-misfolding disease. HFSP journal. 2008 Dec;2(6):354-64.

[34]   Nishitoh H, Kadowaki H, Nagai A, Maruyama T, Yokota T, Fukutomi H, et al. ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. Genes & development. 2008 Jun 1;22(11):1451-64.

[35]   Wang J, Xu G, Borchelt DR. High molecular weight complexes of mutant superoxide dismutase 1: age-dependent and tissue-specific accumulation. Neurobiology of disease. 2002 Mar;9(2):139-48.

[36]   Johnston JA, Dalton MJ, Gurney ME, Kopito RR. Formation of high molecular weight complexes of mutant Cu, Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences of the United States of America. 2000 Nov 7;97(23):12571-6.

[37]   Deng HX, Hentati A, Tainer JA, Iqbal Z, Cayabyab A, Hung WY, et al. Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. Science (New York, NY. 1993 Aug 20;261(5124):1047-51.

[38]   Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, et al. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. Nature genetics. 1996 May;13(1):43-7.

[39]   Kondo T, Reaume AG, Huang TT, Carlson E, Murakami K, Chen SF, et al. Reduction of CuZn-superoxide dismutase activity exacerbates neuronal cell injury and edema formation after transient focal cerebral ischemia. J Neurosci. 1997 Jun 1;17(11):4180-9.

[40]   Kawase M, Murakami K, Fujimura M, Morita-Fujimura Y, Gasche Y, Kondo T, et al. Exacerbation of delayed cell injury after transient global ischemia in mutant mice with CuZn superoxide dismutase deficiency. Stroke; a journal of cerebral circulation. 1999 Sep;30(9):1962-8.

[41]   Ho YS, Gargano M, Cao J, Bronson RT, Heimler I, Hutz RJ. Reduced fertility in female mice lacking copper-zinc superoxide dismutase. The Journal of biological chemistry. 1998 Mar 27;273(13):7765-9.

[42]   Flood DG, Reaume AG, Gruner JA, Hoffman EK, Hirsch JD, Lin YG, et al. Hindlimb motor neurons require Cu/Zn superoxide dismutase for maintenance of neuromuscular junctions. The American journal of pathology. 1999 Aug;155(2):663-72.

[43]   Shefner JM, Reaume AG, Flood DG, Scott RW, Kowall NW, Ferrante RJ, et al. Mice lacking cytosolic copper/zinc superoxide dismutase display a distinctive motor axonopathy. Neurology. 1999 Oct 12;53(6):1239-46.

[44]   Muller FL, Song W, Liu Y, Chaudhuri A, Pieke-Dahl S, Strong R, et al. Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. Free radical biology & medicine. 2006 Jun 1;40(11):1993-2004.

[45]   Fischer LR, Glass JD. Axonal degeneration in motor neuron disease. Neuro-degenerative diseases. 2007;4(6):431-42.

[46]   Epstein CJ, Avraham KB, Lovett M, Smith S, Elroy-Stein O, Rotman G, et al. Transgenic mice with increased Cu/Zn-superoxide dismutase activity: animal model of dosage effects in Down syndrome. Proceedings of the National Academy of Sciences of the United States of America. 1987 Nov;84(22):8044-8.

[47]   Turner BJ, Talbot K. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. Progress in neurobiology. 2008 May;85(1):94-134.

[48]   Dal Canto MC, Gurney ME. Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). Brain research. 1995 Apr 3;676(1):25-40.

[49]   Jonsson PA, Graffmo KS, Brannstrom T, Nilsson P, Andersen PM, Marklund SL. Motor neuron disease in mice expressing the wild type-like D90A mutant superoxide dismutase-1. Journal of neuropathology and experimental neurology. 2006 Dec;65(12):1126-36.

[50]   Kotulska K, LePecheur M, Marcol W, Lewin-Kowalik J, Larysz-Brysz M, Paly E, et al. Overexpression of copper/zinc-superoxide dismutase in transgenic mice markedly impairs regeneration and increases development of neuropathic pain after sciatic nerve injury. Journal of neuroscience research. 2006 Oct;84(5):1091-7.

[51]   Jaarsma D, Haasdijk ED, Grashorn JA, Hawkins R, van Duijn W, Verspaget HW, et al. Human Cu/Zn superoxide dismutase (SOD1) overexpression in mice causes mitochondrial vacuolization, axonal degeneration, and premature motoneuron death and accelerates motoneuron disease in mice expressing a familial amyotrophic lateral sclerosis mutant SOD1. Neurobiology of disease. 2000 Dec;7(6 Pt B):623-43.

[52]   Xing J, Yu Y, Rando TA. The modulation of cellular susceptibility to oxidative stress: protective and destructive actions of Cu,Zn-superoxide dismutase. Neurobiology of disease. 2002 Aug;10(3):234-46.

[53]   Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science (New York, NY. 1994 Jun 17;264(5166):1772-5.

[54]   Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez A, et al. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. Experimental neurology. 2004 Feb;185(2):232-40.

[55]   Hall ED, Oostveen JA, Gurney ME. Relationship of microglial and astrocytic activation to disease onset and progression in a transgenic model of familial ALS. Glia. 1998 Jul;23(3):249-56.

[56]   Zang DW, Yang Q, Wang HX, Egan G, Lopes EC, Cheema SS. Magnetic resonance imaging reveals neuronal degeneration in the brainstem of the superoxide dismutase 1 transgenic mouse model of amyotrophic lateral sclerosis. The European journal of neuroscience. 2004 Oct;20(7):1745-51.

[57]   Gurney ME. Transgenic animal models of familial amyotrophic lateral sclerosis. Journal of neurology. 1997 May;244 Suppl 2:S15-20.

[58]   Chiu AY, Zhai P, Dal Canto MC, Peters TM, Kwon YW, Prattis SM, et al. Age-dependent penetrance of disease in a transgenic mouse model of familial amyotrophic lateral sclerosis. Molecular and cellular neurosciences. 1995 Aug;6(4):349-62.

[59]   Jonsson PA, Graffmo KS, Andersen PM, Brannstrom T, Lindberg M, Oliveberg M, et al. Disulphide-reduced superoxide dismutase-1 in CNS of transgenic amyotrophic lateral sclerosis models. Brain. 2006 Feb;129(Pt 2):451-64.

[60]   Simpson EP, Yen AA, Appel SH. Oxidative Stress: a common denominator in the pathogenesis of amyotrophic lateral sclerosis. Current opinion in rheumatology. 2003 Nov;15(6):730-6.

[61]   Beckman JS, Carson M, Smith CD, Koppenol WH. ALS, SOD and peroxynitrite. Nature. 1993 Aug 12;364(6438):584.

[62]   Wiedau-Pazos M, Goto JJ, Rabizadeh S, Gralla EB, Roe JA, Lee MK, et al. Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. Science (New York, NY. 1996 Jan 26;271(5248):515-8.

[63]   Estevez AG, Crow JP, Sampson JB, Reiter C, Zhuang Y, Richardson GJ, et al. Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. Science (New York, NY. 1999 Dec 24;286(5449):2498-500.

[64]   Andrus PK, Fleck TJ, Gurney ME, Hall ED. Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis. Journal of neurochemistry. 1998 Nov;71(5):2041-8.

[65]   Bruijn LI, Beal MF, Becher MW, Schulz JB, Wong PC, Price DL, et al. Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout amyotrophic lateral sclerosis (ALS)-like disease implicate tyrosine nitration as an aberrant in vivo property of one familial ALS-linked superoxide dismutase 1 mutant. Proceedings of the National Academy of Sciences of the United States of America. 1997 Jul 8;94(14):7606-11.

[66]   Deng HX, Shi Y, Furukawa Y, Zhai H, Fu R, Liu E, et al. Conversion to the amyotrophic lateral sclerosis phenotype is associated with intermolecular linked insoluble aggregates of SOD1 in mitochondria. Proceedings of the National Academy of Sciences of the United States of America. 2006 May 2;103(18):7142-7.

[67]   Bruijn LI, Houseweart MK, Kato S, Anderson KL, Anderson SD, Ohama E, et al. Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. Science (New York, NY. 1998 Sep 18;281(5384):1851-4.

[68]   Ray SS, Nowak RJ, Strokovich K, Brown RH, Jr., Walz T, Lansbury PT, Jr. An intersubunit disulfide bond prevents in vitro aggregation of a superoxide dismutase-1 mutant linked to familial amytrophic lateral sclerosis. Biochemistry. 2004 May 4;43(17):4899-905.

[69]   Sato T, Nakanishi T, Yamamoto Y, Andersen PM, Ogawa Y, Fukada K, et al. Rapid disease progression correlates with instability of mutant SOD1 in familial ALS. Neurology. 2005 Dec 27;65(12):1954-7.

[70]   Shinder GA, Lacourse MC, Minotti S, Durham HD. Mutant Cu/Zn-superoxide dismutase proteins have altered solubility and interact with heat shock/stress proteins in models of amyotrophic lateral sclerosis. The Journal of biological chemistry. 2001 Apr 20;276(16):12791-6.

[71]   Liu J, Shinobu LA, Ward CM, Young D, Cleveland DW. Elevation of the Hsp70 chaperone does not effect toxicity in mouse models of familial amyotrophic lateral sclerosis. Journal of neurochemistry. 2005 May;93(4):875-82.

[72]   Gilchrist CA, Gray DA, Stieber A, Gonatas NK, Kopito RR. Effect of ubiquitin expression on neuropathogenesis in a mouse model of familial amyotrophic lateral sclerosis. Neuropathology and applied neurobiology. 2005 Feb;31(1):20-33.

[73]   Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science (New York, NY. 2006 Oct 6;314(5796):130-3.

[74]   Pesiridis GS, Lee VM, Trojanowski JQ. Mutations in TDP-43 link glycine-rich domain functions to amyotrophic lateral sclerosis. Human molecular genetics. 2009 Oct 15;18(R2):R156-62.

[75]   Geser F, Robinson JL, Malunda JA, Xie SX, Clark CM, Kwong LK, et al. Pathological 43-kDa transactivation response DNA-binding protein in older adults with and without severe mental illness. Archives of neurology. Oct;67(10):1238-50.

[76]   Lagier-Tourenne C, Cleveland DW. Rethinking ALS: the FUS about TDP-43. Cell. 2009 Mar 20;136(6):1001-4.

[77] Ticozzi N, LeClerc AL, van Blitterswijk M, Keagle P, McKenna-Yasek DM, Sapp PC, et al. Mutational analysis of TARDBP in neurodegenerative diseases. Neurobiology of aging. Nov;32(11):2096-9.

[78] Wegorzewska I, Baloh RH. TDP-43-based animal models of neurodegeneration: new insights into ALS pathology and pathophysiology. Neuro-degenerative diseases.8(4):262-74.

[79] Strong MJ, Volkening K, Hammond R, Yang W, Strong W, Leystra-Lantz C, et al. TDP43 is a human low molecular weight neurofilament (hNFL) mRNA-binding protein. Molecular and cellular neurosciences. 2007 Jun;35(2):320-7.

[80] Hasegawa M, Arai T, Nonaka T, Kametani F, Yoshida M, Hashizume Y, et al. Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Annals of neurology. 2008 Jul;64(1):60-70.

[81] Dormann D, Capell A, Carlson AM, Shankaran SS, Rodde R, Neumann M, et al. Proteolytic processing of TAR DNA binding protein-43 by caspases produces C-terminal fragments with disease defining properties independent of progranulin. Journal of neurochemistry. 2009 Aug;110(3):1082-94.

[82] Tan CF, Eguchi H, Tagawa A, Onodera O, Iwasaki T, Tsujino A, et al. TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutation. Acta neuropathologica. 2007 May;113(5):535-42.

[83] Kwiatkowski TJ, Jr., Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, et al. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science (New York, NY. 2009 Feb 27;323(5918):1205-8.

[84] Van Den Bosch L, Van Damme P, Bogaert E, Robberecht W. The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis. Biochimica et biophysica acta. 2006 Nov-Dec;1762(11-12):1068-82.

[85] Alexianu ME, Ho BK, Mohamed AH, La Bella V, Smith RG, Appel SH. The role of calcium-binding proteins in selective motoneuron vulnerability in amyotrophic lateral sclerosis. Annals of neurology. 1994 Dec;36(6):846-58.

[86] Grosskreutz J, Van Den Bosch L, Keller BU. Calcium dysregulation in amyotrophic lateral sclerosis. Cell calcium. Feb;47(2):165-74.

[87] Van Damme P, Van Den Bosch L, Van Houtte E, Callewaert G, Robberecht W. GluR2-dependent properties of AMPA receptors determine the selective vulnerability of motor neurons to excitotoxicity. Journal of neurophysiology. 2002 Sep;88(3):1279-87.

[88]　　Kawahara Y, Ito K, Sun H, Kanazawa I, Kwak S. Low editing efficiency of GluR2 mRNA is associated with a low relative abundance of ADAR2 mRNA in white matter of normal human brain. The European journal of neuroscience. 2003 Jul;18(1):23-33.

[89]　　Van Damme P, Braeken D, Callewaert G, Robberecht W, Van Den Bosch L. GluR2 deficiency accelerates motor neuron degeneration in a mouse model of amyotrophic lateral sclerosis. Journal of neuropathology and experimental neurology. 2005 Jul;64(7):605-12.

[90]　　Tateno M, Sadakata H, Tanaka M, Itohara S, Shin RM, Miura M, et al. Calcium-permeable AMPA receptors promote misfolding of mutant SOD1 protein and development of amyotrophic lateral sclerosis in a transgenic mouse model. Human molecular genetics. 2004 Oct 1;13(19):2183-96.

[91]　　Beers DR, Ho BK, Siklos L, Alexianu ME, Mosier DR, Mohamed AH, et al. Parvalbumin overexpression alters immune-mediated increases in intracellular calcium, and delays disease onset in a transgenic model of familial amyotrophic lateral sclerosis. Journal of neurochemistry. 2001 Nov;79(3):499-509.

[92]　　Van Den Bosch L, Vandenberghe W, Klaassen H, Van Houtte E, Robberecht W. Ca(2+)-permeable AMPA receptors and selective vulnerability of motor neurons. Journal of the neurological sciences. 2000 Nov 1;180(1-2):29-34.

[93]　　Carriedo SG, Sensi SL, Yin HZ, Weiss JH. AMPA exposures induce mitochondrial Ca(2+) overload and ROS generation in spinal motor neurons in vitro. J Neurosci. 2000 Jan 1;20(1):240-50.

[94]　　Pioro EP, Majors AW, Mitsumoto H, Nelson DR, Ng TC. 1H-MRS evidence of neurodegeneration and excess glutamate + glutamine in ALS medulla. Neurology. 1999 Jul 13;53(1):71-9.

[95]　　Alexander GM, Deitch JS, Seeburger JL, Del Valle L, Heiman-Patterson TD. Elevated cortical extracellular fluid glutamate in transgenic mice expressing human mutant (G93A) Cu/Zn superoxide dismutase. Journal of neurochemistry. 2000 Apr;74(4):1666-73.

[96]　　Rothstein JD, Martin LJ, Kuncl RW. Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. The New England journal of medicine. 1992 May 28;326(22):1464-8.

[97]　　Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, et al. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). Proceedings of the National Academy of Sciences of the United States of America. 2002 Feb 5;99(3):1604-9.

[98] Trotti D, Rolfs A, Danbolt NC, Brown RH, Jr., Hediger MA. SOD1 mutants linked to amyotrophic lateral sclerosis selectively inactivate a glial glutamate transporter. Nature neuroscience. 1999 Sep;2(9):848.

[99] Yang Y, Gozen O, Watkins A, Lorenzini I, Lepore A, Gao Y, et al. Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. Neuron. 2009 Mar 26;61(6):880-94.

[100] Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, et al. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature. 2005 Jan 6;433(7021):73-7.

[101] Atsumi T. The ultrastructure of intramuscular nerves in amyotrophic lateral sclerosis. Acta neuropathologica. 1981;55(3):193-8.

[102] Afifi AK, Aleu FP, Goodgold J, MacKay B. Ultrastructure of atrophic muscle in amyotrophic lateral sclerosis. Neurology. 1966 May;16(5):475-81.

[103] Wiedemann FR, Winkler K, Kuznetsov AV, Bartels C, Vielhaber S, Feistner H, et al. Impairment of mitochondrial function in skeletal muscle of patients with amyotrophic lateral sclerosis. Journal of the neurological sciences. 1998;156(1):65-72.

[104] Higgins CM, Jung C, Xu Z. ALS-associated mutant SOD1G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes. BMC neuroscience. 2003 Jul 15;4:16.

[105] Sasaki S, Warita H, Murakami T, Abe K, Iwata M. Ultrastructural study of mitochondria in the spinal cord of transgenic mice with a G93A mutant SOD1 gene. Acta neuropathologica. 2004 May;107(5):461-74.

[106] Duffy LM, Chapman AL, Shaw PJ, Grierson AJ. Review: The role of mitochondria in the pathogenesis of amyotrophic lateral sclerosis. Neuropathology and applied neurobiology. Jun;37(4):336-52.

[107] Liu J, Lillo C, Jonsson PA, Vande Velde C, Ward CM, Miller TM, et al. Toxicity of familial ALS-linked SOD1 mutants from selective recruitment to spinal mitochondria. Neuron. 2004 Jul 8;43(1):5-17.

[108] Bergemalm D, Jonsson PA, Graffmo KS, Andersen PM, Brannstrom T, Rehnmark A, et al. Overloading of stable and exclusion of unstable human superoxide dismutase-1 variants in mitochondria of murine amyotrophic lateral sclerosis models. J Neurosci. 2006 Apr 19;26(16):4147-54.

[109] Okouchi M, Ekshyyan O, Maracine M, Aw TY. Neuronal apoptosis in neurodegeneration. Antioxidants & redox signaling. 2007 Aug;9(8):1059-96.

[110] Pasinelli P, Borchelt DR, Houseweart MK, Cleveland DW, Brown RH, Jr. Caspase-1 is activated in neural cells and tissue with amyotrophic lateral sclerosis-associated mutations in copper-zinc superoxide dismutase. Proceedings of the National Academy of Sciences of the United States of America. 1998 Dec 22;95(26):15763-8.

[111] Durham HD, Roy J, Dong L, Figlewicz DA. Aggregation of mutant Cu/Zn superoxide dismutase proteins in a culture model of ALS. Journal of neuropathology and experimental neurology. 1997 May;56(5):523-30.

[112] Bacman SR, Bradley WG, Moraes CT. Mitochondrial involvement in amyotrophic lateral sclerosis: trigger or target? Molecular neurobiology. 2006 Apr;33(2):113-31.

[113] Pasinelli P, Houseweart MK, Brown RH, Jr., Cleveland DW. Caspase-1 and -3 are sequentially activated in motor neuron death in Cu,Zn superoxide dismutase-mediated familial amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences of the United States of America. 2000 Dec 5;97(25):13901-6.

[114] Boston-Howes W, Gibb SL, Williams EO, Pasinelli P, Brown RH, Jr., Trotti D. Caspase-3 cleaves and inactivates the glutamate transporter EAAT2. The Journal of biological chemistry. 2006 May 19;281(20):14076-84.

[115] Guegan C, Vila M, Rosoklija G, Hays AP, Przedborski S. Recruitment of the mitochondrial-dependent apoptotic pathway in amyotrophic lateral sclerosis. J Neurosci. 2001 Sep 1;21(17):6569-76.

[116] Inoue H, Tsukita K, Iwasato T, Suzuki Y, Tomioka M, Tateno M, et al. The crucial role of caspase-9 in the disease progression of a transgenic ALS mouse model. The EMBO journal. 2003 Dec 15;22(24):6665-74.

[117] Pasinelli P, Belford ME, Lennon N, Bacskai BJ, Hyman BT, Trotti D, et al. Amyotrophic lateral sclerosis-associated SOD1 mutant proteins bind and aggregate with Bcl-2 in spinal cord mitochondria. Neuron. 2004 Jul 8;43(1):19-30.

[118] Kostic V, Jackson-Lewis V, de Bilbao F, Dubois-Dauphin M, Przedborski S. Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. Science (New York, NY. 1997 Jul 25;277(5325):559-62.

[119] Gould TW, Buss RR, Vinsant S, Prevette D, Sun W, Knudson CM, et al. Complete dissociation of motor neuron death from motor dysfunction by Bax deletion in a mouse model of ALS. J Neurosci. 2006 Aug 23;26(34):8774-86.

[120] Pramatarova A, Laganiere J, Roussel J, Brisebois K, Rouleau GA. Neuron-specific expression of mutant superoxide dismutase 1 in transgenic mice does not lead to motor impairment. J Neurosci. 2001 May 15;21(10):3369-74.

[121]   Beers DR, Henkel JS, Xiao Q, Zhao W, Wang J, Yen AA, et al. Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences of the United States of America. 2006 Oct 24;103(43):16021-6.

[122]   Gong YH, Parsadanian AS, Andreeva A, Snider WD, Elliott JL. Restricted expression of G86R Cu/Zn superoxide dismutase in astrocytes results in astrocytosis but does not cause motoneuron degeneration. J Neurosci. 2000 Jan 15;20(2):660-5.

[123]   Clement AM, Nguyen MD, Roberts EA, Garcia ML, Boillee S, Rule M, et al. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. Science (New York, NY. 2003 Oct 3;302(5642):113-7.

[124]   Barbeito AG, Mesci P, Boillee S. Motor neuron-immune interactions: the vicious circle of ALS. J Neural Transm. Aug;117(8):981-1000.

[125]   Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. Nature neuroscience. 2007 May;10(5):608-14.

[126]   Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Annals of neurology. 1995 Jul;38(1):73-84.

[127]   Vargas MR, Pehar M, Cassina P, Beckman JS, Barbeito L. Increased glutathione biosynthesis by Nrf2 activation in astrocytes prevents p75NTR-dependent motor neuron apoptosis. Journal of neurochemistry. 2006 May;97(3):687-96.

[128]   Lepore AC, Rauck B, Dejea C, Pardo AC, Rao MS, Rothstein JD, et al. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. Nature neuroscience. 2008 Nov;11(11):1294-301.

[129]   Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature neuroscience. 2007 Nov;10(11):1387-94.

[130]   McGeer PL, Kawamata T, Walker DG, Akiyama H, Tooyama I, McGeer EG. Microglia in degenerative neurological disease. Glia. 1993 Jan;7(1):84-92.

[131]   Turner MR, Cagnin A, Turkheimer FE, Miller CC, Shaw CE, Brooks DJ, et al. Evidence of widespread cerebral microglial activation in amyotrophic lateral sclerosis: an [11C](R)-PK11195 positron emission tomography study. Neurobiology of disease. 2004 Apr;15(3):601-9.

[132] Almer G, Vukosavic S, Romero N, Przedborski S. Inducible nitric oxide synthase up-regulation in a transgenic mouse model of familial amyotrophic lateral sclerosis. Journal of neurochemistry. 1999 Jun;72(6):2415-25.

[133] Xiao Q, Zhao W, Beers DR, Yen AA, Xie W, Henkel JS, et al. Mutant SOD1(G93A) microglia are more neurotoxic relative to wild-type microglia. Journal of neurochemistry. 2007 Sep;102(6):2008-19.

[134] Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiological reviews. 2007 Jan;87(1):245-313.

[135] Marden JJ, Harraz MM, Williams AJ, Nelson K, Luo M, Paulson H, et al. Redox modifier genes in amyotrophic lateral sclerosis in mice. The Journal of clinical investigation. 2007 Oct;117(10):2913-9.

[136] Harraz MM, Marden JJ, Zhou W, Zhang Y, Williams A, Sharov VS, et al. SOD1 mutations disrupt redox-sensitive Rac regulation of NADPH oxidase in a familial ALS model. The Journal of clinical investigation. 2008 Feb;118(2):659-70.

[137] Poloni M, Facchetti D, Mai R, Micheli A, Agnoletti L, Francolini G, et al. Circulating levels of tumour necrosis factor-alpha and its soluble receptors are increased in the blood of patients with amyotrophic lateral sclerosis. Neuroscience letters. 2000 Jun 30;287(3):211-4.

[138] Cereda C, Baiocchi C, Bongioanni P, Cova E, Guareschi S, Metelli MR, et al. TNF and sTNFR1/2 plasma levels in ALS patients. Journal of neuroimmunology. 2008 Feb;194(1-2):123-31.

[139] Hensley K, Fedynyshyn J, Ferrell S, Floyd RA, Gordon B, Grammas P, et al. Message and protein-level elevation of tumor necrosis factor alpha (TNF alpha) and TNF alpha-modulating cytokines in spinal cords of the G93A-SOD1 mouse model for amyotrophic lateral sclerosis. Neurobiology of disease. 2003 Oct;14(1):74-80.

[140] Weydt P, Yuen EC, Ransom BR, Moller T. Increased cytotoxic potential of microglia from ALS-transgenic mice. Glia. 2004 Nov 1;48(2):179-82.

[141] Gowing G, Dequen F, Soucy G, Julien JP. Absence of tumor necrosis factor-alpha does not affect motor neuron disease caused by superoxide dismutase 1 mutations. J Neurosci. 2006 Nov 1;26(44):11397-402.

[142] Stommel EW, Cohen JA, Fadul CE, Cogbill CH, Graber DJ, Kingman L, et al. Efficacy of thalidomide for the treatment of amyotrophic lateral sclerosis: a phase II open label clinical trial. Amyotroph Lateral Scler. 2009 Oct-Dec;10(5-6):393-404.

[143]   Dodge JC, Haidet AM, Yang W, Passini MA, Hester M, Clarke J, et al. Delivery of AAV-IGF-1 to the CNS extends survival in ALS mice through modification of aberrant glial cell activity. Mol Ther. 2008 Jun;16(6):1056-64.

[144]   Inoue K. The function of microglia through purinergic receptors: neuropathic pain and cytokine release. Pharmacology & therapeutics. 2006 Jan;109(1-2):210-26.

[145]   D'Ambrosi N, Finocchi P, Apolloni S, Cozzolino M, Ferri A, Padovano V, et al. The proinflammatory action of microglial P2 receptors is enhanced in SOD1 models for amyotrophic lateral sclerosis. J Immunol. 2009 Oct 1;183(7):4648-56.

[146]   Yiangou Y, Facer P, Durrenberger P, Chessell IP, Naylor A, Bountra C, et al. COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. BMC neurology. 2006;6:12.

[147]   Urushitani M, Sik A, Sakurai T, Nukina N, Takahashi R, Julien JP. Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis. Nature neuroscience. 2006 Jan;9(1):108-18.

[148]   Liu Y, Hao W, Dawson A, Liu S, Fassbender K. Expression of amyotrophic lateral sclerosis-linked SOD1 mutant increases the neurotoxic potential of microglia via TLR2. The Journal of biological chemistry. 2009 Feb 6;284(6):3691-9.

[149]   Sasaki S, Iwata M. Impairment of fast axonal transport in the proximal axons of anterior horn neurons in amyotrophic lateral sclerosis. Neurology. 1996 Aug;47(2):535-40.

[150]   Sasaki S, Warita H, Abe K, Iwata M. Impairment of axonal transport in the axon hillock and the initial segment of anterior horn neurons in transgenic mice with a G93A mutant SOD1 gene. Acta neuropathologica. 2005 Jul;110(1):48-56.

[151]   Williamson TL, Cleveland DW. Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. Nature neuroscience. 1999 Jan;2(1):50-6.

[152]   Murakami T, Nagano I, Hayashi T, Manabe Y, Shoji M, Setoguchi Y, et al. Impaired retrograde axonal transport of adenovirus-mediated E. coli LacZ gene in the mice carrying mutant SOD1 gene. Neuroscience letters. 2001 Aug 10;308(3):149-52.

[153]   Ligon LA, LaMonte BH, Wallace KE, Weber N, Kalb RG, Holzbaur EL. Mutant superoxide dismutase disrupts cytoplasmic dynein in motor neurons. Neuroreport. 2005 Apr 25;16(6):533-6.

[154] Kieran D, Hafezparast M, Bohnert S, Dick JR, Martin J, Schiavo G, et al. A mutation in dynein rescues axonal transport defects and extends the life span of ALS mice. The Journal of cell biology. 2005 May 23;169(4):561-7.

[155] Teuchert M, Fischer D, Schwalenstoecker B, Habisch HJ, Bockers TM, Ludolph AC. A dynein mutation attenuates motor neuron degeneration in SOD1(G93A) mice. Experimental neurology. 2006 Mar;198(1):271-4.

[156] Morfini G, Pigino G, Beffert U, Busciglio J, Brady ST. Fast axonal transport misregulation and Alzheimer's disease. Neuromolecular medicine. 2002;2(2):89-99.

[157] Trushina E, Dyer RB, Badger JD, 2nd, Ure D, Eide L, Tran DD, et al. Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro. Molecular and cellular biology. 2004 Sep;24(18):8195-209.

[158] Schmitt-John T, Drepper C, Mussmann A, Hahn P, Kuhlmann M, Thiel C, et al. Mutation of Vps54 causes motor neuron disease and defective spermiogenesis in the wobbler mouse. Nature genetics. 2005 Nov;37(11):1213-5.

[159] Rothstein JD. Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. Annals of neurology. 2009 Jan;65 Suppl 1:S3-9.

[160] Benatar M. Lost in translation: treatment trials in the SOD1 mouse and in human ALS. Neurobiology of disease. 2007 Apr;26(1):1-13.

[161] Ludolph AC, Bendotti C, Blaugrund E, Hengerer B, Loffler JP, Martin J, et al. Guidelines for the preclinical in vivo evaluation of pharmacological active drugs for ALS/MND: report on the 142nd ENMC international workshop. Amyotroph Lateral Scler. 2007 Aug;8(4):217-23.

[162] Gurney ME, Cutting FB, Zhai P, Doble A, Taylor CP, Andrus PK, et al. Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. Annals of neurology. 1996 Feb;39(2):147-57.

[163] Reinholz MM, Merkle CM, Poduslo JF. Therapeutic benefits of putrescine-modified catalase in a transgenic mouse model of familial amyotrophic lateral sclerosis. Experimental neurology. 1999 Sep;159(1):204-16.

[164] Jiang F, DeSilva S, Turnbull J. Beneficial effect of ginseng root in SOD-1 (G93A) transgenic mice. Journal of the neurological sciences. 2000 Nov 1;180(1-2):52-4.

[165] Crow JP, Calingasan NY, Chen J, Hill JL, Beal MF. Manganese porphyrin given at symptom onset markedly extends survival of ALS mice. Annals of neurology. 2005 Aug;58(2):258-65.

[166]   Bensimon G, Lacomblez L, Meininger V. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. The New England journal of medicine. 1994 Mar 3;330(9):585-91.

[167]   Zinman L, Cudkowicz M. Emerging targets and treatments in amyotrophic lateral sclerosis. Lancet neurology. May;10(5):481-90.

[168]   Clinical   trial   ceftriaxone   in   subjects   with   ALS.   July   5   h, www.clinicaltrials.gov/ct2/show/NCT00349622?term=,   ceftriaxone+and+als&rank=1. (accessed Feb 17.

[169]   Hargitai J, Lewis H, Boros I, Racz T, Fiser A, Kurucz I, et al. Bimoclomol, a heat shock protein co-inducer, acts by the prolonged activation of heat shock factor-1. Biochemical and biophysical research communications. 2003 Aug 1;307(3):689-95.

[170]   Kieran D, Kalmar B, Dick JR, Riddoch-Contreras J, Burnstock G, Greensmith L. Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. Nature medicine. 2004 Apr;10(4):402-5.

[171]   Cudkowicz ME, Shefner JM, Simpson E, Grasso D, Yu H, Zhang H, et al. Arimoclomol at dosages up to 300 mg/day is well tolerated and safe in amyotrophic lateral sclerosis. Muscle & nerve. 2008 Jul;38(1):837-44.

[172]   Phase II/III randomized p-ctoa, in SOD1 positive familial amyotrophic lateral sclerosis. June   4,   http://www.clinicaltrials.gov/ct2/show/NCT00706147?term=nct0070, 6147&rank=1 (accessed Feb 17.

[173]   Shefner JM, Cudkowicz ME, Schoenfeld D, Conrad T, Taft J, Chilton M, et al. A clinical trial of creatine in ALS. Neurology. 2004 Nov 9;63(9):1656-61.

[174]   Atassi N, Ratai EM, Greenblatt DJ, Pulley D, Zhao Y, Bombardier J, et al. A phase I, pharmacokinetic, dosage escalation study of creatine monohydrate in subjects with amyotrophic lateral sclerosis. Amyotroph Lateral Scler. Dec;11(6):508-13.

[175]   Bordet T, Buisson B, Michaud M, Drouot C, Galea P, Delaage P, et al. Identification and characterization of cholest-4-en-3-one, oxime (TRO19622), a novel drug candidate for amyotrophic lateral sclerosis. The Journal of pharmacology and experimental therapeutics. 2007 Aug;322(2):709-20.

[176]   versus SaecoTaa-ottr, lateral pitopsefa, sclerosis (ALS) (MITOTARGET). March 23 hw, clinicaltrials.gov/ct2/show/NCT00868166?term=NCT00868166&ran, k=1.

[177] Danzeisen R, Schwalenstoecker B, Gillardon F, Buerger E, Krzykalla V, Klinder K, et al. Targeted antioxidative and neuroprotective properties of the dopamine agonist pramipexole and its nondopaminergic enantiomer SND919CL2x [(+)2-amino-4,5,6,7-tetrahydro-6-Lpropylamino-benzathiazole dihydrochloride]. The Journal of pharmacology and experimental therapeutics. 2006 Jan;316(1):189-99.

[178] bozik M. A 12-week phase 2 study of the safety, tolerability, and clinical effects of KNS-760704 in ALS subjects. Amyotroph Lateral Scler. 2009;10(suppl1):28-9.

[179] Locatelli F, Corti S, Papadimitriou D, Fortunato F, Del Bo R, Donadoni C, et al. Fas small interfering RNA reduces motoneuron death in amyotrophic lateral sclerosis mice. Annals of neurology. 2007 Jul;62(1):81-92.

[180] Ralph GS, Radcliffe PA, Day DM, Carthy JM, Leroux MA, Lee DC, et al. Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. Nature medicine. 2005 Apr;11(4):429-33.

[181] Raoul C, Abbas-Terki T, Bensadoun JC, Guillot S, Haase G, Szulc J, et al. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. Nature medicine. 2005 Apr;11(4):423-8.

[182] Papadeas ST, Maragakis NJ. Advances in stem cell research for Amyotrophic Lateral Sclerosis. Current opinion in biotechnology. 2009 Oct;20(5):545-51.

[183] Deshpande DM, Kim YS, Martinez T, Carmen J, Dike S, Shats I, et al. Recovery from paralysis in adult rats using embryonic stem cells. Annals of neurology. 2006 Jul;60(1):32-44.

[184] Lorenzl S, Narr S, Angele B, Krell HW, Gregorio J, Kiaei M, et al. The matrix metalloproteinases inhibitor Ro 28-2653 [correction of Ro 26-2853] extends survival in transgenic ALS mice. Experimental neurology. 2006 Jul;200(1):166-71.

[185] Koh SH, Kim Y, Kim HY, Hwang S, Lee CH, Kim SH. Inhibition of glycogen synthase kinase-3 suppresses the onset of symptoms and disease progression of G93A-SOD1 mouse model of ALS. Experimental neurology. 2007 Jun;205(2):336-46.

[186] Kaufmann P, Levy G, Montes J, Buchsbaum R, Barsdorf AI, Battista V, et al. Excellent inter-rater, intra-rater, and telephone-administered reliability of the ALSFRS-R in a multicenter clinical trial. Amyotroph Lateral Scler. 2007 Feb;8(1):42-6.

[187] Kaufmann P, Levy G, Thompson JL, Delbene ML, Battista V, Gordon PH, et al. The ALSFRSr predicts survival time in an ALS clinic population. Neurology. 2005 Jan 11;64(1):38-43.

[188] Aggarwal SP, Zinman L, Simpson E, McKinley J, Jackson KE, Pinto H, et al. Safety and efficacy of lithium in combination with riluzole for treatment of amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. Lancet neurology. May;9(5):481-8.

[189] Nusslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. Nature. 1980 Oct 30;287(5785):795-801.

[190] Pathi S, Pagan-Westphal S, Baker DP, Garber EA, Rayhorn P, Bumcrot D, et al. Comparative biological responses to human Sonic, Indian, and Desert hedgehog. Mechanisms of development. 2001 Aug;106(1-2):107-17.

[191] Ingham PW, Nakano Y, Seger C. Mechanisms and functions of Hedgehog signalling across the metazoa. Nature reviews. Jun;12(6):393-406.

[192] Beachy PA, Cooper MK, Young KE, von Kessler DP, Park WJ, Hall TM, et al. Multiple roles of cholesterol in hedgehog protein biogenesis and signaling. Cold Spring Harbor symposia on quantitative biology. 1997;62:191-204.

[193] Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, et al. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 1996 Oct 3;383(6599):407-13.

[194] Thayer SP, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature. 2003 Oct 23;425(6960):851-6.

[195] Dellovade T, Romer JT, Curran T, Rubin LL. The hedgehog pathway and neurological disorders. Annual review of neuroscience. 2006;29:539-63.

[196] Ribes V, Briscoe J. Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: the role of negative feedback. Cold Spring Harbor perspectives in biology. 2009 Aug;1(2):a002014.

[197] Litingtung Y, Chiang C. Control of Shh activity and signaling in the neural tube. Dev Dyn. 2000 Oct;219(2):143-54.

[198] Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, et al. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell. 1993 Dec 31;75(7):1417-30.

[199] Briscoe J, Pierani A, Jessell TM, Ericson J. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell. 2000 May 12;101(4):435-45.

[200] Marti E, Takada R, Bumcrot DA, Sasaki H, McMahon AP. Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. Development (Cambridge, England). 1995 Aug;121(8):2537-47.

[201] Dessaud E, Yang LL, Hill K, Cox B, Ulloa F, Ribeiro A, et al. Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. Nature. 2007 Nov 29;450(7170):717-20.

[202] Litingtung Y, Chiang C. Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. Nature neuroscience. 2000 Oct;3(10):979-85.

[203] Rubin JB, Choi Y, Segal RA. Cerebellar proteoglycans regulate sonic hedgehog responses during development. Development (Cambridge, England). 2002 May;129(9):2223-32.

[204] Keeler RF. Cyclopamine and related steroidal alkaloid teratogens: their occurrence, structural relationship, and biologic effects. Lipids. 1978 Oct;13(10):708-15.

[205] Gaffield W, Keeler RF. Induction of terata in hamsters by solanidane alkaloids derived from Solanum tuberosum. Chemical research in toxicology. 1996 Mar;9(2):426-33.

[206] Cooper MK, Porter JA, Young KE, Beachy PA. Teratogen-mediated inhibition of target tissue response to Shh signaling. Science (New York, NY. 1998 Jun 5;280(5369):1603-7.

[207] Chen JK, Taipale J, Cooper MK, Beachy PA. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes & development. 2002 Nov 1;16(21):2743-8.

[208] Stanton BZ, Peng LF. Small-molecule modulators of the Sonic Hedgehog signaling pathway. Molecular bioSystems. Jan;6(1):44-54.

[209] Frank-Kamenetsky M, Zhang XM, Bottega S, Guicherit O, Wichterle H, Dudek H, et al. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. Journal of biology. 2002 Nov 6;1(2):10.

[210] Ingham PW. Transducing Hedgehog: the story so far. The EMBO journal. 1998 Jul 1;17(13):3505-11.

[211] McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Current topics in developmental biology. 2003;53:1-114.

[212] Bai CB, Stephen D, Joyner AL. All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. Developmental cell. 2004 Jan;6(1):103-15.

[213]   Jacob J, Briscoe J. Gli proteins and the control of spinal-cord patterning. EMBO reports. 2003 Aug;4(8):761-5.

[214]   Persson M, Stamataki D, te Welscher P, Andersson E, Bose J, Ruther U, et al. Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. Genes & development. 2002 Nov 15;16(22):2865-78.

[215]   Zimmerman H. Beitrage zur kenntniss einiger drusen und epithelien. Arch Mikr Anat. 1898;52:552-706.

[216]   Wong SY, Reiter JF. The primary cilium at the crossroads of mammalian hedgehog signaling. Current topics in developmental biology. 2008;85:225-60.

[217]   Christensen ST, Pedersen LB, Schneider L, Satir P. Sensory cilia and integration of signal transduction in human health and disease. Traffic (Copenhagen, Denmark). 2007 Feb;8(2):97-109.

[218]   Arima T, Shibata Y, Yamamoto T. A deep-etching study of the guinea pig tracheal cilium with special reference to the ciliary transitional region. Journal of ultrastructure research. 1984 Oct;89(1):34-41.

[219]   Qin H, Diener DR, Geimer S, Cole DG, Rosenbaum JL. Intraflagellar transport (IFT) cargo: IFT transports flagellar precursors to the tip and turnover products to the cell body. The Journal of cell biology. 2004 Jan 19;164(2):255-66.

[220]   Rosenbaum JL, Witman GB. Intraflagellar transport. Nature reviews. 2002 Nov;3(11):813-25.

[221]   Gilula NB, Satir P. The ciliary necklace. A ciliary membrane specialization. The Journal of cell biology. 1972 May;53(2):494-509.

[222]   Wang Q, Pan J, Snell WJ. Intraflagellar transport particles participate directly in cilium-generated signaling in Chlamydomonas. Cell. 2006 May 5;125(3):549-62.

[223]   Schermer B, Hopker K, Omran H, Ghenoiu C, Fliegauf M, Fekete A, et al. Phosphorylation by casein kinase 2 induces PACS-1 binding of nephrocystin and targeting to cilia. The EMBO journal. 2005 Dec 21;24(24):4415-24.

[224]   Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF. Vertebrate Smoothened functions at the primary cilium. Nature. 2005 Oct 13;437(7061):1018-21.

[225]   Peters A PSL. The Fine Structure of the Nervous System: Neurons and Their Supporting Cells, Third Edition. New York, Oxford University Press 1991.

[226] Narita K, Kawate T, Kakinuma N, Takeda S. Multiple primary cilia modulate the fluid transcytosis in choroid plexus epithelium. Traffic (Copenhagen, Denmark). Feb;11(2):287-301.

[227] Banizs B, Pike MM, Millican CL, Ferguson WB, Komlosi P, Sheetz J, et al. Dysfunctional cilia lead to altered ependyma and choroid plexus function, and result in the formation of hydrocephalus. Development (Cambridge, England). 2005 Dec;132(23):5329-39.

[228] Sawamoto K, Wichterle H, Gonzalez-Perez O, Cholfin JA, Yamada M, Spassky N, et al. New neurons follow the flow of cerebrospinal fluid in the adult brain. Science (New York, NY. 2006 Feb 3;311(5761):629-32.

[229] Tobin JL, Beales PL. The nonmotile ciliopathies. Genet Med. 2009 Jun;11(6):386-402.

[230] Ansley SJ, Badano JL, Blacque OE, Hill J, Hoskins BE, Leitch CC, et al. Basal body dysfunction is a likely cause of pleiotropic Bardet-Biedl syndrome. Nature. 2003 Oct 9;425(6958):628-33.

[231] Alton DJ, McDonald P. Urographic findings in the Bardet-Biedl syndrome, formerly the Laurence-Moon-Biedl syndrome. Radiology. 1973 Dec;109(3):659-63.

[232] Hildebrandt F, Zhou W. Nephronophthisis-associated ciliopathies. J Am Soc Nephrol. 2007 Jun;18(6):1855-71.

[233] Slavotinek AM, Stone EM, Mykytyn K, Heckenlively JR, Green JS, Heon E, et al. Mutations in MKKS cause Bardet-Biedl syndrome. Nature genetics. 2000 Sep;26(1):15-6.

[234] Katsanis N, Ansley SJ, Badano JL, Eichers ER, Lewis RA, Hoskins BE, et al. Triallelic inheritance in Bardet-Biedl syndrome, a Mendelian recessive disorder. Science (New York, NY. 2001 Sep 21;293(5538):2256-9.

[235] Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science (New York, NY. 1997 Aug 22;277(5329):1109-13.

[236] Han YG, Kim HJ, Dlugosz AA, Ellison DW, Gilbertson RJ, Alvarez-Buylla A. Dual and opposing roles of primary cilia in medulloblastoma development. Nature medicine. 2009 Sep;15(9):1062-5.

[237] Garcia-Garcia MJ, Eggenschwiler JT, Caspary T, Alcorn HL, Wyler MR, Huangfu D, et al. Analysis of mouse embryonic patterning and morphogenesis by forward genetics. Proceedings of the National Academy of Sciences of the United States of America. 2005 Apr 26;102(17):5913-9.

[238]   Louvi A, Grove EA. Cilia in the CNS: the quiet organelle claims center stage. Neuron. Mar 24;69(6):1046-60.

[239]   Cheung HO, Zhang X, Ribeiro A, Mo R, Makino S, Puviindran V, et al. The kinesin protein Kif7 is a critical regulator of Gli transcription factors in mammalian hedgehog signaling. Science signaling. 2009;2(76):ra29.

[240]   Wilson CW, Nguyen CT, Chen MH, Yang JH, Gacayan R, Huang J, et al. Fused has evolved divergent roles in vertebrate Hedgehog signalling and motile ciliogenesis. Nature. 2009 May 7;459(7243):98-102.

[241]   Goetz SC, Anderson KV. The primary cilium: a signalling centre during vertebrate development. Nature reviews. May;11(5):331-44.

[242]   Humke EW, Dorn KV, Milenkovic L, Scott MP, Rohatgi R. The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. Genes & development. Apr 1;24(7):670-82.

[243]   Traiffort E, Charytoniuk DA, Faure H, Ruat M. Regional distribution of Sonic Hedgehog, patched, and smoothened mRNA in the adult rat brain. Journal of neurochemistry. 1998 Mar;70(3):1327-30.

[244]   Lai K, Kaspar BK, Gage FH, Schaffer DV. Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nature neuroscience. 2003 Jan;6(1):21-7.

[245]   Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake Pepinsky R, et al. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. Nature medicine. 2001 Jun;7(6):706-11.

[246]   Hynes M, Porter JA, Chiang C, Chang D, Tessier-Lavigne M, Beachy PA, et al. Induction of midbrain dopaminergic neurons by Sonic hedgehog. Neuron. 1995 Jul;15(1):35-44.

[247]   Miao N, Wang M, Ott JA, D'Alessandro JS, Woolf TM, Bumcrot DA, et al. Sonic hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult In vitro. J Neurosci. 1997 Aug 1;17(15):5891-9.

[248]   Rafuse VF, Soundararajan P, Leopold C, Robertson HA. Neuroprotective properties of cultured neural progenitor cells are associated with the production of sonic hedgehog. Neuroscience. 2005;131(4):899-916.

[249]   Tsuboi K, Shults CW. Intrastriatal injection of sonic hedgehog reduces behavioral impairment in a rat model of Parkinson's disease. Experimental neurology. 2002 Jan;173(1):95-104.

[250] Pepinsky RB, Shapiro RI, Wang S, Chakraborty A, Gill A, Lepage DJ, et al. Long-acting forms of Sonic hedgehog with improved pharmacokinetic and pharmacodynamic properties are efficacious in a nerve injury model. Journal of pharmaceutical sciences. 2002 Feb;91(2):371-87.

[251] Calcutt NA, Allendoerfer KL, Mizisin AP, Middlemas A, Freshwater JD, Burgers M, et al. Therapeutic efficacy of sonic hedgehog protein in experimental diabetic neuropathy. The Journal of clinical investigation. 2003 Feb;111(4):507-14.

[252] Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, et al. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron. 2003 Sep 11;39(6):937-50.

[253] Boucher N BE, Albers DS, Guy K, et al.,. Treatment with hedgehog agonist reduces apomorphine induced rotations in 6-OHDA. Society of Neuroscience Abstract program. 2004;563.11.

[254] Dellovade TL AD, Godon L, Smith D, Xu G, et al.,. In vivo therapeutic efficiacy of small molecule hedgehog agonist. Society of Neuroscience Abstract program. 2002;487.18.

[255] Allendoerfer KL BE, Albers DS, Mathur A, Dudek H, Dellovade TL, et al.,. CNS astrocytes respond to hedgehog pathway agonist in vivo and in vitro. Society of Neuroscience Abstract program. 2004;104.10.

------CHAPTER 2------

Sonic Hedgehog is Cytoprotective against Oxidative Challenge in a Cellular Model of Amyotrophic Lateral Sclerosis

Randy Peterson · John Turnbull

This manuscript investigated the possible role of Shh in cellular resistance against an oxidative challenge in a defined cell-line model. Utilizing the immortalized hippocampal cell-line HT-22 functional studies were conducted to elucidate the effects of an applied exogenous treatment of Shh pathway modulators would have on the ability of the cells to withstand an oxidative stress in the form of a hydrogen peroxide challenge. From this initial proof-of-concept study we deduced that Shh pathway attenuation resulted in a sensitization of cells to oxidative stress, and conversely that Shh pathway activation in SOD1^{G93A} expressing cells conferred a greater ability to resist oxidative stress. Also transient expression of SOD1^{G93A} resulted in a decrease in key Gli factors resulting in a down regulation of Gli mediated transcriptional activation of a Luciferase reporter.

Sonic Hedgehog is Cytoprotective against Oxidative Challenge in a Cellular Model of Amyotrophic Lateral Sclerosis

Randy Peterson · John Turnbull

Abstract We have previously demonstrated that primary cilia on spinal motor neurons are reduced in G93A SOD1 (mSOD) mice, a mouse model of amyotrophic lateral sclerosis (ALS). Sonic hedgehog (Shh) signaling involves the primary cilium and Shh has been shown to be cytoprotective in models of other neurodegenerative diseases. Thus, the Shh signaling pathway may bear further study in ALS. Accordingly, we established that interference with the Shh pathway (with the Shh antagonist cyclopamine or with miRNA 3245p) sensitized HT22 cells, while augmentation of the Shh pathway (with Shh or the Shh agonist purmorphamine) protected cells against hydrogen peroxide (H_2O_2) challenge. We ectopically expressed mSOD, human wild-type SOD1 (wtSOD), or an empty vector in HT22 cells. Compared to empty vector, wtSOD decreased cell death and mSOD increased cell death in response to H_2O_2 challenge. Treatment with cyclopamine or miRNA 3245p sensitized all three transfections to H_2O_2 challenge. Treatment with recombinant human Shh or purmorphamine decreased cell death after H_2O_2 challenge, an effect more pronounced in mSOD cells. Compared with empty vector, overexpression of wtSOD increased Shh and Gli transcript levels and increased activity in a Gli-responsive reporter assay. Overexpression of mSOD did not change Shh transcript levels, but decreased Gli transcript levels, especially Gli3, and reduced activity in a Gli reporter assay. These results suggest that overexpression of mSOD but not wtSOD reduces signaling in the Shh pathway and renders mSOD cells more susceptible to H_2O_2 challenge, and that treatment with Shh or Shh agonists is cytoprotective to mSOD cells. Shh or Shh agonists merit further consideration as potential therapy in ALS.

Keywords Amyotrophic lateral sclerosis · Sonic hedgehog · Oxidative stress · HT22 cell culture

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, mostly without known cause (sporadic ALS). A small minority of patients inherit the disease (familial ALS), and in some of these families, mutations in the SOD1 gene have been found (Rosen 1993). To date, over 130 disease-causing mutations in SOD1 have been identified (http://alsod.iop.kcl.ac.uk/), spanning all five exons. Mutations in SOD1 result in a toxic gain of function, which leads to neurotoxicity (Pasinelli and Brown 2006). Clinically and pathologically, sporadic and familial ALS are largely indistinguishable, and there is some hope that findings in animal and cellular models of familial SOD1 ALS will be relevant to all ALS.

The mechanisms of neurotoxicity and cell death in ALS remain obscure, even with familial disease and known mutation. Several pathological mechanisms may be in common, including impaired axonal transport and reduced trophic support, excitotoxicity, oxidative stress, mitochondrial dysfunction, inflammation, accelerated ageing, errors in RNA processing, and terminally, apoptosis (Rothstein 2009). However, in spite of reasonable therapeutic rationale, all treatments of ALS to date targeting the above

processes have been disappointing, and alternative approaches are needed.

One new approach arises from the unlikely consideration that molecules with a primary role in embryogenesis might also play a role in senescence and neurodegeneration. One such molecule is the developmental morphogen sonic hedgehog (Shh), which has long been studied as a factor essential to the development and sustenance of neural progenitors and the specification of many neuronal subtypes including motor neurons, dopaminergic neurons, and basal forebrain neurons during neurogenesis (Briscoe 2009). However, it has become apparent that the Shh pathway might play an important role in the maintenance of adult neurons.

In models of Parkinson's disease, Shh protects terminally differentiated dopaminergic neurons in culture from N-methyl-4-phenylpyridinium mediated toxicity (Miao et al. 1997). Local infusion of Shh mitigates the deleterious effects of striatal 6-OH dopamine injections in rats (Tsuboi and Shults 2002; Hurtado-Lorenzo et al. 2004), and similar results are seen following Shh delivered by adenovirus vector (Dass et al. 2005). Shh improves peripheral neuropathy in adult diabetic rats (Calcutt et al. 2003), reduces malonate-induced damage in a Huntington model (Dellovade et al. 2006, referenced in Dellovade et al. 2006), and reduces brain damage in a rat focal ischemia model (Dellovade et al. 2006). Conversely, inhibition of Shh increases apoptosis in smooth muscle cells in the rat corporis cavernosa by 12-fold (Bond et al. 2008). Zebrafish heterozygous for the Shh gene show accelerated death of retinal cone neurons (Stenkamp et al. 2008).

Shh signaling is complex (Stecca and Ruiz i Altaba 2002), with multiple points of possible aberration in disease and multiple points of possible therapeutic intervention. The receptor for Shh is a 12 pass transmembrane receptor of the Patched family, which in the unactivated state represses the G protein coupled receptor Smoothened (Smo) and which when bound by Shh de-represses Smo, which can then translocate to the primary cilium and become active (Farzan et al. 2008). Smo ultimately acts on three downstream zinc finger transcription factors, named (after their activity in glioma) Gli1, Gli2, Gli3, whose physiological activity depends on their phosphorylation state. Several intermediate factors modulate the effect of Smo on the Glis including suppressor of fused and Rab23 (abnormal in Charcot Marie Tooth 2B).

Phosphorylation of the Gli factors results from the action of four kinases: fused, PKA, GSK-3, and casein kinase 1. When Smo is held inactive by patched (i.e., no Shh signal), Gli1 is phosphorylated and digested by the proteasome, Gli2 phosphorylated and largely digested, and Gli3 is only partially digested and the N terminal fragment (Gli3R) translocates to the nucleus where it binds to and strongly represses Gli target promoters. Activated Smo (i.e., Shh signal) prevents the phosphorylation and proteasomal cleavage of Gli proteins, and full-length Gli3 translocates to the nucleus and activates the transcription of Gli1 and Gli2, and these in turn activate Shh targets (Humke et al. 2010).

The Gli proteins act on their cognate promoters to alter the expression of several genes, with tissue specificity. Among others, the Shh/Gli pathway may enhance expression of CyclinD1, IGF2, N-Myc, Bcl2, TIMP3, Bmi1, Snail, FOXF1, FOXM1, FOXE1, NR2F2, FGF10, PKC (epsilon), and VEGF and inhibit expression of Plakoglobin and TSC22 (Chari and McDonnell 2007). Although transcription from Gli promoters may sometimes occur through 'non-canonical' pathways (Lauth and Toftgard 2007), it is felt that most Gli activation occurs as above through the Shh pathway and the primary cilium.

Interestingly, surviving spinal motor neurons in G93A SOD1 (mSOD) mice show a marked reduction in primary cilia (Ma et al. 2011). Since the Shh pathway might be altered in ALS and since Shh is neuroprotective in some neurodegenerative disease models as above, we hypothesized that modulation of Shh activity might alter cell survival in face of cytotoxic challenge in cellular models of ALS. HT22 cells have been widely used to study neurotoxicity in vitro. They are relatively easy to transfect, in our case with different SOD1 constructs. They lack glutamate receptors and are particularly appropriate for the study of oxidative stress and free radical damage. Previous studies have shown that oxidative stress and free radical damage are important in the pathogenesis of sporadic and SOD1 familial ALS. Glutathione (GSH) levels are decreased (Lanius et al. 1993), and there are increases in free radical by-products such as 8-hydroxy-2 -deoxyguanosine (8-OHdG), 4-hydroxy-2-$trans$-noneal (HNE), and protein carbonyls (Shaw et al. 1995; Ferrante et al. 1997; Hall et al. 1998; Pedersen et al. 1998).

Consequently, in this study, we have examined the levels of cell death induced by increasing concentrations of hydrogen peroxide (H_2O_2) in HT22 cells transfected with human wild-type SOD1 (wtSOD), mSOD, or empty vector. For each condition, we examined the effect of manipulating (up or down) Shh signaling on the H_2O_2 dose–response curves thus obtained. We studied the mRNA expression levels of different components of the Shh signaling pathway in HT22 cells transfected with wtSOD, mSOD, or empty (GFP) vector, and finally, we measured the corresponding effect in a Gli-responsive reporter assay. Prior to undertaking the above experiments, we established a dose–response range for inhibition of Shh signaling with cyclopamine in untransfected HT22 cells and verified the effect seen with cyclopamine was similar to that seen with siRNA interference of the Shh signaling pathway. We also

established that, in untransfected HT22 cells, the beneficial effect of Shh plateaued over a range of concentrations.

Experimental Procedures

Cell Culture

HT22 cells (a gift from Dr. Robert Cummings, University of Western Ontario) were maintained in Dulbecco's modified Eagle's medium (DMEM; Centre for Gene Therapeutics, McMaster University, Hamilton ON, Canada) containing 10% fetal bovine serum (PAA Laboratories), 100 µg/l penicillin and 100 µg/l streptomycin (Life Technologies), in a humidified cell culture cabinet under 5% CO_2 95% O_2 at 37°C. For transfection assays, 10^4 cells were seeded per well of a 96-well plate in 100 µl of 10% DMEM.

DNA Transfections

pEGFP-N1, pWTSOD1-N1, and pSOD1^{G93A}-N1 (all a gift from Dr. Heather Durham, McGill University) were used to transfect HT22 cells for this study. pEGFP-N1 is a plasmid expressing only EGFP. pWTSOD1-N1 and pSOD1^{G93A}-N1 express human WT SOD1 and human mSOD, respectively, as well as eGFP. Briefly, per 96-well plate, 2.5 µg of plasmid DNA resuspended in double distilled water (ddH$_2$O) was mixed with 10 µl of FUGENE HD (Roche Diagnostics) in 250 µl of OPTIMEM (Life Technologies). Transfection complexes were then allowed to form for 20 min at room temperature, after which the entire volume was added to 4.25 ml of 10% DMEM. Media/DNA complexes (50 µl) were subsequently dispensed into each well. With this protocol, the transfection efficiency was relatively high (empty vector: 80.4%±6.04, wtSOD: 82.9%±6.07, mSOD: 81.5%±4.18, further details can be found in Fig. S1).

microRNA Transfections

miRNA 324-5p was purchased from Life Technologies/ Applied Biosystems and resuspended as per manufacturer's directions. The mature sequence of miR324-5p is CGCAUCC CCUAGGGCAUUGGUGU. Briefly, 2.5 µl of 50 µM stock of miR324-5p (50 nM final concentration) was mixed with 12.5 µl of FUGENE HD (Roche Diagnostics) resulting in a 5:1 ratio of FUGENE HD: miRNA. Expression of the negative control, scrabble miRNA was achieved from the pcDNA 6.2GW/EmGFP-miR-Neg control plasmid (Life Technologies) following the same procedure outline previously. The resulting mature sequence is UUUACAUGACGCACCUCUGCA.

Pharmacological Treatments and Oxidative Challenge

24 h Post-transfection, cells were treated with either recombinant human Shh protein (Leinco), purmorphamine (Stemgent), or cyclopamine (Sigma Aldrich). The recombinant Shh protein encompassed amino acids Cys24-Gly197 of the N-terminal portion with Ile-Ile substituted for Cys24, and was resuspended in pure ddH$_2$O without any carrier protein. Stock solutions of both purmorphamine, and cyclopamine were diluted in pure dimethyl sulfoxide (DMSO). 24 h post-treatment and 48 h post-transfection, cells were subsequently exposed to an H_2O_2 challenge. Cells were exposed to a titre range of H_2O_2 concentrations (0 µM, 300 µM, 400 µM, 500 µM, 750 µM, 900 µM, 1 mM, 1.25 mM in complete media; each concentration tested in triplicate), for a period of 24 h after which viability was measured via MTT assay.

Viability Assay

Viability of surviving cells was assessed by their ability to metabolize 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich) into a dark blue formazan salt (Morgan 1998). After exposure to H_2O_2, 5 µl of 5 mg/ml MTT was added to each well and allowed to incubate for 2 h. After incubation, an equal well volume of DMSO was added to burst the cells. Absorbance (570 nm with 690 nm reference) was measured using a multiwell plate reader (Tecan).

Reverse Transcription and Real-Time Quantitative PCR

Total RNA was isolated from cells 48 h post-transfection, using the Aurum total RNA Mini Kit (BIO-RAD) following the manufacture's protocol. Isolated RNA was subsequently used for quantitative, real-time PCR and relative gene expression levels were analyzed using MxPro3000P software (Stratagene). Further details are available in Fig. S2.

Gli-Responsive Luciferase Reporter Assay

HT22 cells were co-transfected with the various SOD1 plasmids individually, along with a GLI-responsive luciferase construct which encoded the firefly luciferase reporter gene under the control of a minimal (m) Cytomegalovirus (mCMV) promoter and six tandem repeats of the Gli transcriptional response element (SA Biosciences/Qiagen). After 48 h, cells were lysed and luciferase activity was measured using Dual-Glo Luciferase Assay kit (Promega) as outlined in the protocol without deviation.

Data Analysis

Naive HT22 cells and cells transiently transfected with wtSOD1, mSOD1, or empty vector were treated with Shh,

purmorphamine, cyclopamine, or mock. For each experimental condition, exposing cells to a range of increasing concentrations of H_2O_2 resulted in a sigmodial dose–response curve, bracketing the survival range of HT22 cells from no effect on survival to complete lethality. Each curve was then fitted to a sigmodial dose–response curve with variable slope, using the curve fitting algorithms of PRISM (version 4, GraphPad software, San Diego, CA, equivalent to a four parameter logistic equation), which returned the LC_{50} and the standard deviation of the LC_{50}. All measures were performed in triplicate, and each experiment was repeated two times. The cyclopamine dose–response curve (to estimate the optimal inhibitory dose of cyclopamine) was analysed using Dunnett's t-test (comparing all cyclopamine levels against control). For all transient transfection experiments, we performed a two-factor analysis of variance (ANOVA; SPSS). The effect of transfection in untreated cells (Fig. 2) was undertaken with a Tukey's HSD post hoc correction for multiple comparisons. The effect of treatment (Fig. 3) in the three transfection groups was analysed by planned orthogonal contrasts (SPSS). The relative expression level of various Shh pathway components along with luciferase activity, among transfected cells were analysed using a one-way ANOVA with Tukey's HSD correction for multiple comparisons.

Results

Downregulation of the Shh Pathway Increases Susceptibility to H_2O_2 Challenge in Untransfected HT22 Cells

We examined the effect of downregulation of the Shh pathway on sensitivity to oxidative challenge in HT22 cells. We did this in two ways. We used the Shh antagonist cyclopamine, and we knocked down Smo and Gli utilizing a miRNA. Cyclopamine in increasing doses progressively increased the sensitivity of HT22 cells to H_2O_2 challenge and reduced the calculated LC_{50} (Fig. 1a). Of the doses tested, the concentration of cyclopamine which elicited a maximal response was 25 μM, and subsequent experiments using cyclopamine as an antagonist were done at this concentration.

The micro RNA miR324-5p has previously been shown to downregulate the activity of two key members of the Shh pathway, namely, Smo and Gli1 (Ferretti et al. 2008). We transiently transfected miR324-5p into HT22 cells. 24 h Post-transfection, cells were challenged with increasing concentrations of H_2O_2. Cellular viability was markedly decreased in cells which were transfected with miR324-5p but not in cells expressing a scrambled miRNA or mock treatment (Fig. 1b).

In untransfected HT22 cells, pre-treatment with Shh increased survival after H_2O_2 challenge, but further increasing concentrations of Shh did not appreciably increase the LC_{50} (data not shown), suggesting a ceiling effect. We used Shh 1 μg/ml for all subsequent treatments. Similarly, in untransfected HT22 cells, purmorphamine at a dose of 5 μM provided optimal benefit, and further increasing the dose beyond this led to increased cell death. We used purmorphamine at 5 μM in subsequent experiments.

Expression of wtSOD or mSOD Alters HT22 Susceptibility to Free-Radical Challenge

We examined the effect of ectopic transient expression of the various constructs would have on the sensitivity of HT22 cells to oxidative challenge. Compared to empty vector, wtSOD expression significantly increased resistance to H_2O_2 challenge while mSOD had the opposite effect, significantly reducing resistance to H_2O_2 challenge (Fig. 2).
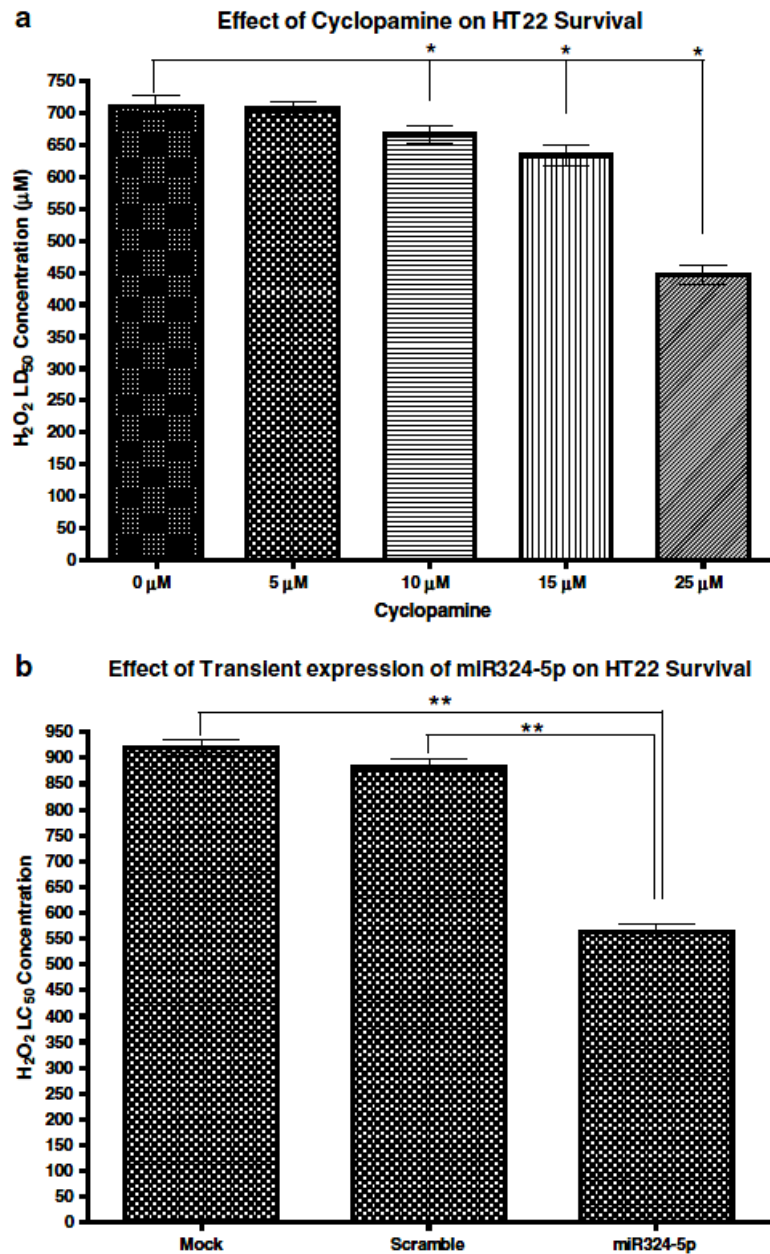
Cyclopamine Reduces Resistance to Oxidative Challenge, While Shh or the Shh Agonist Purmorphamine Increase Resistance to Oxidative Challenge, in Transiently Transfected HT22 Cells

We examined the effect of pharmacological manipulation of the Shh pathway in transiently transfected HT22 cells (Figs. 3a–c, Table S2a and b). Shh showed a significant protective effect in all groups (Fig. 3a). The effect was most pronounced in the mSOD cells, and least pronounced in wtSOD cells. Purmorphamine produced similar results (Fig. 3b). The effect of Shh agonism was greatest in mSOD and empty vector-only cells, and there was no effect in wtSOD cells. Cyclopamine sensitized all transfected cell groups to oxidative challenge (Fig. 3c).

Relative Expression Levels of Shh Pathway Genes in Transiently Transfected HT22 Cells

We examined the relative mRNA levels of various Shh pathway members in transiently transfected HT22 cells. We isolated total RNA and measured relative mRNA levels by qRT-PCR 48 h post-transfection (paralleling the time course of the oxidative challenge experiments; Fig. 4). Differential gene expression levels were observed in three of six key pathway genes. The relative mRNA expression levels of PTCH, Smo, and Gli1 in wtSOD and mSOD did not differ from cells expressing the empty vector. However, wtSOD expressing cells had a significant increase in the relative levels of Shh and Gli2 mRNA, while mSOD expressing cells had no change in Shh or Gli1. mSOD1 expression did result in a decrease in the levels of Gli2 and especially Gli3.

Fig. 1 a Cyclopamine increases the susceptibility of HT22 cells to H_2O_2 challenge. HT22 cells were pre-treated with four concentrations of cyclopamine or vehicle for 24 h, and then challenged with increasing concentrations of H_2O_2 (each in triplicate) to establish an H_2O_2 dose response, determined at 24 h. Values represent the LC_{50} of H_2O_2 (mean $\pm$ SD) calculated from the survival curves, for each concentration of cyclopamine and control. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison post hoc test (*$p<0.01$). **b** miR324-5p increases the susceptibility of HT22 cells to H_2O_2 challenge. HT22 cells were transfected with miR324-5p, scrambled miRNA, or no miRNA (*mock*) and challenged 24 h later with a dose range of H_2O_2 concentrations. Values represent the LC_{50} of H_2O_2 (mean $\pm$ SD) calculated for each condition. Significance was determined by a one-way ANOVA with a Tukey's multiple comparison post hoc test (**$p<0.001$)

a Effect of Cyclopamine on HT22 Survival

b Effect of Transient expression of miR324-5p on HT22 Survival

Effect of wtSOD or mSOD Expression on Gli-Mediated Activation of a Luciferase Reporter

We examined the effect of transient transfection of wtSOD or mSOD on the output from a Gli-responsive light reporter plasmid. The luciferase reporter construct consists of a firefly luciferase reporter gene under the control of an mCMV promoter along with several tandem repeats of the Gli transcriptional response element and is sensitive to overall Gli levels in the cell. 48 h post co-transfection, cells were lysed and resulting luminescence was measured (Fig. 5). Overall, expression of wtSOD significantly increased Gli-directed luciferase activity, confirming the qPCR Shh and Gli mRNA results. In contradistinction, expression of mSOD reduced Gli-mediated transcriptional activation of the luciferase reporter when compared with empty vector alone and even more so when compared with wtSOD.

Fig. 2 Expression of wtSOD increases resistance of HT22 cells to H_2O_2 challenge, and expression of mSOD increases susceptibility to H_2O_2 challenge. HT22 cells were transfected with plasmids expressing empty vector, wtSOD, or mSOD, and the cells were challenged with increasing concentrations of H_2O_2 24 h later to establish a dose–response curve. Values represent the LC_{50} of H_2O_2 (mean ± SD) calculated for each condition. Significance was determined by a one-way ANOVA with a Tukey's multiple comparison post hoc test ($*p<0.01$, $**p<0.001$)

Effect of Transient expression of SOD1 constructs on HT22 Survival
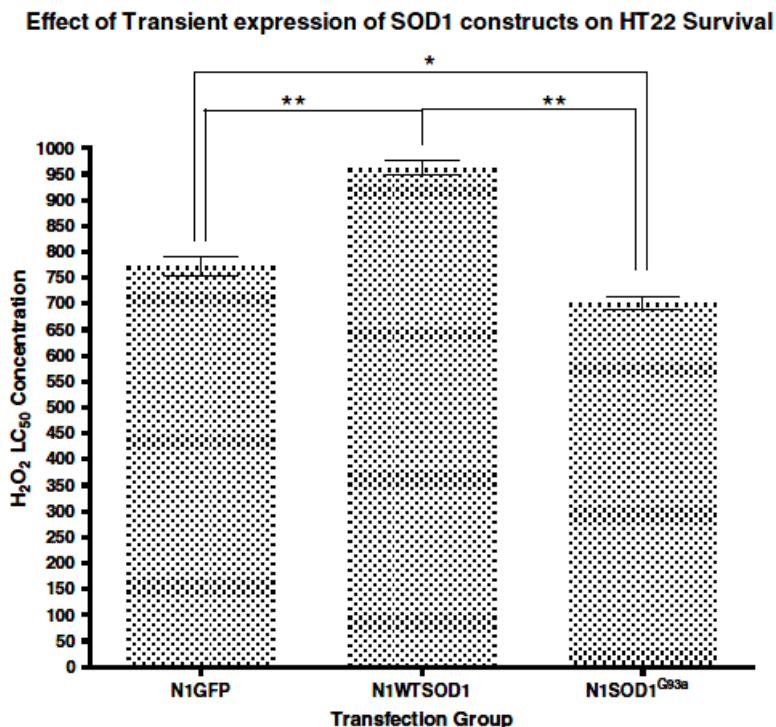
Discussion

It has long been recognized that Shh plays an essential role in the development of the nervous system, but the realization that it might also be a factor in the senescence and degeneration of mature neurons is more recent. However, Shh is cytoprotective against neurotoxic challenge in several neurodegenerative disease models, as set out in the "Introduction." Stenkamp et al. (2008) made the important observation that Shh hemizygote zebrafish showed retinal cone loss only in late adult life, thereby linking reduced levels of a developmental morphogen with late onset neurodegeneration. Most Shh signaling is processed through the primary cilium, and we have recently shown that spinal motor neurons in mSOD mice express primary cilia less frequently than control motor neurons. It is on this background that we decided to examine the role of Shh signaling in a cell culture model of ALS.

We first demonstrated that Shh modulates H_2O_2 toxicity in untransfected HT22 cells. Antagonism of the Shh pathway with cyclopamine (Fig. 1a) and with miR3245p (Fig. 1b) increased susceptibility to H_2O_2 challenge. Cyclopamine is a natural compound that binds directly to Smo and inhibits downstream Shh signaling (Stanton and Peng 2010), while miR3245p decreases Smo and Gli1 mRNA and protein levels (Ferretti et al. 2008). The improvement in cell survival with Shh and with a Shh agonist, and perhaps more importantly, the exacerbation of

cell death following impairment of Shh signaling, by two different methods, argues that the Shh pathway remains operative in these mature cells, and serves to enhance cell survival in the face of oxidative challenge. Increasing levels of cyclopamine led to increased cell death, while the protective effect of Shh and purmorphamine seemed to plateau with increasing levels, demonstrating a ceiling effect to Shh neuroprotection against oxidative challenge.

We transfected HT22 cells with empty vector, wtSOD, or mSOD constructs and challenged the transfected cells with H_2O_2. Pre-treatment with cyclopamine resulted in reduced cell survival in all three transfection groups, but pre-treatment with Shh or purmorphamine increased cell survival mainly in empty vector and mSOD groups. We sought an explanation for these results by measuring components of the Shh signaling pathway. We measured the relative effect of wtSOD or mSOD transfection on mRNA levels within the Shh pathway, and we measured the downstream effect of Gli function using a Gli-responsive light reporter construct. [We did examine Gli protein levels via Western immunoblotting, but multiple bands were seen, due to alternative splicing of Gli transcript, phosphorylation, and partial proteolysis. Gli1 has two alternatively spliced isoforms and Gli2 has four (Cohen 2010). It was not clear which bands retain bioactivity, and as such we substituted a functional assay of Gli activity.

Compared to cells expressing GFP only, cells expressing wtSOD had an increase in Shh mRNA. The reason for this
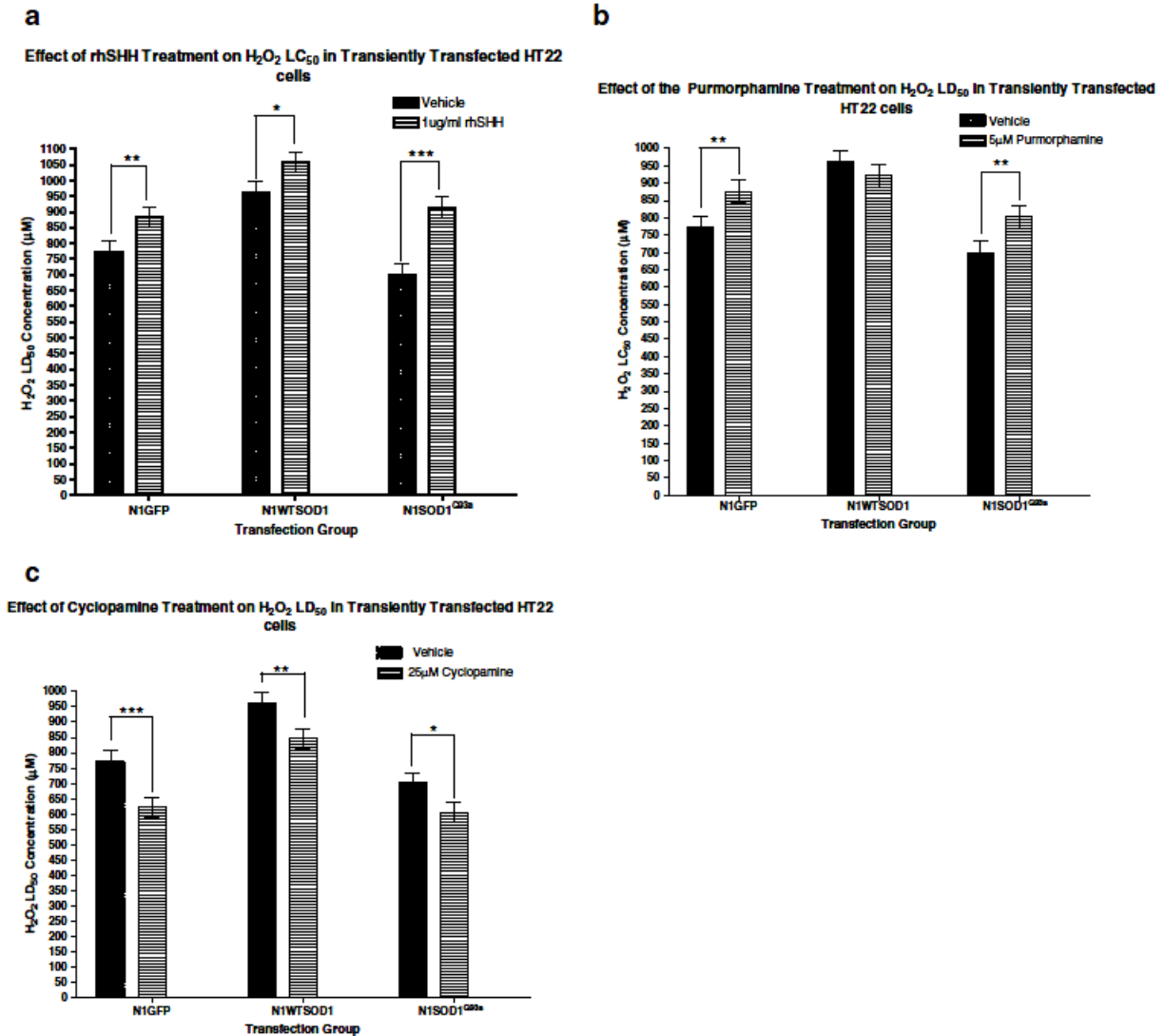
a

Effect of rhSHH Treatment on H$_2$O$_2$ LC$_{50}$ in Transiently Transfected HT22 cells

b

Effect of the Purmorphamine Treatment on H$_2$O$_2$ LD$_{50}$ in Transiently Transfected HT22 cells

c

Effect of Cyclopamine Treatment on H$_2$O$_2$ LD$_{50}$ in Transiently Transfected HT22 cells

Fig. 3 **a** Shh increases resistance of transiently transfected HT22 to H$_2$O$_2$ challenge. 24 h post-transfection cells were treated with 1ug/ml recombinant Shh for an additional 24 h prior to challenge with a range of H$_2$O$_2$ concentrations. Values represent the LC$_{50}$ of H$_2$O$_2$ (mean ± SD) calculated for each condition. Significance was determined by a two-way ANOVA with a planned orthogonal contrast post hoc test (*$p<$ 0.05, **$p<$0.01, ***$p<$0.001). **b** Purmorphamine increases resistance of transiently transfected HT22 to H$_2$O$_2$ challenge. 24 h post-transfection cells were treated with 5 μM Shh agonist purmorphamine for an additional 24 h to challenge with a range of H$_2$O$_2$ concentrations.

Values represent the LC$_{50}$ of H$_2$O$_2$ (mean ± SD) calculated for each condition. Significance was determined by a two-way ANOVA with a planned orthogonal contrast post hoc test (*$p<$0.05, **$p<$0.01, ***$p<$ 0.001). **c** Cyclopamine pre-treatment sensitizes transiently transfected HT22 to H$_2$O$_2$ challenge. 24 h post-transfection cells were treated with 25 μM of the Shh antagonist cyclopamine for an additional 24 h prior to challenge with a range of H$_2$O$_2$ concentrations. Values represent the LC$_{50}$ of H$_2$O$_2$ (mean ± SD) calculated for each condition. Significance was determined by a two-way ANOVA with a planned orthogonal contrast post hoc test. (*$p<$0.05, **$p<$0.01, ***$p<$0.001)

increase is unknown; however it was accompanied by an increase in the transcription of downstream Gli members as expected, especially Gli2, and an increase in activity in the Gli-responsive light reporter assay. Expression of wtSOD increased the resistance of HT22 cells to H$_2$O$_2$ challenge. This in itself is not unexpected as SOD1 is a major cellular

defence against oxidative toxicity (Lee et al. 2001). It was less expected that wtSOD would increase background levels of Shh, which would furnish another explanation for the relative resistance of wtSOD transfected cells.

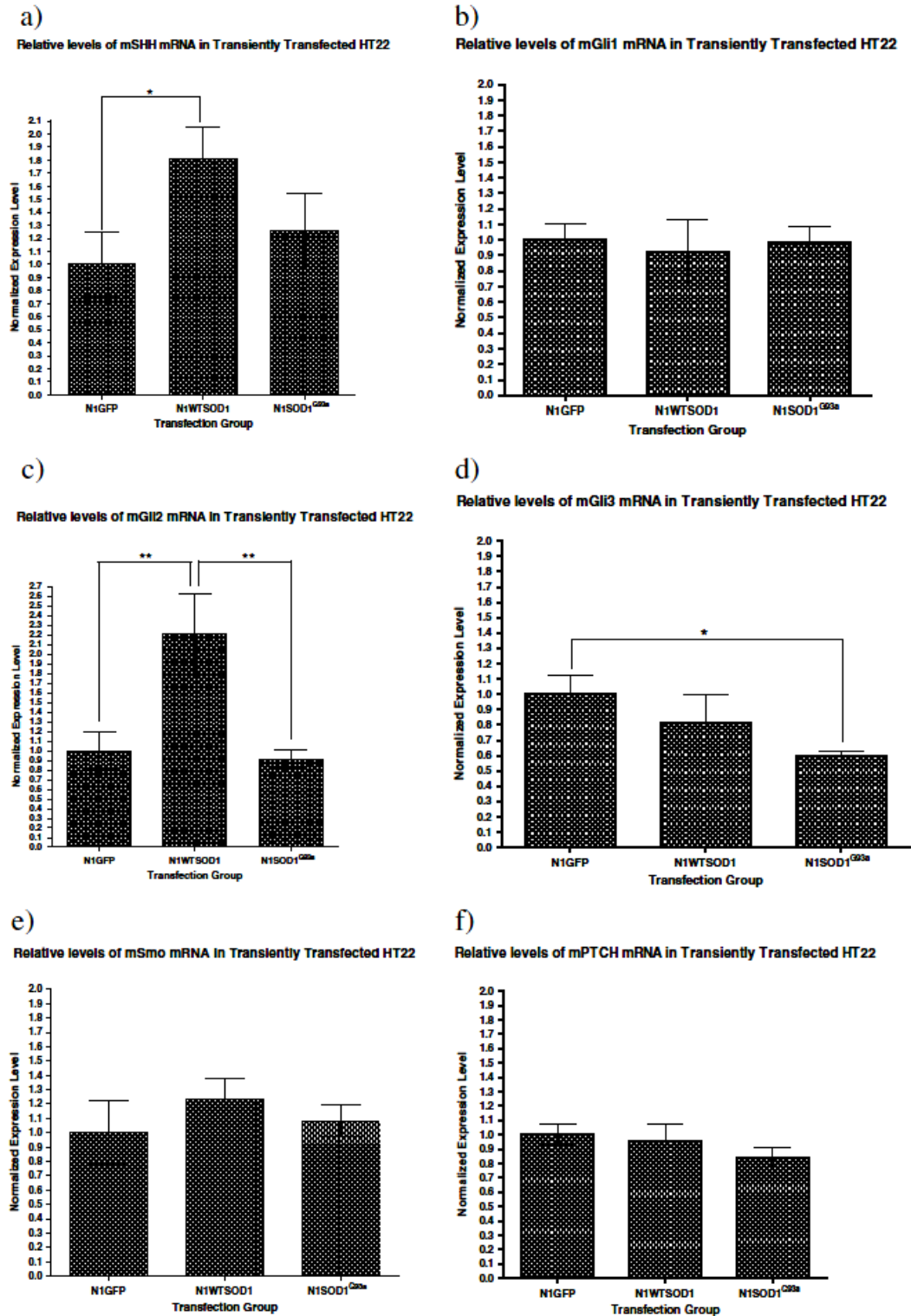Compared to cells expressing GFP only, cells transfected with mSOD showed no change in Shh message, but

a)

Relative levels of mSHH mRNA in Transiently Transfected HT22

b)

Relative levels of mGli1 mRNA in Transiently Transfected HT22

c)

Relative levels of mGli2 mRNA in Transiently Transfected HT22

d)

Relative levels of mGli3 mRNA in Transiently Transfected HT22

e)

Relative levels of mSmo mRNA in Transiently Transfected HT22

f)

Relative levels of mPTCH mRNA in Transiently Transfected HT22

Fig. 4 a–f Differential mRNA expression of Shh pathway genes in transiently transfected HT22 cells. 48 h. post-transfection total RNA was collected and relative mRNA levels was determined by qRT-PCR.

Values are expressed as mean ± SD. Significance was determined by a one-way ANOVA with a Tukey's multiple comparison post-hoc test ($*p<0.05$, $**p<0.01$)

Gli Mediated Luciferase Activity in Transfected HT22 cells
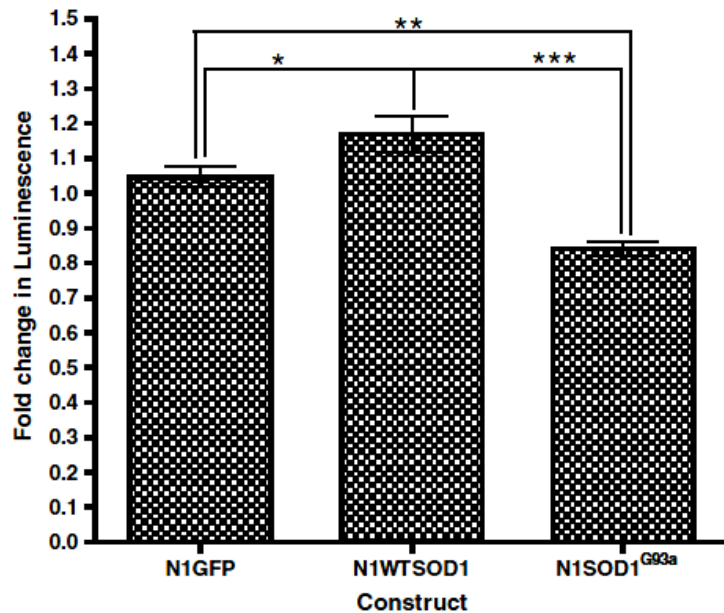
downstream Gli member transcripts were decreased, espe-
cially Gli3, and the output of the Gli-responsive reporter
assay was decreased. A paradoxical decrease in down-
stream Gli members in light of a rise in Shh has been
reported in multiple sclerosis, and attributed to a down-
regulating effect of IFNγ acting in a non-canonical fashion
on Gli transcription (Wang et al. 2008). Glial activation and
inflammation is a universal accompaniment in complex
ALS models, but would not be seen in pure HT22 cell
culture, and additional mechanisms may be operative.

One might anticipate that the effect of increasing Shh
might be less pronounced when starting Shh levels are
higher (e.g., wtSOD cells) and more pronounced when the
starting levels are lower (e.g., mSOD cells), and this
appears to be true. Shh and purmorphamine exerted their
greatest effect in mSOD cells, and had little or no effect in
wtSOD cells, where starting Shh and Gli levels are
increased. This translates into the significant interaction
term in the two-factor ANOVA (Table S2b, $F=4.65$; $p=0.01$).
Since neither the overexpression of mutant SOD1 nor the
downregulation of the Shh pathway with cyclopamine
appreciably alter the growth characteristics or the viability
of HT22 cells in the unchallenged state (Fig. S2), the Shh
pathway in mature cells may play a significant role in the
resistance to exogenous challenge, especially in cells with
pre-existing compromise.

Although Shh levels are unstudied in human motor
neuron disease, there are reasons to hypothesize that Shh
signaling could be impaired, either through a decline in
ambient Shh levels or a decline in the cellular responses to
existing Shh levels. In the adult, Shh continues to play a
role in the differentiation and survival of resident neural
stem cells (Palma et al. 2005; Cai et al. 2008), but this
activity is regionally restricted and depends on Shh release by
astrocytes in those brain areas supporting neurogenesis — the
subventricular zone and the hippocampal subgranular zone —
and transplantation of astrocytes from these areas enables
neurogenesis in other brain areas (Jiao and Chen 2008). This
suggests that Shh release from local astrocytes is important,
with regional variability. Even in health, astrocytes in the
anterior horn may release lower levels of Shh than those in
the subventricular or subgranular zones, and it is well
established that astrocytes in the anterior horn are affected
in ALS (Schiffer and Fiano 2004), which could further
depress Shh levels. Astrocytes are abnormal when examined
at necropsy, and restricted expression of mSOD in motor
neurons is insufficient to cause motor neuron disease in
mice; rather, there is a requirement for glial involvement,
both astroglial and microglial (Mir et al. 2009). With respect
to Shh signaling, impairment of astrocyte function could
directly reduce Shh levels, and microglial activation could
raise IFNγ levels, further reducing Gli effect, as above.

Diseased motor neurons may become less responsive to
Shh signals. Most Shh signaling involves the primary
cilium, and ciliation is greatly reduced in surviving spinal
motor neurons in mSOD mice (Ma et al. 2011). Also, as
cellular differentiation proceeds, changes occur in the
methylation state of Gli promoters, and in histone modifi-

cation, that restrict the ability of cells to respond to fluctuations in Gli signals (Cheng and Bishop 2002; Chari and McDonnell 2007). It is possible but unproven that these processes are also perturbed in ALS.

HT22 cells have been widely used to model many neurodegenerative diseases and have several advantages. They lend themselves readily to the study of oxidative stress and free radical challenge as in our experiments, and oxidative stress is felt to be a critical element in the neuropathological cascade in ALS (Barber and Shaw 2010), seen in fALS and sALS and in all animal models of the disease. However, glutamate excitotoxicity is another important contributor to the pathology of ALS, but here HT22 cells are a less suitable model, as they do not express glutamate receptors (Maher and Davis 1996). From one viewpoint, this is advantageous, as it permits the study of oxidative and free radical challenge independent of excitotoxicity.

However, future studies could examine the effect of Shh in mitigating excitotoxic glutamate challenge in motor neuron cells lines expressing glutamate receptors (such as primary motor neuron cultures or NSC34 cells).

As well, at least some actions of Shh require retinoic acid (RA). In a peripheral nerve crush model, Shh is induced in the DRG of wildtype mice but not RA receptor (RAR) null knockouts, and in motor neurons, Shh is insufficient to induce neurite outgrowth alone and requires RA (So et al. 2006). RA, by itself, is neuroprotective in a rat cerebral ischemia model, an effect possibly related to upregulation of neurogranin (Li, et al 2008). In the differentiation of neural-competent mesenchymal stem cells, RA alone and Shh alone have little effect, but together are effective (Kondo et al 2005). Future experiments could examine the combined effect of RA and Shh in reducing oxidative or excitotoxic damage as above, in addition to any beneficial role they might play in the stimulation and differentiation of resident stem cells in situ.

To summarize our key findings, reducing Shh signaling with cyclopamine or miR324-5p increases cell death in HT22 cells exposed to oxidative challenge, while Shh or the Shh agonist purmorphamine reduces cell death. Downstream Gli signaling is reduced in mSOD-transfected HT22 cells, and cell death is reduced in these cells when pre-treated with Shh or purmorphamine. Further consideration of the therapeutic potential of Shh agonists, alone or in combination with other synergistic factors, is warranted.

References

ALS Online Genetic Database. http://alsod.iop.kcl.ac.uk/

Barber SC, Shaw PJ (2010) Oxidative stress in ALS: key role in motor neuron injury and therapeutic target. Free Radic Biol Med 48:629–641

Bond C, Tang Y, Podlasek CA (2008) Neural influences on sonic hedgehog and apoptosis in the rat penis. Biol Reprod 78:947–956

Briscoe J (2009) Making a grade: sonic hedgehog signalling and the control of neural cell fate. EMBO J 28:457–465

Cai C, Thorne J, Grabel L (2008) Hedgehog serves as a mitogen and survival factor during embryonic stem cell neurogenesis. Stem Cells (Dayton, Ohio) 26:1097–1108

Calcutt NA, Allendoerfer KL, Mizisin AP, Middlemas A, Freshwater JD, Burgers M, Ranciato R, Delcroix JD, Taylor FR, Shapiro R, Strauch K, Dudek H, Engber TM, Galdes A, Rubin LL, Tomlinson DR (2003) Therapeutic efficacy of sonic hedgehog protein in experimental diabetic neuropathy. J Clin Invest 111:507–514

Chari NS, McDonnell TJ (2007) The sonic hedgehog signaling network in development and neoplasia. Adv Anat Pathol 14:344–352

Cheng SY, Bishop JM (2002) Suppressor of Fused represses Gli-mediated transcription by recruiting the SAP18-mSin3 corepressor complex. Proc Natl Acad Sci USA 99:5442–5447

Cohen MM Jr (2010) Sonic hedgehog signaling update. Am J Med Genet A 152A(8):1875–1914

Dass B, Iravani MM, Huang C, Barsoum J, Engber TM, Galdes A, Jenner P (2005) Sonic hedgehog delivered by an adeno-associated virus protects dopaminergic neurones against 6-OHDA toxicity in the rat. J Neural Transm 112:763–778

Dellovade T, Romer JT, Curran T, Rubin LL (2006) The hedgehog pathway and neurological disorders. Ann Rev Neurosci 29:539–563

Farzan SF, Singh S, Schilling NS, Robbins DJ (2008) The adventures of sonichedgehog in development and repair: III. Hedgehog processing and biological activity. Am J Physiol 294:G844–G849

Ferrante RJ, Shinobu LA, Schulz JB, Matthews RT, Thomas CE, Kowall NW, Gurney ME, Beal MF (1997) Increased 3-nitrotyrosine and oxidative damage in mice with a human copper/zinc superoxide dismutase mutation. Ann Neurol 42:326–334

Ferretti E, De Smaele E, Miele E, Laneve P, Po A, Pelloni M, Paganelli A, Di Marcotullio L, Caffarelli E, Screpanti I, Bozzoni I, Gulino A (2008) Concerted microRNA control of Hedgehog signalling in cerebellar neuronal progenitor and tumour cells. EMBO J 27:2616–2627

Hall ED, Andrus PK, Oostveen JA, Fleck TJ, Gurney ME (1998) Relationship ofoxygen radical-induced lipid peroxidative damage to disease onset and progression in a transgenic model of familial ALS. J Neurosci Res 53:66–77

Humke EW, Dorn KV, Milenkovic L, Scott MP, Rohatgi R (2010) The output of Hedgehog signaling is controlled by the dynamic association between suppressor of fused and the Gli proteins. Genes Dev 24:670–682

Hurtado-Lorenzo A, Millan E, Gonzalez-Nicolini V, Suwelack D, Castro MG, Lowenstein PR (2004) Differentiation and transcription factor gene therapy in experimental Parkinson's disease: sonic hedgehog and Gli-1, but not Nurr-1, protect nigrostriatal cell bodies from 6-OHDA-induced neurodegeneration. Mol Ther 10:507–524

Jiao J, Chen DF (2008) Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. Stem cells (Dayton, Ohio) 26:1221–1230

Kondo T, Johnson SA, Yoder MC, Romand R, Hashino E (2005) Sonic hedgehog and retinoic acid synergistically promote sensory fate specification from bone marrow-derived pluripotent stem cells. Proc Natl Acad Sci USA 102:4789–4794

Lanius RA, Krieger C, Wagey R, Shaw CA (1993) Increased [35S]glutathione binding sites in spinal cords from patients with sporadic amyotrophic lateral sclerosis. Neurosci Lett 163:89–92

Lauth M, Toftgard R (2007) Non-canonical activation of GLI transcription factors: implications for targeted anti-cancer therapy. Cell Cycle (Georgetown, TX) 6:2458–2463

Lee M, Hyun DH, Halliwell B, Jenner P (2001) Effect of over-expression of wild-type and mutant Cu/Zn-superoxide dismutases on oxidative stress and cell death induced by hydrogen peroxide, 4-hydroxynonenal or serum deprivation: potentiation of injury by ALS-related mutant superoxide dismutases and protection by Bcl-2. J Neurochem 78:209–220

Li L, Li Y, Ji X, Zhang B, Wei H, Luo Y (2008) The effects of retinoic acid on the expression of neurogranin after experimental cerebral ischemia. Brain Res 1226:234–240

Ma X, Peterson R, Turnbull J (2011) Adenylyl cyclase type 3, a marker of primary cilia, is reduced in primary cell culture and in lumbar spinal cord in situ in G93ASOD1 mice. BMC Neurosci 12(1):71

Maher P, Davis JB (1996) The role of monoamine metabolism in oxidative glutamate toxicity. J Neurosci 16:6394–6401

Miao N, Wang M, Ott JA, D'Alessandro JS, Woolf TM, Bumcrot DA, Mahanthappa NK, Pang K (1997) Sonic hedgehog promotes survival of specific CNS neuron populations and protects these cells from Toxic insults in vitro. J Neurosci 17(15):5891–5899

Mir M, Asensio VJ, Tolosa L, Gou-Fabregas M, Soler RM, Llado J, Olmos G (2009) Tumor necrosis factor alpha and interferon gamma cooperatively induce oxidative stress and motoneuron death in rat spinal cord embryonic explants. Neuroscience 162:959–971

Morgan DM (1998) Tetrazolium (MTT) assay for cellular viability and activity. Methods Mol Biol (Clifton, NJ) 79:179–183

Palma V, Lim DA, Dahmane N, Sanchez P, Brionne TC, Herzberg CD, Gitton Y, Carleton A, Alvarez-Buylla A, Ruiz i Altaba A (2005) Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. Development (Cambridge, England) 132:335–344

Pasinelli P, Brown RH (2006) Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nature Rev 7:710–723

Pedersen WA, Fu W, Keller JN, Markesbery WR, Appel S, Smith RG, Kasarskis E, Mattson MP (1998) Protein modification by the lipid peroxidation product 4-hydroxynonenal in the spinal cords of amyotrophic lateral sclerosis patients. Ann Neurol 44:819–824

Rosen DR (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362 (6415):59–62

Rothstein JD (2009) Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. Ann Neurol 65(Suppl 1):S3–S9

Schiffer D, Fiano V (2004) Astrogliosis in ALS: possible interpreta-tions according to pathogenetic hypotheses. Amyotroph Lateral Scler Other Motor Neuron Disord 5:22–25

Shaw IC, Fitzmaurice PS, Mitchell JD, Lynch PG (1995) Studies on cellular free radical protection mechanisms in the anterior horn from patients with amyotrophic lateral sclerosis. Neurodegeneration 4:391–396

So PL, Yip PK, Bunting S, Wong LF, Mazarakis ND, Hall S, McMahon S, Maden M, Corcoran JP (2006) Interactions between retinoic acid, nerve growth factor and sonic hedgehog signalling pathways in neurite outgrowth. Dev Biol 298:167–175

Stanton BZ, Peng LF (2010) Small-molecule modulators of the sonic hedgehog signaling pathway. Mol Biosyst 6:44–54

Stecca B, Ruiz i Altaba A (2002) The therapeutic potential of modulators of the hedgehog–Gli signaling pathway. J Biol 1:9

Stenkamp DL, Satterfield R, Muhunthan K, Sherpa T, Vihtelic TS, Cameron DA (2008) Age-related cone abnormalities in zebrafish with genetic lesions in sonic hedgehog. Invest Ophthalmol Vis Sci 49:4631–4640

Tsuboi K, Shults CW (2002) Intrastriatal injection of sonic hedgehog reduces behavioral impairment in a rat model of Parkinson's disease. Exp Neurol 173:95–104

Wang Y, Imitola J, Rasmussen S, O'Connor KC, Khoury SJ (2008) Paradoxical dysregulation of the neural stem cell pathway sonic hedgehog–Gli1 in autoimmune encephalomyelitis and multiple sclerosis. Ann Neurol 64:417–427

------CHAPTER 3------

Adenylyl Cyclase type 3, a marker of primary cilia, is reduced in primary cell culture and in lumbar spinal cord in situ in G93A SOD1 mice

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This manuscript represented the first study to survey for the appearance of the primary cilia in the spinal cord of SOD1^{G93A} mice. Specifically we identified the presence of Adenylyl Cyclase 3 (ACIII) positive cilia on spinal cord motor neurons, both in primary culture and *in situ* from histological sections of the lumber spine. Our findings indicate that there is a decrease in the relative proportion of ciliated motor neurons between wild-type and SOD1^{G93A} mice and that this decline in ciliated motor neurons increases with disease progression.

Adenylyl Cyclase type 3, a marker of primary cilia, is reduced in primary cell culture and in lumbar spinal cord in situ in G93A SOD1 mice

Xiaoxing Ma, Randy Peterson and John Turnbull[*]

Abstract

Background: The primary cilium is a solitary organelle important in cellular signaling, that projects from the cell surface of most growth-arrested or post-mitotic cells including neurons in the central nervous system. We hypothesized that primary cilial dysfunction might play a role in the pathogenesis of Amyotrophic Lateral Sclerosis (ALS), and as a first step, report on the prevalence of primary cilial markers on cultured motor neurons from the lumbar spinal cord of embryonic wildtype (WT) and transgenic G93A SOD1 mice, and on motor neurons in situ in the lumbar spinal cord.

Results: At 7 days in culture there is no difference in the proportion of G93A SOD1 and WT motor neurons staining for the cilial marker ACIII. However, at 21 days there is a large relative drop in the proportion of ciliated G93A SOD1 motor neurons. In situ, at 40 days there was a slight relative drop in the proportion of ciliated motor neurons in G93A SOD1 mice. At 98 days of age there was no change in motor neuron ciliation in WT mice, but there was motor neuron loss and a large reduction in the proportion of surviving motor neurons bearing a primary cilium in G93A SOD1 mice.

Conclusions: In primary culture and in situ in G93A SOD1 mice there is a large reduction in the proportion of motor neurons bearing a primary cilium.

Background

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterized chiefly by progressive and ultimately fatal weakness of voluntary muscle, and is presently defined on clinical grounds. Most cases are without known cause. Some cases are familial (FALS) and of these, a minority are associated with known mutation, in genes encoding SOD1 [1], Alsin [2], Dynactin 1 [3], VAPB [4], angiogenin [5], TDP43 [6,7], or FUS [8,9]. Mutation in genes more commonly associated with other diseases can rarely present as ALS (eg. Kennedy's disease [10], Spastin [11]), as can viral infection (eg. HIV [12] and HTLV [13]). In no case do we fully understand how known mutations lead to ALS, and it is presently unclear how multiple known and unknown triggers can lead to a similar disease phenotype. Several pathological mechanisms may be in common, including impaired axonal transport and reduced trophic support, excitotoxicity, oxidative stress, mitochondrial dysfunction, inflammation, accelerated ageing, errors in RNA processing, and terminally, apoptosis [14]. However, no intervention targeting any of these processes singly has proven successful in substantially mitigating the disease process. It is possible that ALS is complex and will require a multifaceted approach for successful treatment. Alternatively, it is possible that one or several presently unknown causes may lead to a more economical understanding of the disease pathophysiology, and open new avenues for treatment.

The most common model of ALS in present use is the G93A SOD1 mouse, a transgenic model resulting from significant over-expression of a mutant human Cu/Zn superoxide dismutase (SOD1) associated with familial ALS. Overexpression of G93A SOD1 in mice causes a progressive hind limb paralysis, which resembles human ALS in clinical and pathological features [15].

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The primary (solitary) cilium is a single microtubule-based organelle that projects from the surface of nearly all post-mitotic or growth-arrested cells and acts as a cellular signaling antenna. Many receptors and ion channels are expressed on the membrane of primary cilia, with expression profiles that differ between organisms and between cell types within an organism. Modified primary cilia on specialized cells transduce the sensory inputs of vision, taste and smell. Intercellular signaling pathways involving the primary cilium include sonic hedgehog (Shh), Wnt, and platelet-derived growth factor (PDGF) among others [16]. These signaling pathways are important in development, and more recently, they have been implicated in cellular homeostasis and resistance to exogenous challenge [17-20]. Previous studies [21] have demonstrated primary cilia on different types of neurons in several species, including granule cells in rat dentate gyrus, major neuron types in rat and guinea pig cerebellum, the hamster paraventricular hypothalamic nucleus, and human neocortex. Immunohistochemical studies using the cilial marker adenylyl cyclase 3 (ACIII) have demonstrated primary cilia on neurons in the mouse hippocampus, cerebral cortex, forebrain, and in the brainstem [22]. The role of primary cilia on spinal motor neurons is less studied.

Since several important signaling pathways converge on the primary cilium, and since some of these pathways are neuroprotective, we questioned whether dysfunction of the primary cilium on spinal motor neurons might be associated with ALS. Accordingly, we used ACIII staining in G93A SOD1 mice to examine the expression of primary cilia on motor neurons in dissociated primary cell culture and in the lumbar spinal cord in situ at two different ages. To date, there has been little work on the expression of primary cilia on motor neurons in the spinal cord even of normal mice, and we are unaware of any studies of primary cilia in hmSOD1 mice. We show here that nearly all motor neurons in the spinal cord of adult wildtype (WT) mice stain avidly for ACIII but motor neurons from embryonic G93A SOD1 mice in primary cell culture, and lumbar motor neurons in situ in G93A SOD1 mice, show greatly reduced expression of ACIII. In the discussion we speculate how absence or dysfunction of the primary cilium might contribute to diseases of the motor neuron.

Methods

Mixed primary murine spinal cord cultures

Primary motor neuron cultures were grown from embryonic mice at 14 days gestation, as previously described [23]. Eight to 12 embryos were used per pregnant dam. Briefly, spinal cords were dissected from E14 embryos and were processed individually. A segment of the body was kept for genotyping. Lumbar spinal cord was cut into small pieces and dissociated in 1% trypsin (Sigma) for 15 minutes. After trypsinization an equal volume of trypsin inhibitor (Sigma) was added and the mixture was lightly triturated until a single cell suspension was achieved. The cell suspension was then transferred to Neurobasal medium containing 1% GLUTAMAX (Invitrogen) and centrifuged at 400 g for 5 minutes without brake. The supernatant was discarded and the cell pellet resuspended in complete Neurobasal medium containing 1% GLUTAMAX, 3% horse serum, 1X B-27 supplement (all from Invitrogen), 5 ng/ml ciliary neurotrophic factor (CNTF) and 5 ng/ml brain-derived neurotrophic factor (BDNF) (Leinco). 5×10^4 cells per chamber were plated on poly-D-lysine (Sigma) coated 8-chamber slides (Labtek) and grown in a 37°C incubator in 5% CO_2 environment. Half of the culture volume was replaced every third day.

Immunofluorescent staining of primary cells in culture

At the indicated time points cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 10 minutes, after which cells were washed three times with PBS. Cells were permeabilized with methanol for 10 minutes at -20°C. Fixed permeabilized cells were blocked with 5% goat serum (Invitrogen)/0.3% Triton X-100 (Sigma) in PBS for 1 hour at room temperature. Cells were subsequently hybridized to rabbit anti-ACIII (1:1000, Santa Cruz Biotechnology) and mouse anti SMI 32 (1:1000, Abcam) in 1% goat serum/0.3% Triton X-100 overnight at 4°C. Secondary antibodies used were goat anti-rabbit IgG Alexa Fluor 488 (1:1000, Invitrogen) and goat anti-mouse IgG Alexa Fluor 568 (1:1000, Invitrogen). Secondary antibodies were hybridized in 1% goat serum/0.3% Triton X-100 in PBS at room temperature for 1 hour. Slides were then allowed to air dry in the dark, and then ProLong anti-fade with DAPI (Invitrogen) was added to the slides prior to the addition of the coverslip. Slides were allowed to cure prior to sealing with nail polish.

Animals and experimental design for in situ analysis of primary cilia

Two age groups, 40 day old and 98 day old transgenic G93A SOD1 mice (B6SJL-TgN[SOD1-G93A]1Gur) were used in the present study. For the 40 day old study, 35 day old animals, WT (littermates, n = 6; 3 males, 3 females) and G93A SOD1 transgenics (n = 5; 3 males, 2 females) were purchased directly from Jackson Laboratory (Bar Harbor, ME), and acclimatized for 5 days before sacrifice. Ninety eight day old animals were taken from a breeding colony established from breeding pairs of male transgenic G93A SOD1 mice bred with female WT B6SJL mice (JAX). Animals, WT (littermates, n = 5;

2 males, 3 females) and G93A SOD1 transgenics (n = 6; 3 males, 3 females) were housed 5 per cage with a 12-h light/dark cycle. All mice were fed standard murine chow and water ad libitum, and food intake was recorded weekly for each cage. Experimental protocols were approved by the McMaster University Animal Research Ethics Board and were carried out in accordance with guidelines of the National Institutes of Health and the Canadian Council on Animal Care.

Genotyping

DNA was isolated from embryos or from tail snip using a Qiagen blood and tissue preparation kit (Qiagen). 250 ng of total DNA was used to determine the presence or absence of the hmG93A SOD1 transgene using primers and conditions outlined on the JAX website.

Tissue preparation

Mice were anesthetized with isoflurane inhalation and perfused transcardially with 50 ml of phosphate buffered saline (PBS), followed by 50 ml of 4% PFA. The spinal column was removed and fixed at 4°C overnight, then transferred into a 30% sucrose solution until saturated. Next, lumbar spinal cords were carefully removed using a dissection microscope, frozen, embedded in optimal cutting temperature (OCT) solution, and stored at -80°C until sectioning. A cryostat was used to cut transverse sections, 40 µm, throughout the entire L3 segment of the spinal cord. The L3 segment was identified using the coordinates of Watson et al [24]. Transverse sections were kept at -20°C in a cryoprotectant containing 25% glycerin, 25% ethylene glycol, and 0.05 M phosphate buffer. We examined every sixth section for immunohistochemistry.

Optimization for double labeling of motor neurons and primary cilia

Commonly used markers to identify motor neurons include anti-Choline acetyltransferase (anti-ChAT) and the 200 kD neurofilament marker SMI 32. In a pilot study, we found that ChAT staining was indistinct in the anterior horns of 98 day old G93A SOD1 mice, and while SMI32 antibody strongly labels neuronal cytoplasmic processes, it is not well suited to counterstaining for a somatic structure like the primary cilium. To circumvent similar problems, previous studies have used a combination of a neuronal cell body stain (eg Nissl) and the size of neurons (>20 µm in diameter) to identify motor neurons [25]. Adopting a similar approach, we used the somatic and nuclear neuronal marker (NeuN) [26] in combination with cell size >20 µm, to identify motor neurons in the ventral horn of the spinal cord. We confirmed that all NeuN positive cells in the ventral horn larger than 20 µm stained for ChAT (Additional file 1).

In a second pilot experiment, we examined the suitability of available primary cilia markers. Some of these did not reliably stain motor neuronal primary cilia, and others, such as acetylated alpha tubulin, stained so diffusely that identification and quantification of primary cilia would have been impossible. Antibodies to both ACIII and melanin-concentrating hormone receptor 1 (MCH1R) stained motor neuronal primary cilia with little background or cytosolic staining, such that reliable counts were possible, and of these ACIII gave the more robust staining. We thus examined primary cell cultures derived from the lumbar spinal cord of embryonic G93A SOD1 and WT mice at 2 time points early and late in culture, as well as the lumbar spinal cord of WT and G93A SOD1 mice before and after the development of signs of paralysis, for the prevalence of ACIII as a marker of ciliated motor neurons. Based on previous observations [27], G93A SOD1 mice at 40 days showed no clinical signs of disease and did not show motor neuron loss in the spinal cord. However, G93A SOD1 mice at 98 days showed clinical signs and obvious motor neuron loss, most particularly in the third lumbar spinal cord segment (L3). This segment corresponds to the spinal innervation of the quadriceps and adductor muscles and is easily identified [28]; as such, this spinal level and these two age groups were chosen to examine the effect of hmSOD1 on cilial expression.

Immunofluorescent staining

A one-in-six series of sections throughout the entire lumbar L3 segment cut at 40 µm was examined. This resulted in about 5-7 sections examined for the L3 region for each animal. Immunofluorescent double labeling for ACIII and NeuN was done on free floating sections as previously described [29]. Briefly, after rinsing in PBS and blocking with 5% normal goat serum (Vector Laboratories), sections were incubated overnight at 4°C in a cocktail of rabbit polyclonal anti-ACIII (1:200, Santa Cruz Biotechnology, Santa Cruz, USA) and mouse monoclonal anti-NeuN (1:500, Chemicon, Temecula, USA). Next day, sections were rinsed in PBS and incubated for 4 h at 4°C in a cocktail of Alexa Fluor 488 goat anti-rabbit antibody (1:500, Molecular Probes, Carlsbad, USA) and Alexa Fluor 568 goat anti-mouse highly cross-adsorbed antibody (1:500, Molecular Probes, Carlsbad, USA). Sections were then rinsed several times and mounted on slides and coverslipped with ProLong gold antifade reagent with 4,6 diamidino-2-phenylindole (DAPI) (Molecular Probes, Carlsbad, USA).

Analysis of primary cilia in large and small neurons

Immunofluorescent stained cross-sections at L3 (5-7 sections per animal, 5-6 animals per group) were analyzed with widefield deconvolution microscopy (Leica

DMI 6000B, Germany). For each section, images, using a 20X objective lens at multiple consecutive focal planes spaced at 1 μm intervals (Z-stack), were captured with a digital camera (Hamamatsu Orca ER-AG) using Volocity 4 Acquisition Software. The counting areas in the L3 segment derived from Watson et al [24] are shown in Additional file 2. We counted all neurons greater than 20 μm in diameter throughout the Z-stack in the entire lamina IX of the anterior horn on both sides of the cord (as outlined in red on Additional file 2). We counted random fields in lamina VII (as outlined in yellow in Additional file 2), where interneurons reside. No cells > 20 μm were found in this area. Only cells with the nucleus visible were counted. In each section, the number of cells single-labeled with NeuN or double-labeled with NeuN and ACIII was tabulated, and the proportion of double labeled cells to all NeuN labeled cells (ie the proportion of neurons bearing a primary cilium) was calculated.

Statistical analysis

For the cell culture experiments, the main analysis was the difference in the proportion of motor neurons bearing a primary cilium between cells derived from G93A SOD1 and WT mice, at 7 and at 21 days. This was calculated using the two-tailed z statistic for the difference between proportions, with the usual assumption for this number of samples that the Binomial distribution can be approximated by the Normal distribution. Some observations of the in situ experiment are descriptive in nature; further statistical analysis of the in situ experiment is complex. At a first level of analysis we calculated a Levene's F statistic for the equality of variances (SPSS), and then used a t test for equal or unequal variances (as appropriate), to compare SOD1 and WT animals. This is a commonly accepted approach, but lacks sophistication as the underlying data are proportions, and the actual units of analysis are cells (WT cells or G93A SOD1 cells). Thus, we further analyzed the cell proportion data using binary logistic regression (SPSS), with the presence or absence of a primary cilium as the dependent variable, and neuron size (motor neuron vs interneuron), SOD1 status (WT vs transgenic), age (40 d vs 98 d), and sex (M vs F) as predictive variables, and as well we examined the interaction between predictive variables. Significant differences were defined as $p \leq 0.05$, two tailed where applicable.

Results
Primary neuronal cultures

Primary cilia on motor neurons in culture could be robustly identified by co-staining with SMI32 and ACIII (figure 1). (Throughout this paper, we have used the term co-localization to imply the co-localization of cilial
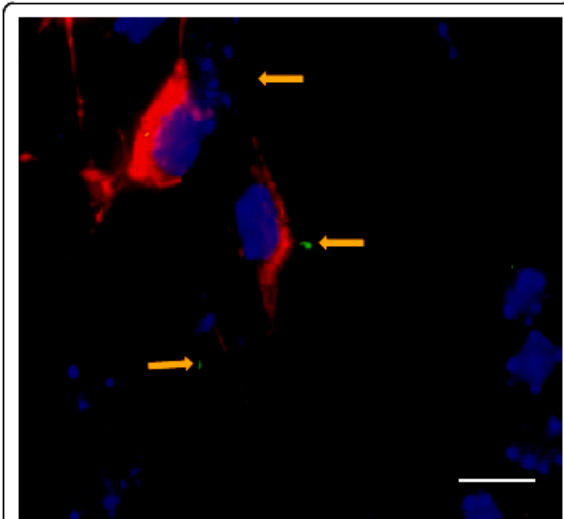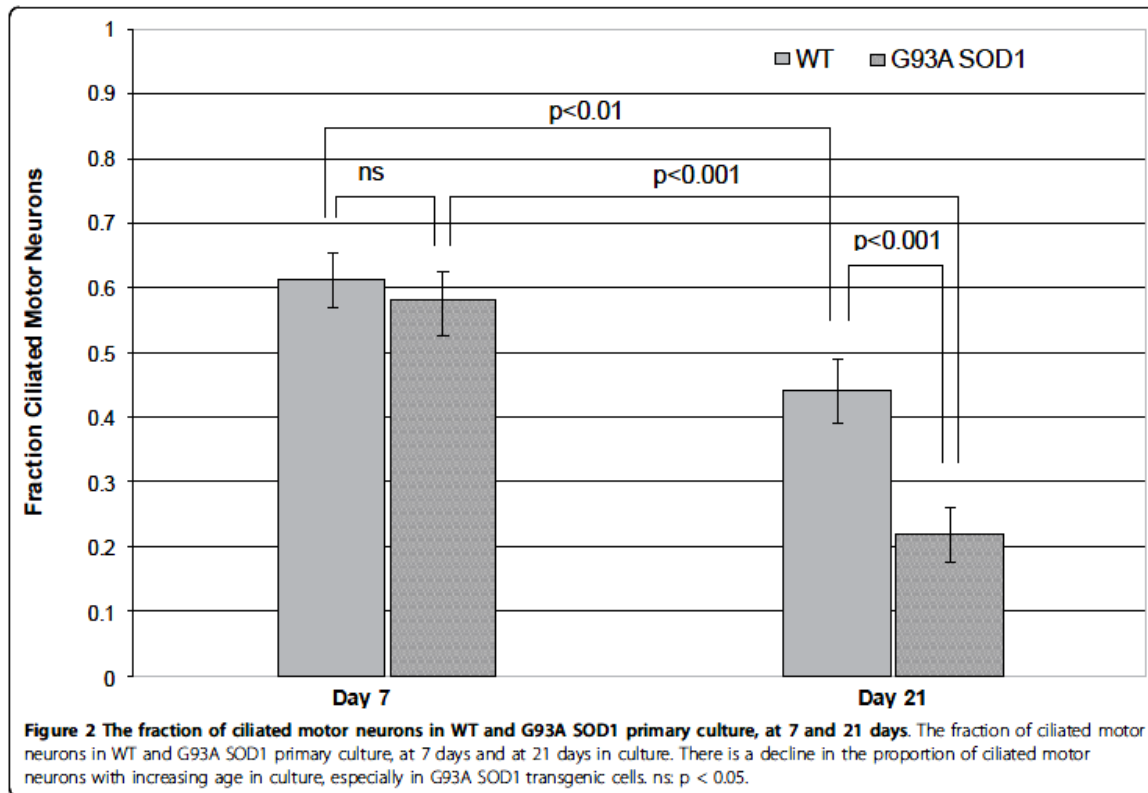

Figure 1 Cells from embryonic mouse lumbar spinal cord in primary mixed culture. Motor neurons are stained with SMI32 (red), primary cilia are stained with ACIII (green), and the nuclear stain DAPI is blue. In the center of the photomicrograph, a ciliated motor neuron is seen. Above it and below it, two cilia are seen, not associated with motor neurons. Scale Bar = 10 μM. Yellow arrows indicate ACIII labeled primary cilia

and neuronal stains to the same cell). After 7 days in mixed primary culture, the percentage of cells staining positive for SMI32 (motor neurons) did not differ between WT cells (13.9 ± 4.62%) and G93A SOD1 cells (12.8 ± 3.66%). Sixty one of 100 WT cells staining positive for SMI32 expressed a primary cilium, compared with 57 of 100 G93A SOD1 cells (p = 0.56). At 21 days, there was no difference in the percentage of motor neurons in cultured WT (12.3 ± 2.35%) and G93A SOD1 cells (9.5 ± 4.15%). There was a drop in ciliated motor neurons, especially in G93A SOD1 cells (44 of 100 WT cells positive for SMI32 expressed a primary cilium, vs 22 of 100 G93A SOD1 cells, p = 0.001). This is illustrated in figure 2. Interestingly, this drop was seen more in motor neurons than other cell types in these mixed cultures (not shown).

In situ distribution of ACIII positive primary cilia

Since no previous reports have discussed ciliated cells in the G93A SOD1 mouse lumbar cord, we first examined the general distribution of ACIII labeling on all cells in the L3 spinal cord, for both WT and G93A SOD1 mice at 40 and 98 days of age. At low magnification, primary cilia were detected on cells throughout L3, including in spinal laminae I through X (Additional file 3a, c). Ciliated cells were particularly abundant in laminae I-VI of dorsal horns and lamina VII of ventral horns. At higher magnification, primary cilia typically displayed a

Figure 2 The fraction of ciliated motor neurons in WT and G93A SOD1 primary culture, at 7 and 21 days. The fraction of ciliated motor neurons in WT and G93A SOD1 primary culture, at 7 days and at 21 days in culture. There is a decline in the proportion of ciliated motor neurons with increasing age in culture, especially in G93A SOD1 transgenic cells. ns: $p < 0.05$.

rod-like shape with bright and homogenous staining for ACIII (Additional file 3b, d.). Although the length of primary cilia was not formally measured, it was apparent that the length of the primary cilium varied across regions of the spinal cord, with longer primary cilia in spinal laminae V, VI, VII, VIII and shorter cilia in spinal laminae I, II, III, IV, and IX. Thus, there is regional heterogeneity of primary cilia with respect to length, as previously reported by Fuchs [21] in an ultrastructural study. The general appearance and general distribution of ACIII did not differ between WT mice and G93A mice, at either age.

Primary cilia project from neurons

We studied the co-localization of ACIII staining with NeuN staining, in the spinal cords of WT and G93A SOD1 mice at both age groups. Most NeuN positive cells co-stained with ACIII in the spinal cord, for both WT and G93A SOD1 mice, at both ages. Figures 3 showed representative low magnification microscopic channel merged images of double stained (ACIII labeled primary cilia, dot-like structures in green, and NeuN positive neurons labeled in red), in the ventral and dorsal horn of L3 of WT 98 day old mice. At higher magnification, most of ACIII positive primary cilia, rod-like structures in green, were shown to co-localize with red NeuN staining, for all mice (figure inserts in 3a and 3b). Thus, most neurons throughout the spinal cord possess a primary cilium, and most primary cilia are associated with neurons.

In lamina IX, most NeuN positive cells are large (> 20 μm). That is, most neurons are motor neurons. Figure 4a shows representative images of large motor neurons co-localized with ACIII labeled primary cilia (yellow arrow), in WT 40 day and WT 98 day mice. In laminae V, VI, VIII, VIII, most neurons are small (< 20 μm), and most of these small NeuN labeled neurons co-labeled with ACIII (Figure 4b), suggesting that interneurons possess primary cilia. Occasionally small NeuN positive cells did not co-label with ACIII. Also, some ACIII positive cells did not co-stain with NeuN, suggesting that ACIII labeled primary cilia may project from glial cells.

Quantification of primary cilia in Lamina VII

At 40 days there is no difference in the average number of L3 neurons (NeuN positive cells) in lamina VII between WT and G93A SOD1 mice (158.2 ± 29.4 vs

Figure 3 ACIII labeled primary cilia project from NeuN labeled neurons in the spinal cord. Representative microscopic images show overall co-localization of ACIII (green) and NeuN (red) in the ventral horn (A) and dorsal horn (B) of L3 spinal cord in 98d-old WT mice. At higher power, inserts in A and B show numerous ACIII labeled rod-like primary cilia (green) projecting from NeuN labeled neurons (red).

162.2 ± 14.3; p = 0.79). There is no difference in the proportion of ciliated neurons between WT and SOD1 mice (0.93 ± 0.031 WT vs 0.91 ± 0.040 SOD1; p = 0.65 using t test for equal variances (F = 0.151; p = 0.71)). The proportion of ciliated small neurons for each animal at 40 days is shown in Additional file 4.

At 98 days the number of small neurons was slightly less than at 40 days, and in addition there was a slight non-significant reduction in neuron numbers in SOD1 as compared to WT mice (120.4 ± 24.1 vs 86.5 ± 21.5; p = 0.34). There was a modest relative decline in the proportion of ACIII and NeuN double labeled cells in G93A SOD1 mice that did not reach significance (0.96 ± 0.012 WT vs 0.81 ± 0.073 SOD1; p = 0.09 using t test for unequal variances (F = 11.8; p = 0.007)). The proportion of ciliated small neurons at 98 days is shown for each animal in Additional file 5.

Quantification of primary cilia on motor neurons in lamina IX

At 40 days, there was no difference in average L3 anterior horn motor neuron number between WT and G93A SOD1 mice (66.6 ± 12.5 WT vs 50.8 ± 8.8 SOD1; p = 0.36). However, there was a non-significant reduction in the proportion of motor neurons bearing a primary cilium in the G93A SOD1 group (0.73 ± 0.041 WT vs 0.65 ± 0.048 SOD1; p = 0.267 using t test for equal variance (F = 0.026; p = 0.876)). The proportion of ciliated motor neurons is shown for each animal in figure 5.

At 98 days, there was a significant reduction in average L3 motor neuron numbers between WT and SOD1 animals (70.4 ± 11.1 vs 29.5 ± 5.4; p = 0.006). In addition, there was a major reduction in the proportion of remaining motor neurons bearing a primary cilium (0.79 ± 0.02 WT vs 0.47 ± 0.11 SOD1; p = 0.03 using t test for unequal proportions (F = 8.837; p = 0.016)). The proportion of ciliated motor neurons for each animal at is shown graphically in figure 6. This is also illustrated in representative photomicrographs, in a WT mouse where all three motor neurons bear primary cilia (figure 7a), whereas none among three motor neurons had a primary cilium in a G93A SOD1 mouse (figure 7b). There is considerably more variability in the cilial proportions in G93A SOD1 mice, as evidenced by the F statistics above, with marked variability even within different regions within the same animal and larger standard deviations in the proportions.
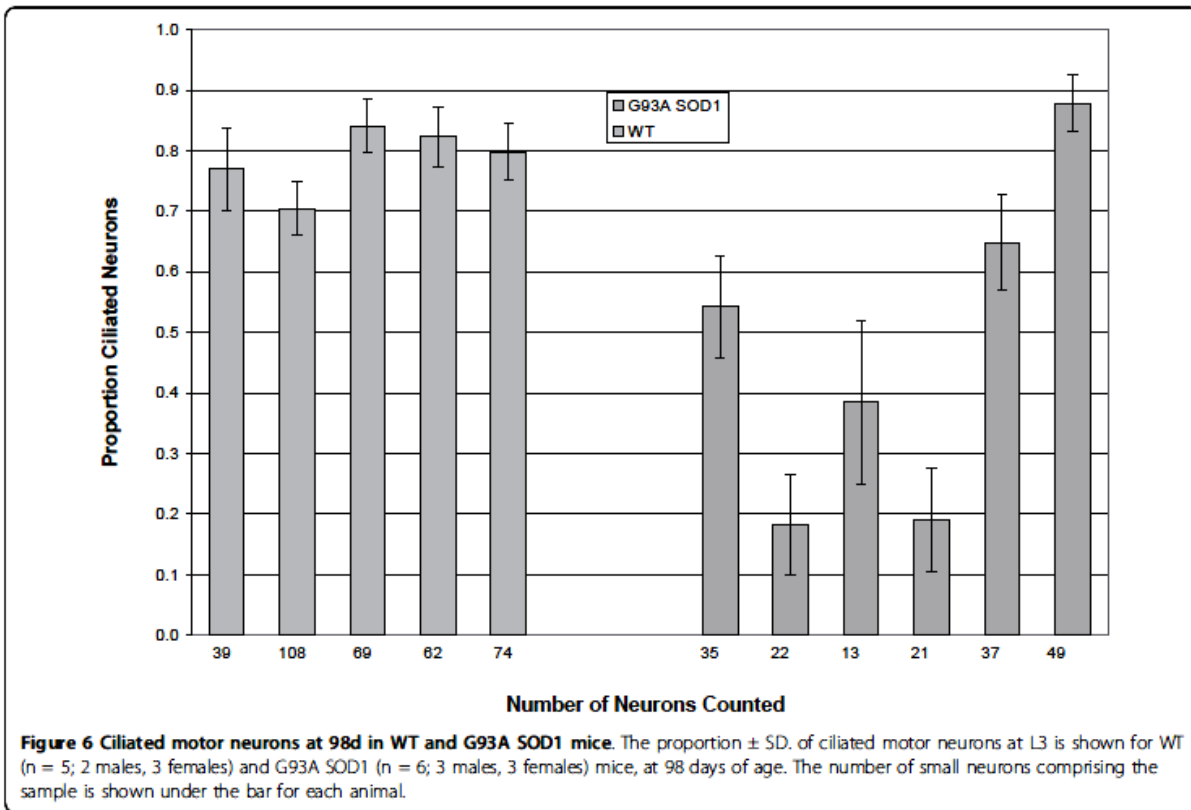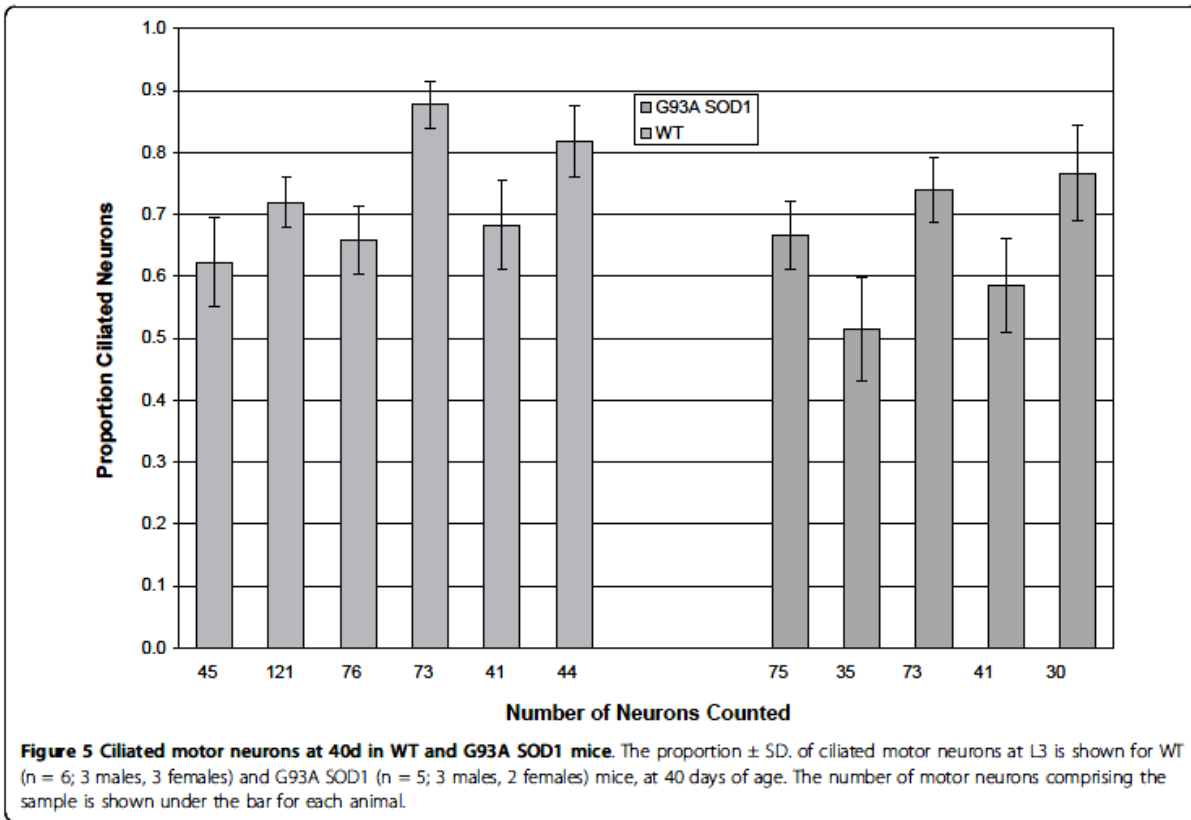
A logistic regression of this dataset, with presence or absence of a cilium as the dependent variable, and age, transgenic status, neuron size (ie >20 μm), and sex as predictive variables, shows that presence of a mutant transgene is a highly significant predictor of reduced ciliation (p < 0.001), as is large neuron size (p < 0.001). There is a strong interaction between transgene status and age (p < 0.001; reflecting the drop in ciliation in aged transgenics but not in aged WT mice). Otherwise, age adds no independent predictive value (p = 0.248). Female sex is minimally and non-significantly predictive (p = 0.165) of greater ciliation.
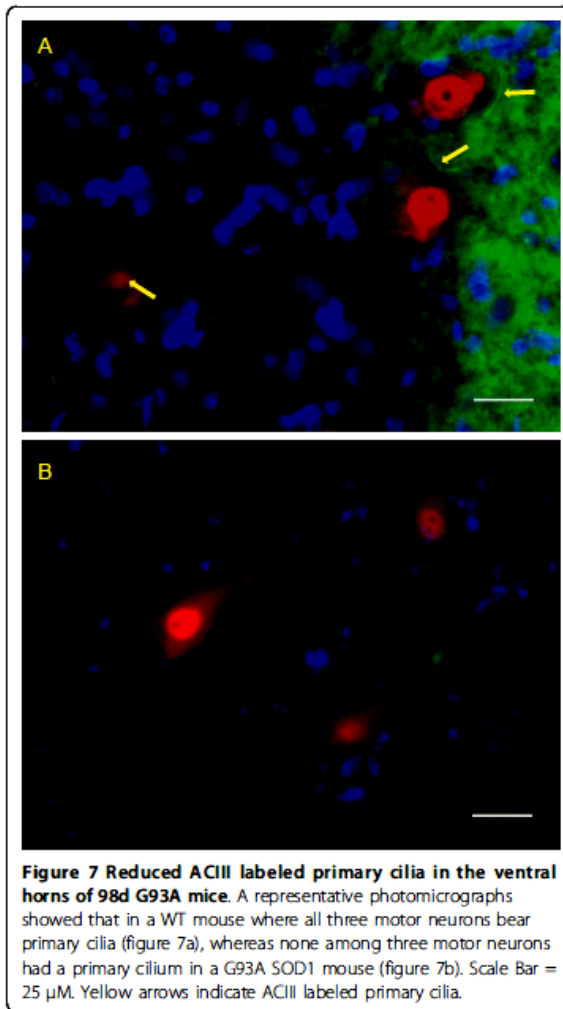
Discussion

It is common practice to demonstrate primary cilia using immunohistochemical staining for ACIII [22,30], and on this basis we conclude that over-expression of a disease-causing SOD1 mutation significantly reduces the expression of primary cilia on motor neurons late in primary culture and in the lumbar spinal cord of adult animals in situ. However, it might be more conservative to conclude that there is a reduction in ACIII cilial staining, as it is theoretically possible that the ACIII staining has disappeared from an otherwise intact primary
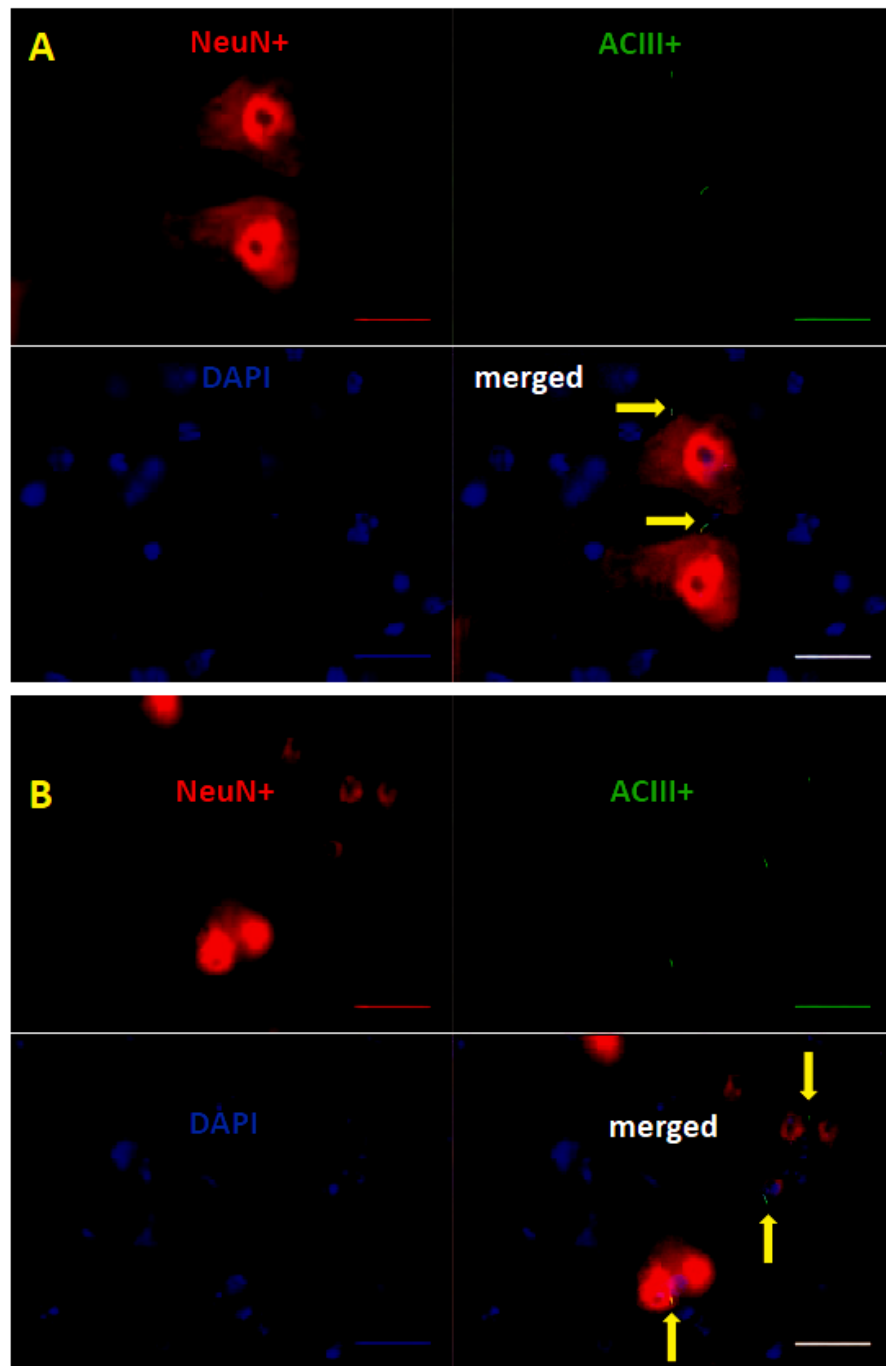
Figure 4 Ciliated motor neurons and ciliated small neurons in 98d WT mice. A photomicrograph of ACIII labeled primary cilium (green), NeuN labeled neurons (red), and two motor neurons double labeled with ACIII and NeuN (merged channel) are shown in the ventral horn of a WT-98d mouse (A). In the lower panel, ACIII labeled primary cilia were co-localized with NeuN small neurons in lamina VII of WT-98d mice. Scale Bar = 25 µM. Yellow arrows indicate ACIII labeled primary cilia.

Figure 5 Ciliated motor neurons at 40d in WT and G93A SOD1 mice. The proportion ± SD. of ciliated motor neurons at L3 is shown for WT (n = 6; 3 males, 3 females) and G93A SOD1 (n = 5; 3 males, 2 females) mice, at 40 days of age. The number of motor neurons comprising the sample is shown under the bar for each animal.

Figure 6 Ciliated motor neurons at 98d in WT and G93A SOD1 mice. The proportion ± SD. of ciliated motor neurons at L3 is shown for WT (n = 5; 2 males, 3 females) and G93A SOD1 (n = 6; 3 males, 3 females) mice, at 98 days of age. The number of small neurons comprising the sample is shown under the bar for each animal.

Figure 7 Reduced ACIII labeled primary cilia in the ventral horns of 98d G93A mice. A representative photomicrographs showed that in a WT mouse where all three motor neurons bear primary cilia (figure 7a), whereas none among three motor neurons had a primary cilium in a G93A SOD1 mouse (figure 7b). Scale Bar = 25 µM. Yellow arrows indicate ACIII labeled primary cilia.

identification of cilia, and size greater than 20 µm, as a surrogate for direct identification of motor neurons. The indirect identification of motor neurons could introduce error if some motor neurons in the hmSOD1 mice were pyknotic (and thereby not identified as motor neurons by size). However, there were very few NeuN positive cells less than 20 µm in lamina IX in either WT or G93A SOD1 mice, and thus pyknotic motor neurons would have to have lost their NeuN staining (which has been reported in neurons in an experimental stroke model [26]).

We used 40 µm sections and only counted cells where the nucleus could be identified in totality by imaging at multiple planes. It is possible that a primary cilium was present on some cells but out of section, and the cell thus misidentified as lacking a primary cilium. This error would not be large, and indeed, if there were somatic shrinkage with disease, it would bias against finding a difference in G93A SOD1 mice.

Even in diseased animals, some neurons appeared healthy and had normal appearing primary cilia, and thus there was considerable variability in cilial staining. This variability raises the possibility that the disease process is patchy with respect to cilial pathology, that there are differences in susceptibility between types of motor neurons, or that there is a differential expression of hmSOD1 between individual motor neurons in this model. (There is some evidence that the transgene copy number may not be fully stable in G93A SOD1 mice [31] but nothing is known of cellular mosaicism.)

In spite of these caveats, our cell culture results and our animal studies are mutually consistent, and consistent with the hypothesis that there is a reduction in the proportion of ciliated motor neurons in G93A SOD1 mice. Neurotoxic SOD1 mutations could affect primary cilial structure and function in several ways. There might be intra-ciliary protein accretion and/or altered intraflagellar transport, and in this light, mSOD1-induced changes in the normal anatomical barriers to the exchange of proteins between cytosolic and ciliary compartments might be important (ie the ciliary necklace and transitional fibers [32]).

A major unanswered question that will await further experimentation is whether the reduction in ciliated motor neurons simply reflects the underlying diseased state of these cells, or whether the loss may be more directly and causally related to the ALS disease process. There are several reasons to believe that a loss of primary cilia could be detrimental. Most directly related to this work, the loss of ACIII would be expected to disrupt cAMP second messenger signaling, in this case from an unknown G protein coupled receptor and unknown ligand [33].

cilium. There could be important consequences in either event, and moreover, since our study focused on immunohistochemical alterations only, the potential importance of these findings would be broadened if there were additional deficits in cilial function in motor neurons still retaining ACIII staining.

Some technical issues bear discussion. Because of restrictions imposed by antibody suitability, species, and the availability of secondary stains, we chose to identify motor neurons indirectly. Usual stains for motor neurons, eg SMI32 or ChAT, result in a stain that is diffused and poorly suited to counterstaining for cilia. In consequence, we used an indirect method for identifying ciliated motor neurons. We first verified that nearly all large neurons (greater than 20 µm) in the anterior horn co-express ChAT and the neuronal stain NeuN, and then used NeuN, which is better suited to the

Moreover, much, indeed perhaps all, Shh signaling occurs through the primary cilium, and disruption of the primary cilium alters the balance between canonical and non-canonical Wnt pathways [16]. Both Shh and Wnt have been shown to exert neuroprotective function [17-20,34,35]. Similarly, other signaling pathways involve the primary cilium and may be neuroprotective, including MCH1, SSTR3, and PDGF [36-38]. Loss of cilial signaling (or reduced cilial signaling due to reduced cilial stability and increased cilial turnover) could render motor neurons more susceptible to neurotoxic challenge.

Last, the primary cilium derives from the maternal kinetophore, is expressed only on growth-arrested cells [16] (possibly for this reason), and perhaps not surprisingly many cilial signaling pathways have been implicated in cell cycle control. Specifically, Shh, Wnt, PDGF, and SSTR3 have all been implicated in cell cycle control [39-43]. Disruption of primary cilial function might alter the response of post-mitotic motor neurons to disease-induced cell cycle re-entry signals [44,45], with possible adverse consequences [46].

Conclusions

Primary cilia are abundant and widely distributed in motor neurons in primary culture from normal embryonic mice and in the lumbar spinal cord of adult mice. In G93A SOD1 mice, the proportion of ciliated motor neurons drops markedly in primary culture, and in the lumbar spinal cord in situ. Future experiments will determine whether alterations in primary cilia contribute to the pathogenesis of ALS, and by extension, of other neurodegenerative diseases.

Additional material

Additional file 1: Co-localization of ChAT and NeuN staining in motor neurons. A representative image showing colocalization of ChAT (green) and NeuN (red) staining in large (>20 μm) cells in the ventral horn of L3 of 98d WT mice. Scale bar 25 μm.

Additional file 2: Sampling areas in the L3 cord. The L3 cord was used to draw the sampling areas (shown on one side for illustration purposes but counted on both sides), using the mouse atlas of Watson et al. Motor neurons were located in lamina IX, outlined in red, and interneurons in lamina VII (yellow).

Additional file 3: Distribution of ACIII staining for primary cilia in L3 cord. Representative images show that ACIII labeled primary cilia (dot or rod-shaped structures in green) are widely distributed in the ventral horn (A) and in the dorsal horn (C) of wild type 98d mice. Red arrows indicate ACIII labeled primary cilia.

Additional file 4: Ciliated small neurons at 40d in WT and G93A SOD1 mice. The proportion ± SD. of ciliated small neurons at L3 is shown for WT (n = 6; 3 males, 3 females) and G93A SOD1 (n = 5; 3 males, 2 females) mice, at 40 days of age. The number of small neurons comprising the sample is shown under the bar for each animal.

Additional file 5: Ciliated small neurons at 98d in WT and G93A SOD1 mice. The proportion ± SD. of ciliated small neurons at L3 is shown for WT (n = 5; 2 males, 3 females) and G93A SOD1 (n = 6; 3 males, 3 females) mice, at 98 days of age. The number of small neurons comprising the sample is shown under the bar for each animal.

References
1. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, et al: Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 1993, 362(6415):59-62.
2. Yang Y, Hentati A, Deng HX, Dabbagh O, Sasaki T, Hirano M, Hung WY, Ouahchi K, Yan J, Azim AC, et al: The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. *Nat Genet* 2001, 29(2):160-165.
3. Munch C, Sedlmeier R, Meyer T, Homberg V, Sperfeld AD, Kurt A, Prudlo J, Peraus G, Hanemann CO, Stumm G, et al: Point mutations of the p150 subunit of dynactin (DCTN1) gene in ALS. *Neurology* 2004, 63(4):724-726.
4. Nishimura AL, Mitne-Neto M, Silva HC, Richieri-Costa A, Middleton S, Cascio D, Kok F, Oliveira JR, Gillingwater T, Webb J, et al: A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am J Hum Genet* 2004, 75(5):822-831.
5. Greenway MJ, Andersen PM, Russ C, Ennis S, Cashman S, Donaghy C, Patterson V, Swingler R, Kieran D, Prehn J, et al: ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. *Nat Genet* 2006, 38(4):411-413.
6. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, et al: Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 2006, 314(5796):130-133.
7. Arai T, Hasegawa M, Akiyama H, Ikeda K, Nonaka T, Mori H, Mann D, Tsuchiya K, Yoshida M, Hashizume Y, et al: TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun* 2006, 351(3):602-611.
8. Vance C, Rogelj B, Hortobagyi T, De Vos KJ, Nishimura AL, Sreedharan J, Hu X, Smith B, Ruddy D, Wright P, et al: Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 2009, 323(5918):1208-1211.
9. Kwiatkowski TJ Jr, Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, Davis A, Gilchrist J, Kasarskis EJ, Munsat T, et al: Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* 2009, 323(5918):1205-1208.
10. Parboosingh JS, Figlewicz DA, Krizus A, Meininger V, Azad NA, Newman DS, Rouleau GA: Spinobulbar muscular atrophy can mimic ALS: the importance of genetic testing in male patients with atypical ALS. *Neurology* 1997, 49(2):568-572.
11. Meyer T, Schwan A, Dullinger JS, Brocke J, Hoffmann KT, Nolte CH, Hopt A, Kopp U, Andersen P, Epplen JT, et al: Early-onset ALS with long-term survival associated with spastin gene mutation. *Neurology* 2005, 65(1):141-143.

12. MacGowan DJ, Scelsa SN, Waldron M: **An ALS-like syndrome with new HIV infection and complete response to antiretroviral therapy.** *Neurology* 2001, **57**(6):1094-1097.

13. Silva MT, Leite AC, Alamy AH, Chimelli L, Andrada-Serpa MJ, Araujo AQ: **ALS syndrome in HTLV-I infection.** *Neurology* 2005, **65**(8):1332-1333.

14. Lomen-Hoerth C: **Amyotrophic lateral sclerosis from bench to bedside.** *Semin Neurol* 2008, **28**(2):205-211.

15. Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX, *et al*: **Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation.** *Science* 1994, **264**(5166):1772-1775.

16. Gerdes JM, Davis EE, Katsanis N: **The vertebrate primary cilium in development, homeostasis, and disease.** *Cell* 2009, **137**(1):32-45.

17. Calcutt NA, Allendoerfer KL, Mizisin AP, Middlemas A, Freshwater JD, Burgers M, Ranciato R, Delcroix JD, Taylor FR, Shapiro R, *et al*: **Therapeutic efficacy of sonic hedgehog protein in experimental diabetic neuropathy.** *J Clin Invest* 2003, **111**(4):507-514.

18. Cappuccio I, Calderone A, Busceti CL, Biagioni F, Pontarelli F, Bruno V, Storto M, Terstappen GT, Gaviraghi G, Fornai F, *et al*: **Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is required for the development of ischemic neuronal death.** *J Neurosci* 2005, **25**(10):2647-2657.

19. Cai C, Thorne J, Grabel L: **Hedgehog serves as a mitogen and survival factor during embryonic stem cell neurogenesis.** *Stem Cells* 2008, **26**(5):1097-1108.

20. Matrisciano F, Busceti CL, Bucci D, Orlando R, Caruso A, Molinaro G, Cappuccio I, Riozzi B, Gradini R, Motolese M, *et al*: **Induction of the Wnt antagonist Dickkopf-1 is involved in stress-induced hippocampal damage.** *PLoS One* **6**(1):e16447.

21. Fuchs JL, Schwark HD: **Neuronal primary cilia: a review.** *Cell Biol Int* 2004, **28**(2):111-118.

22. Bishop GA, Berbari NF, Lewis J, Mykytyn K: **Type III adenylyl cyclase localizes to primary cilia throughout the adult mouse brain.** *J Comp Neurol* 2007, **505**(5):562-571.

23. Wiese S, Herrmann T, Drepper C, Jablonka S, Funk N, Klausmeyer A, Rogers ML, Rush R, Sendtner M: **Isolation and enrichment of embryonic mouse motoneurons from the lumbar spinal cord of individual mouse embryos.** *Nat Protoc* **5**(1):31-38.

24. Watson C, Paxinos G, Kayalioglu G, Heise C: **Atlas of the Mouse Spinal Cord.** In *The Spinal Cord*. Edited by: Watson C, Paxinos G, Kayalioglu G. San Diego: Academic Press; 2009:308-379.

25. Wengenack TM, Holasek SS, Montano CM, Gregor D, Curran GL, Poduslo JF: **Activation of programmed cell death markers in ventral horn motor neurons during early presymptomatic stages of amyotrophic lateral sclerosis in a transgenic mouse model.** *Brain Res* 2004, **1027**(1-2):73-86.

26. Unal-Cevik I, Kilinc M, Gursoy-Ozdemir Y, Gurer G, Dalkara T: **Loss of NeuN immunoreactivity after cerebral ischemia does not indicate neuronal cell loss: a cautionary note.** *Brain Res* 2004, **1015**(1-2):169-174.

27. Chiu AY, Zhai P, Dal Canto MC, Peters TM, Kwon YW, Prattis SM, Gurney ME: **Age-dependent penetrance of disease in a transgenic mouse model of familial amyotrophic lateral sclerosis.** *Mol Cell Neurosci* 1995, **6**(4):349-362.

28. McHanwell S, Watson C: **Localization of Motoneurons in the Spinal Cord.** In *The Spinal Cord*. Edited by: Watson C, Paxinos G, Kayalioglu G. San Diego: Academic Press; 2009:94-114.

29. van Praag H, Christie BR, Sejnowski TJ, Gage FH: **Running enhances neurogenesis, learning, and long-term potentiation in mice.** *Proc Natl Acad Sci USA* 1999, **96**(23):13427-13431.

30. Berbari NF, Bishop GA, Askwith CC, Lewis JS, Mykytyn K: **Hippocampal neurons possess primary cilia in culture.** *J Neurosci Res* 2007, **85**(5):1095-1100.

31. Scott S, Kranz JE, Cole J, Lincecum JM, Thompson K, Kelly N, Bostrom A, Theodoss J, Al-Nakhala BM, Vieira FG, *et al*: **Design, power, and interpretation of studies in the standard murine model of ALS.** *Amyotroph Lateral Scler* 2008, **9**(1):4-15.

32. Rohatgi R, Snell WJ: **The ciliary membrane.** *Curr Opin Cell Biol* **22**(4):541-546.

33. Defer N, Best-Belpomme M, Hanoune J: **Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase.** *Am J Physiol Renal Physiol* 2000, **279**(3):F400-416.

34. Hashimoto M, Ishii K, Nakamura Y, Watabe K, Kohsaka S, Akazawa C: **Neuroprotective effect of sonic hedgehog up-regulated in Schwann cells following sciatic nerve injury.** *J Neurochem* 2008, **107**(4):918-927.

35. Dai RL, Zhu SY, Xia YP, Mao L, Mei YW, Yao YF, Xue YM, Hu B: **Sonic hedgehog protects cortical neurons against oxidative stress.** *Neurochem Res* **36**(1):67-75.

36. Lee JH, Gleeson JG: **The role of primary cilia in neuronal function.** *Neurobiol Dis* **38**(2):167-172.

37. Antal-Zimanyi I, Khawaja X: **The role of melanin-concentrating hormone in energy homeostasis and mood disorders.** *J Mol Neurosci* 2009, **39**(1-2):86-98.

38. Ishii Y, Oya T, Zheng L, Gao Z, Kawaguchi M, Sabit H, Matsushima T, Tokunaga A, Ishizawa S, Hori E, *et al*: **Mouse brains deficient in neuronal PDGF receptor-beta develop normally but are vulnerable to injury.** *J Neurochem* 2006, **98**(2):588-600.

39. Verani R, Cappuccio I, Spinsanti P, Gradini R, Caruso A, Magnotti MC, Motolese M, Nicoletti F, Melchiorri D: **Expression of the Wnt inhibitor Dickkopf-1 is required for the induction of neural markers in mouse embryonic stem cells differentiating in response to retinoic acid.** *J Neurochem* 2007, **100**(1):242-250.

40. Stanic D, Malmgren H, He H, Scott L, Aperia A, Hokfelt T: **Developmental changes in frequency of the ciliary somatostatin receptor 3 protein.** *Brain Res* 2009, **1249**:101-112.

41. Breunig JJ, Sarkisian MR, Arellano JI, Morozov YM, Ayoub AE, Sojitra S, Wang B, Flavell RA, Rakic P, Town T: **Primary cilia regulate hippocampal neurogenesis by mediating sonic hedgehog signaling.** *Proc Natl Acad Sci USA* 2008, **105**(35):13127-13132.

42. Andrae J, Gallini R, Betsholtz C: **Role of platelet-derived growth factors in physiology and medicine.** *Genes Dev* 2008, **22**(10):1276-1312.

43. Bambakidis NC, Horn EM, Nakaji P, Theodore N, Bless E, Dellovade T, Ma C, Wang X, Preul MC, Coons SW, *et al*: **Endogenous stem cell proliferation induced by intravenous hedgehog agonist administration after contusion in the adult rat spinal cord.** *J Neurosurg Spine* 2009, **10**(2):171-176.

44. Ranganathan S, Bowser R: **Alterations in G(1) to S phase cell-cycle regulators during amyotrophic lateral sclerosis.** *Am J Pathol* 2003, **162**(3):823-835.

45. Cova E, Ghiroldi A, Guareschi S, Mazzini G, Gagliardi S, Davin A, Bianchi M, Ceroni M, Cereda C: **G93A SOD1 alters cell cycle in a cellular model of Amyotrophic Lateral Sclerosis.** *Cell Signal* **22**(10):1477-1484.

46. Yang Y, Herrup K: **Cell division in the CNS: protective response or lethal event in post-mitotic neurons?** *Biochim Biophys Acta* 2007, **1772**(4):457-466.

------CHAPTER 4------

Sonic hedgehog reduces excitotoxicity in cultured mouse motor neurons and improves survival in the G93A SOD1 mouse model of ALS.

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This manuscript represents the culmination of our initial efforts to assess the neuroprotective ability of Shh and its agonist. Utilizing the same primary culture system as in the previous chapter, and mimicking the same experimental paradigm presented in chapter 1 we exposed cells to an excitotoxic challenge and assessed the ability of treated cells to withstand stress. Also, we conducted an *in vivo* trial to ascertain if chronic administration of Shh directly into the central canal of the spinal cord, via intrathecal injection, over a period of 30 days would beneficially extend the life-span of $SOD1^{G93A}$ mice. Our findings concluded that Shh and its agonists purmorphamine and SAG-1 were able to confer resistance to excitotoxic challenge *in vitro* and that intrathecal injection of Shh resulted in a significant increase in life-span in treated mice.

Abstract

Developmental morphogens may retain physiological activity and relevance to mature neurons. We show here that the developmental morphogen Sonic hedgehog (Shh) is cytoprotective against excitotoxic challenge to cultured wildtype and G93A SOD1 spinal motor neurons (an ALS disease model). The Shh agonists purmorphamine and SAG are likewise cytoprotective. Intrathecal Shh infused by osmotic minipump prolongs survival of G93A SOD1 mice, and a polymorphism in the Shh receptor Patched (PTCH1) modulates survival in these animals independent of the treatment effect. Developmental morphogens, and in particular, Shh and Shh agonists, warrant further scrutiny in ALS.

Background

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterized chiefly by progressive and ultimately fatal weakness of voluntary muscle, and is presently defined on clinical grounds. Most cases are without known cause. Some ALS is familial and most causative mutations have now been identified [Rosen 1993, Yang 2001, Munch 2004, Nishimura 2004, Greenway 2006, Neumann 2006, Arai 2006, Vance 2009, Kwiatkowski 2009, Deng 2011, DeJesus-Hernandez 2011, Renton 2011]. However, in no case do we fully understand how any known mutation leads to ALS, and it is also unclear how multiple known and unknown triggers can lead to a similar disease phenotype. Several pathological mechanisms may be in common, including errors in RNA processing, oxidative stress, excitotoxicity, mitochondrial dysfunction, impaired axonal transport and reduced trophic support, inflammation, and terminally, apoptosis [eg Rothstein 2009].

It has been suggested that developmental morphogens with activity in embryogenesis might continue to play a trophic role to mature neurons in adult life, and that underactivity or dysfunction in these pathways might contribute to neurodegeneration in later life [eg Tsuboi 2002, Calcultt 2003, Dass 2005, Dellovade 2006, Farzan 2008, Stenkemp 2008, Dai 2011]. We have considered this possibility in ALS, and we have shown that cultured HT22 cells transfected with a plasmid overexpressing a human mutant SOD1 express reduced levels of mRNA for the Sonic Hedgehog (Shh) associated DNA-binding gli proteins (that transduce most Shh action), and these cells show reduced output from a gli reporter [Peterson 2011]. Shh added to the growth medium protects these HT22 cells against oxidative challenge [Peterson 2011]. Much if not all Shh signalling occurs in the primary cilium, and we have also shown that primary cilia are reduced in motor neurons cultured from embryonic G93A SOD1 mice, and in motor neurons in situ of the lumbar spinal cord of G93A SOD1 mice as they age [Ma 2011].

We show here that Shh, and the Shh agonists purmorphamine and SAG, protect motor neurons cultured from embryonic wildtype and G93A SOD1 mouse spinal cord against excitotoxic challenge, and that subarachnoid infusion of Shh prolongs survival in G93A SOD1 mice. We also show that survival in this model is modified by a polymorphism in the gene for the Shh receptor Patched (PTCH1).

Material and Methods

In Vitro experiments

Primary motor neuron cultures were derived from embryonic fetal mice at 14 days gestation as previously described [12]. Briefly, spinal cords were dissected from E14 embryos and were processed individually, with a segment of the body kept for genotyping. Each spinal cord was

minced into small pieces and dissociated in 1% trypsin (Sigma) for 15 min. After trypsinization, an equal volume of trypsin inhibitor (Sigma) was added and the mixture was lightly triturated until a single cell suspension was achieved. Cell suspensions were then transferred to Neurobasal media containing 1% Glutamax (Invitrogen) and centrifuged at 400g for 5 minutes without brake. Supernatants were then decanted and cell pellets were resuspended in complete Neurobasal media containing 1% Glutamax, 3% Horse Serum, 1X B-27 supplement (all from Invitrogen), 5ng/ml CNTF and 5ng/ml BDNF (Leinco). 5×10^4 cells were plated on poly-D-Lysine (Sigma) coated 96 well plates (BD Bioscience) and grown in a $37^{\circ}C$ incubator in 5% CO_2 environment. Half of the culture volume was replaced with fresh media every third day. On the 6th day in vitro (DIV) cultures were treated with a 1/400 dilution (v/v) of 1-β-D-Arabinofuranosylcytosine (EMD Biosciences) to halt glial growth, after which the media was changed back to complete media.

SOD1 genotyping was undertaken using DNA isolated from embryos using the Qiagen blood and tissue DNA isolation kit (Qiagen). 250ng of total DNA was used in a PCR reaction using primers and conditions as outlined on the JAX website.

Recombinant Shh protein encompassed amino acids Cys24-Gly197 of the N-terminal portion with Ile-Ile substituted for Cys24 (Leinco), and was resuspended in pure ddH_2O without any carrier protein. Stock solutions of purmorphamine (Stemgent) and SAG-1 (EMD biosciences) were diluted in dimethyl sulfoxide (DMSO). DIV10 cultured cells were treated with Shh at 4 concentrations (0, 250, 500, 1000 nM), purmorphamine at 4 concentrations (0, 1, 5, 10 μM), or SAG-1 at 3 concentrations (0, 5, 25 nM). 24 hours post treatment, cultures were treated with 2μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma) to block AMPA/Kainate receptors

and then exposed to a titre range of NMDA concentrations (0, 10, 20, 25, 30, 50, 75, 100 μM) in complete media; each concentration was tested in triplicate. These NMDA concentrations bracketed the survival range of motor neurons from no effect on survival to complete lethality.

After 24 hours, motor neuron death as a proportion of total cell population was measured using a dead cell protease assay in the culture media as outlined by the manufacturer (Cytotox-Glo, Promega). Briefly, after 24 hour exposure to NMDA challenge, 50ul (half) of the culture media was removed from each well and placed in the corresponding well of a 96-well white opaque micro assay plate (Greiner Bio-One), and 50μl of Cytotox-Glo reagent was added. The dead-cell protease activity was then quantified via luminescence as per manufacturer's instructions. The cells not killed by the NMDA challenge (i.e. surviving motor neurons and glia) were quantified by adding 5μl of 10% Triton X to the remaining culture media in each well of the original plate, and the dead-cell protease assay was repeated as above. Motor neuron death, as a percentage of the total cellular population, was calculated as:

$$200A / (A+B),$$

where A= Luminescence signal after NMDA (motor neuron death)

   B= Luminescence signal after Triton X (remaining cell death)

For each pharmacological treatment, we exposed cells to a range of increasing concentrations of NMDA, with each concentration tested in triplicate, and obtained a sigmodial dose response curve. Each curve was then fitted to a sigmodial dose-response curve with variable slope, using the curve fitting algorithms of PRISM (version 4, GraphPad software Inc, San Diego, CA),

(equivalent to a 4 parameter logistic equation), which returned the LC_{50} and the standard deviation of the LC_{50}. Each experiment was repeated a minimum of 3 times.

The different doses of Shh, and Shh agonists, were compared to control using ANOVA with Dunnett's post hoc test for multiple comparisions (Graph Pad, San Diego).

In vivo experiments

All animal experiments and procedures were carried out with the approval of McMaster University's Animal Research Ethics Board (animal utilization protocol # 091143). High-copy G93A SOD1 breeding pairs were obtained from the Jackson Laboratory (B6SJL-Tg(SOD1*G93A)1 Gur/J) and the colony was maintained by crossing hemizygous male carriers with inbred wild-type C57BL/6 dams or later, transgene-negative offspring. Mice were individually housed in the McMaster University Central Animal Facility in a 12-h light/dark cycle. Food and water were provided ad libitum, and provided at the cage floor when needed (because of hindlimb weakness).

Mice were genotyped for the G93A SOD1 transgene using tail snip and primers described on the JAX website as above. At 60 days of age, 35 mice carrying the G93A SOD1 transgene were implanted with an Alzet (Cupertino, CA) osmotic minipump (Alzet #1004, 100ul reservoir; 11 ul/hr flowrate, 28 day infusion) mounted subcutaneously in the dorsal midline. The pump was connected to an intrathecal catheter modified from 0.025 inch o.d. polyethylene tubing (#BB31695-PE/1, Scientific Commodities, Lake Havesu City, Arizona), using a protocol modified from Ohta [13].

Briefly, the tubing was placed on a 0.01" introducer wire (nickel/chromium/steel 0.01, Smallparts, www.Smallparts.com) and then stretched to the size of the introducer over an open

flame. More proximally, 2 small bumps were made close together in the softened tubing to permit measurement and subsequent fixation in place with a stitch. The wire and catheter were then soaked in ethanol to sterilize and the ethanol was allowed to evaporate. Under general anesthesia with isoflurane, the mice were positioned, skin over the back shaved and prepped, and the skin incised along the midline. With practice, the small introducer wire with the tubing could then be angled blindly into the subarachnoid space at the lumbar level and the introducer withdrawn. The proximal catheter was then folded up in the incision to the dorsal upper thoracic region. The osmotic pump, already loaded with either Shh or control CSF, was attached to the catheter and placed in the dorsal midline in a subcutaneous tissue pocket at the level of the forelegs, and then held in place with a stitch. The overlying skin was closed with sutures and staples. Ketoprofen was infused around the surgical site post-operatively for analgesia. A single dose of 0.01 mg ampicillin was given, and if skin infections developed later they were treated with Fucidin at the recommendation of the University Veterinarian.

Even with practice, there is a substantial surgical mortality to this procedure, but if the mice survived for a week or more, there was no additional mortality from that point on. In some cases, there was some trauma to the nerve roots causing a degree of weakness in one or other hindleg, but this was stable and improved with time. As discussed later, most but not all catheters were found to be properly positioned in the subarachnoid space at post-mortem.

The Alzet mini-pumps were loaded with 0, 1.6, 4.7, or 14ug of Shh in 100 ul artificial CSF. Based on a CSF flow rate of 18ul/h in the mouse (Alzet website) and a pump infusion rate of 11 ul/hr, this should give CSF concentrations of Shh of about 0 (control), 0.1, 0.33, and 1ug/ul, for the 28 days the pump is active. We implanted 35 mice, 18 Shh and 17 controls. Unfortunately, 6

Shh and 9 control animals became moribund and had to be euthanized shortly after surgery, which left insufficient Shh animals to consider the three Shh groups separately, and we analysed all Shh-treated animals as a group. We also included a group of 7 un-operated animals to serve as an additional control.

The presence of a PTCH polymorphism at position 1267 was determined by PCR with subsequent sequencing. Briefly, PCR reactions were performed with 250ng genomic DNA samples from tail snipes, 0.2 mM dNTPs, 1 µM of each primer, and 2.5 µl of the 10X PCR Buffer (Life technologies, Burlington Canada). Reaction mixtures were heated at 94°C for 5 min followed by 35 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s) and polymerization (72°C, 1 min) and by a final polymerization at 72°C for 5 min in a thermocycler (Life technologies/ Applied Biosystems). Amplified DNA fragments were purified from agarose gels with the Qiagen MinElute Gel Extraction Kit (Qiagen, Toronto, Canada). We used primers ATCCGGACTCCAGACATCAG (left) and TATTGCTAGGGCCAGAATGC (right), to identify an ACC (T) or AAC (N) polymorphism. Sequencing was done in the McMaster Institute for Molecular Biology and Biotechnology (MOBIX). Sequence electropherograms were displayed and analysed using FinchTV (Geospiza). Only one animal had a 1267 N/N genotype and this animal was analysed along with the 1267 T/N animals.

All mice were examined daily from 45 days of age to endpoint, and after surgery animals were monitored twice daily. Body weight measurements were monitored. A commonly-used surrogate endpoint for death was employed (failure to right within 30 seconds after being placed on the side). The animals were euthanized at that point. At post-mortem, the catheter position was determined. In most animals, the catheter was found appropriately positioned in the subarachnoid

space; in a few animals the catheter was outside the spinal canal. We thus analysed the animals both as intention-to-treat (based on the initial treatment allocation) and on a per-protocol basis (based on whether they actually received any intrathecal treatment).

Kaplan-Meier analysis with log-rank statistics was used to analyze the time-to-event measures (survival) for both the intention-to-treat and per-protocol groups, alone and with gene polymorphism status stratified as a covariate (SPSS 19, IBM). Survival in days is presented as mean $\pm$ s.e.m. Cox regression was used to model and graph the effect of gene polymorphism on survival, entering treatment group, gene status, and an interaction term as variables, and plotting gene status at the mean of the covariates (SPSS 19, IBM). Two-tailed p values less than 0.05 were considered to be statistically significant. For graphical presentation, Kaplan Meier plots were displayed with GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

In Vitro

There is a protective effect of Shh on embryonic motor neurons in culture to NMDA excitotoxic challenge, as shown in figures 1a (wildtype) and 1b (G93A SOD1). There is a clear dose-response effect, with larger doses of Shh conferring greater benefit. The beneficial effect is seen in both wildtype and G93A SOD1 motor neurons, about equally. Similarly, the Shh agonist purmorphamine causes a dose-related increase in resistance to excitotoxic challenge, in both wildtype (figure 1c, left) and G93A SOD1 (figure 1d, right) mice. A second agonist, SAG1, also produces an almost identical picture (figure 1e and 1f).

In Vivo

We restricted our analysis to animals that survived more than 1 week post surgery, as from this point there was no additional unexpected death until the expected death from ALS. We included 2 groups of animals as controls for the in vivo study. Somewhat surprisingly, the un-operated controls fared worse (mean survival 125.0 ± 1.77 days) than the operated controls (mean survival 131.4 ± 2.0 days). The difference reached significance (Kaplan Meier log-rank $p=0.03$), and as a result we used the operated controls only to compare the effect of Shh and Shh agonists on survival.

At post-mortem, the catheter was seen to be in position in 8 of 12 treated animals and in 8 of 8 control animals. We analysed the survival results both on an intention-to-treat analysis and a per-protocol analysis (catheter in place intrathecally). Without controlling for the PTCH gene polymorphism (that is, using the usual way of considering these data), controls survived 131.4 ± 2.0 days, and Shh treated animals survived 140.8 ± 3.3 days in the intention to treat analysis ($p=0.02$) (fig 2a) and 147.6 ± 2.3 days ($p<0.001$) (fig 2b) in the per-protocol analysis. Thus, the results were significant in both cases but particularly so in the per-protocol analysis.

We found a single nucleotide polymorphism in the PTCH1 gene, resulting in either 1267T (ACC) or 1267N (AAC). We have sequencing information on 31 animals G93A SOD1 animals, and of these 1 (3.2%) was AAC/AAC (N/N), 7 (22.5%) were ACC/AAC (T/N), and 23 (74.2%) were ACC/ACC (T/T). Representative sequence electropherograms are shown in figure 3. Since this polymorphism confers sensitivity to K5Hras-induced squamous cell skin cancers in FVB/N mice [14], we reasoned that it might also influence survival of our animals to excitotoxic challenge. This proved to be the case.

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In the intention-to-treat group, a Cox logistic regression analysis showed both treatment group and gene status were significant variables (p=0.01 for both). The Cox plot for the effect of gene status on survival is shown in figure 4. When the gene status was entered as a stratified variable in the Kaplan Meier analysis, in those animals with 1267 T/T genotype, Shh treatment resulted in a mean survival of 143.8 ± 3.5 days compared with control survival of 134 ± 2.9 days, while in those animals with 1267 T/N genotype, Shh treatment resulted in a survival of 131.7 ± 6.2 days compared with 124.5 ± 1.5 days. The overall comparison adjusted for gene status was positive and retained about the same significance (p=0.02), suggesting the groups were balanced.

When the gene status group was controlled for in the per-protocol group, in those animals with 1267 T/T genotype Shh treatment resulted in a survival of 148.1 ± 2.6 days compared with control survival of 134 ± 2.9 days, and in those animals with 1267 T/N genotype Shh treatment resulted in a survival of 144 days (1 animal) compared with 124.5 ± 1.5 days for controls. The overall comparison, adjusted for gene status, remained highly significant (p=0.004). A Cox logistic regression analysis showed both treatment group and gene status were significant variables (p=0.003 for both).

Discussion

In the cell culture experiments, some technical factors bear discussion. We have assumed that cell death caused by NMDA (in the presence of CNQX) reflects excitotoxic motor neuron death. This is commonly assumed [15], but it is possible that there is some death of non-neuronal cells at higher NMDA concentrations. Also, we measured cell death using a dead cell protease assay, and in mixed primary culture, death of larger cells (such as motor neurons) might contribute more to the assay than smaller cells (such as glia). As such, the normalization procedure might

be better viewed as normalization of motor neuron death to initial cellular mass rather than to initial cell numbers. Neither of these considerations should introduce systemic bias.

The cell culture experiments were completed before we discovered the PTCH1 polymorphism, and we do not have PTCH1 genotyping on the in vitro experiments. In principle, since each culture was derived from a single embryo, genetic polymorphism in PTCH1 could be controlled for in future experiments.

Shh, purmorphamine, and SAG1 all promote motor neuron survival against an excitotoxic challenge in a dose-responsive fashion. Shh binds to the Patched receptor and thereby releases Smoothened (Smo) to act downstream. Purmorphamine and SAG1 directly activate Smo. There are effects of Shh not mediated by the canonical pathway that do not involve Smo (eg Barnes [16]). Since all three agents seem to produce the same degree of benefit in our assay, this suggests that the benefit of Shh results from activation of the canonical Shh pathway, and eventual activation of the downstream gli transcription factors. In our previous studies, we felt that these agents preferentially benefited G93A SOD1-transfected HT22 cells exposed to oxidative stress, but in the present study using an excitotoxic challenge, wildtype and transgenic cells benefited equally.

There are many reasons why Shh might provide a survival advantage to motor neurons, as the downstream gli transcription factors influence transcription of many factors [17], several of which have been implicated in neurodegenerative disease including CyclinD1 [18], IGF2 [19], Bcl2 [20], TIMP3 [21], Bmi1 [22], FGF10 [23], VEGF [24], Plakoglobin [25], and TSC22 [26].

The in vivo study was technically demanding, and in spite of practice before undertaking these experiments, a significant number of animals still had to be euthanized shortly after surgery. We

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implanted 35 mice, 18 Shh and 17 control. Six of the Shh animals died within a few days of surgery as did 9 of the control animals. The blind introduction of the wire introducer must in some cases cause sufficient trauma to spinal cord, peripheral roots, or the thecal sac to compromise survival, though the exact cause of the morbidity is unknown.

We used 2 control groups: animals implanted with a pump infusing only artificial CSF, and an un-operated control group. We had expected that the 2 control groups would be identical, but somewhat surprisingly the operated controls survived marginally but significantly longer than the un-operated controls. The reasons for this are unclear. It might be that the presence of an intrathecal catheter caused a small CSF leak and greater flow-through of CSF, perhaps important if the CSF contains substances harmful to motor neurons (eg inflammatory signals, etc). Equally, it is possible that some of the difference is artifactual. Since we analysed only those animals that survived surgery, perhaps the more seriously afflicted animals- those otherwise destined to die at an earlier age- were preferentially culled. (The animals showed no signs of impairment at the age of surgery, as signs of impairment in this model generally onset about 40 days later). In any event, we used only the operated controls in the analysis of our experiment, as this is both appropriate (to control for any survival bias as above) and conservative (since any differences attributed to Shh treatment would have been accentuated had we included the un-operated controls.)

At post-mortem, the catheter was seen to be in position in 8 of 12 treated animals and in 8 of 8 control animals. We analysed the survival results both on an intention-to-treat analysis and a 'per-protocol' analysis (catheter intrathecal). The results were significant in both cases, but the results were quite striking in the per-protocol analysis. In this group, animals lived 18 days

longer for a 28 day infusion which ended some 40-50 days prior. (Indeed, the results may be even more significant, since we do not know if the bioactivity of the Shh was fully maintained while in the pump). We have not been able to change the pump without disrupting the intrathecal catheter, to assess the effects of longer treatment periods.

For our experiments, we obtained high-copy SOD1^{G93A} breeding pairs (B6SJL-Tg(SOD1*G93A)1 Gur/J) from the Jackson Laboratory. We maintained the colony by crossing transgene-positive males with transgene-negative dams. There is a reported polymorphism at position 1267 in the Patched gene (PTCH1) of FVB/N mice (1267N), compared to C57BL/6 mice (1267T), that renders FVB/N mice susceptible to the precocious development of K5Hras-induced cutaneous squamous cell carcinomas [14]. On speculation, we sequenced the PTCH1 gene at this locus in our transgenic G93A SOD1 mice, and somewhat surprisingly, found the same 1267N/T polymorphism with significant frequency (about 25%). We reasoned that this polymorphism, presumably arising from the SJL background, could alter the basal activity of the Shh pathway, or alter the response to Shh or Shh agonists, and we included the polymorphism status of the animals in our analysis.

There are three points to be made here. First, controlling for the polymorphism status in the statistical analysis did not alter the main conclusion that Shh is neuroprotective in this model, in either the intention-to-treat or the per protocol analysis. Second, we found that the PTCH1 polymorphism is an independent predictor of survival in our animals. Third, including an interaction term in the Cox analysis conferred no additional improvement to the model (p=0.66) and we conclude that the effects of Shh were not preferentially seen in one or other of the

polymorphism groups. (However, our numbers are small and a minor interaction remains possible.)

Wakabayashi et al [14] conclude that the PTCH1 1267 N/N genotype confers resistance to apoptotic challenge, that might thereby predispose to the precocious squamous cell tumour formation they observed. One might therefore assume that this genotype would prolong survival in a neurodegenerative disease model, yet we found that these animals performed less well, and explanations for this effect will have to be more complicated that a simple down-regulation in hedgehog signalling that we had initially envisaged. (Wayabayashi et al note that the system is complex even for cancer, and that the 1267 T/T genotype might lead to a relative reduction in squamous cell skin cancers but an increase in basal cell skin cancers.)

Variability in the G93A SOD1 model has plagued ALS research, and Heiman-Patterson et al [27] showed that strain differences (between B6 and SJL backgrounds) significantly alter the progression of disease. Animals expressing G93A SOD1 on a B6 background live much longer than those with a SJL background. It is possible that the polymorphism we have identified accounts for some of this difference. It is also highly probable that the polymorphism frequencies would change as a colony is maintained, and with time one might expect selection pressures to result in a greater allelic percentage of PTCH1 1267T (more like B6) and less PTCH1 1267N (like SJL).

Purmorphamine and SAG1 are oral agents, and might have greater attraction as therapeutics than an agent such as Shh that requires intrathecal administration. However, there is a theoretical risk of carcinogenesis that would be mitigated by lower dose of intrathecal administration. We are presently testing these oral agents in the G93A SOD1 model of ALS. As a final conclusion, we

believe that developmental morphogens, including but not limited to Shh, warrant further

scrutiny in ALS and other neurodegenerative disease models.

References

1. Siddique T, Ajroud-Driss S 2011) Familial amyotrophic lateral sclerosis, a historical perspective. Acta Myol;30(2):117-20.

2. Rothstein JD (2009) Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. Ann Neurol;65:S3-9.

3. Tsuboi K, Shults CW (2002) Intrastriatal injection of sonic hedgehog reduces behavioral impairment in a rat model of Parkinson's disease. Exp Neurol;173:95-104.

4. Calcutt NA, Allendoerrfer KL, Mizisin AP, Middlemas A, Freshwater JD et al. (2003) Therapeutic efficacy of sonic hedgehog protein in experimental diabetic neuropathy. J Clin Invest;111(4):507-514.

5. Dass B, Iravani MM, Huang C, Barsoum J, Engber TM, Galdes A, Jenner P (2005) Sonic hedgehog delivered by an adeno-associated virus protects dopaminergic neurones against 6-OHDA toxicity in the rat. J Neural Transm;112:763-778.

6. Dellovade T, Romer JT, Curran T, Rubin LL (2006) The hedgehog pathway and neurological disorders. Annual review of neuroscience;29:539-563.

7. Farzan SF, Singh S, Schilling NS, Robbins DJ. (2008) The adventures of sonic hedgehog in development and repair. III. Hedgehog processing and biological activity. Am J Physiol;294:G844-849.

8. Stenkamp DL, Satterfield R, Muhunthan K, Sherpa T, Vihtelic TS, Cameron DA (2008) Age-related cone abnormalities in zebrafish with genetic lesions in sonic hedgehog. Invest Ophthalmol Vis Sci;49:4631-40.

9. Dai RL, Zhu SY, Xia YP, Mao L, Mei YW, et al. (2011) Sonic Hedgehog protects cortical neurons against oxidative stress. Neurochem Res;36910:67-75.

10. Peterson R, Turnbull J (2011) Sonic Hedgehog is Cytoprotective against Oxidative Challenge in a Cellular Model of Amyotrophic Lateral Sclerosis. J Mol Neurosci; Oct 7. PMID: 21979788.

11. Ma X, Peterson R, Turnbull J (2011) Adenylyl cyclase type 3, a marker of primary cilia, is reduced in primary cell culture and in lumbar spinal cord in situ in G93A SOD1 mice. BMC Neurosci;12:71-82.

12. Wells K, Anderson DK, Farooqui AA, Horrocks LA (1994) Excitotoxicity of glutamate and four analogs in primary spinal cord cell cultures. Neurochem Int;25(4):377-84.

13. Ohta Y, Nagai M, Nagata T, Murakami T, Nagano I et al. (2006) Intrathecal injection of epidermal growth factor and fibroblast growth factor 2 promotes proliferation of neural precursor cells in the spinal cords of mice with mutant human SOD1 gene. J Neurosci Res;84(5):980-92.

14. Wakabayashi Y, Mao JH, Brown K, Girardi M, Balmain A. (2007) Promotion of Hras-induced squamous cell carcinomas by a polymorphic variant of the Patched gene in FVB mice. Nature;445:761-5.

15. Lau AC, Cui H, Tymianski M. (2007) The use of propidium iodide to assess excitotoxic neuronal death in primary mixed cortical cultures. Methods of molecular biology: Neuroprotection methods and protocols;399:15-29. Borsello T ed, Humana Press.

16. Barnes EA, Kong M, Ollendorff V, Donoghue DJ. (2001) Patched1 interacts with cyclin B1 to regulate cell cycle progression. EMBO J;20(9):2214-23.

17. Chari NS, McDonnell TJ. (2007) The sonic hedgehog signalling network in development and neoplasia. Adv Anat pathol;14(5):344-52.

18. Nguyen MD, Boudreau M, Kriz J, Couillard-Després S, Kaplan DR, Julien JP. (2003) Cell cycle regulators in the neuronal death pathway of amyotrophic lateral sclerosis caused by mutant superoxide dismutase 1. J Neurosci;23(6):2131-40.

19. Kihira T, Suzuki A, Kubo T, Miwa H, Kondo T. (2007) Expression of insulin-like growth factor-II and leukemia inhibitory factor antibody immunostaining on the ionized calcium-binding adaptor molecule 1-positive microglias in the spinal cord of amyotrophic lateral sclerosis patients. Neuropathology;27(3):257-68.

20. Reyes NA, Fisher JK, Austgen K, VandenBerg S, Huang EJ, Oakes SA. (2010) Blocking the mitochondrial apoptotic pathway preserves motor neuron viability and function in a mouse model of amyotrophic lateral sclerosis. J Clin Invest;120(10):3673-9.

21. Hoe HS, Cooper MJ, Burns MP, Lewis PA, van der Brug M, et al. (2007) The metalloprotease inhibitor TIMP-3 regulates amyloid precursor protein and apolipoprotein E receptor proteolysis. J Neurosci;27(40):10895-905.

22. Chatoo W, Abdouh M, David J, Champagne MP, Ferreira J, et al. (2009) The polycomb group gene Bmi1 regulates antioxidant defenses in neurons by repressing p53 pro-oxidant activity. J Neurosci;29(2):529-42.

23. Williams AH, Valdez G, Moresi V, Qi X, McAnally J, et al. (2009) MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. Science;326(5959):1549-54.

24. Lambrechts D, Storkebaum E, Morimoto M, Del-Favero J, Desmet F. (2009) VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat Genet;34(4):383-94.

25. Raurell I, Castaño J, Francí C, García de Herreros A, Duñach M. (2006) Presenilin-1 interacts with plakoglobin and enhances plakoglobin-Tcf-4 association. Implications for the regulation of beta-catenin/Tcf-4-dependent transcription. J Biol Chem;281(3):1401-11.

26. Wu X, Yamada-Mabuchi M, Morris EJ, Tanwar PS, Dobens L, et al. (2008) The Drosophila homolog of human tumor suppressor TSC-22 promotes cellular growth, proliferation, and survival. Proc Natl Acad Sci USA;105(14):5414-9.

27. Heiman-Patterson TD, Deitch JS, Blankenhorn EP, Erwin KL, Perreault MJ et al. (2005) Background and gender effects on survival in the TgN(SOD1-G93A)1Gur mouse model of ALS. J Neurol Sci;236(1-2):1-7.

Acknowledgement
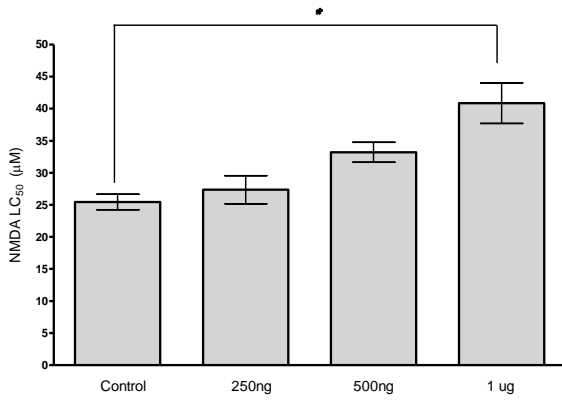
Figure 1a. Sonic Hedgehog in WT motor neurons
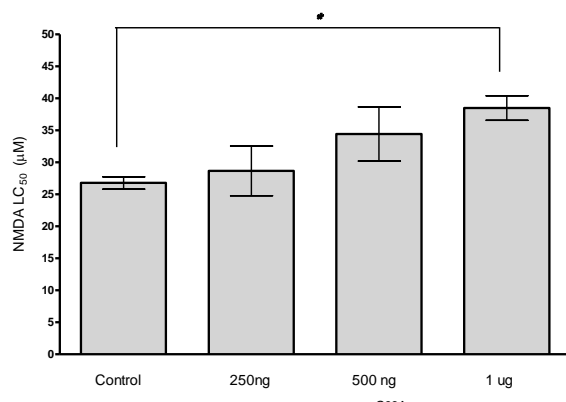
Figure 1b. Sonic Hedgehog in SOD1 [G93A] Motor Neurons
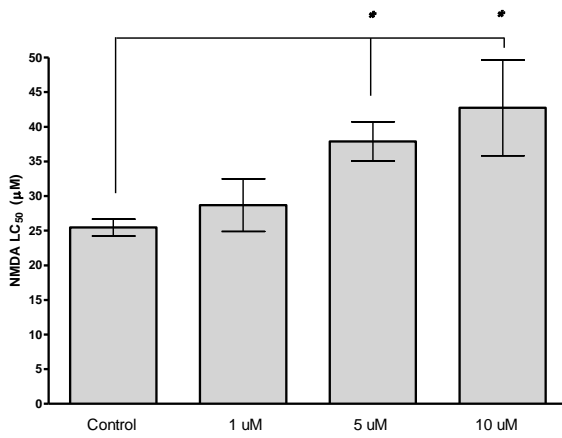
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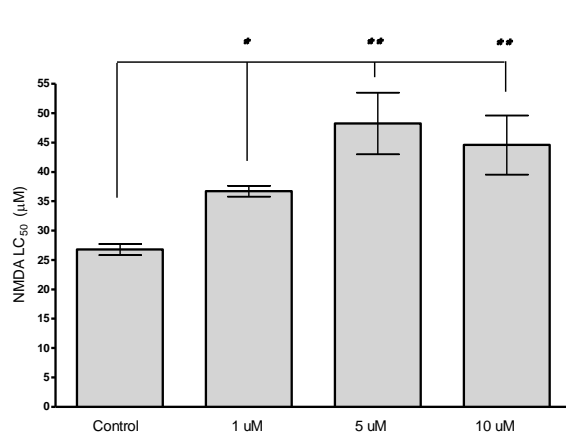
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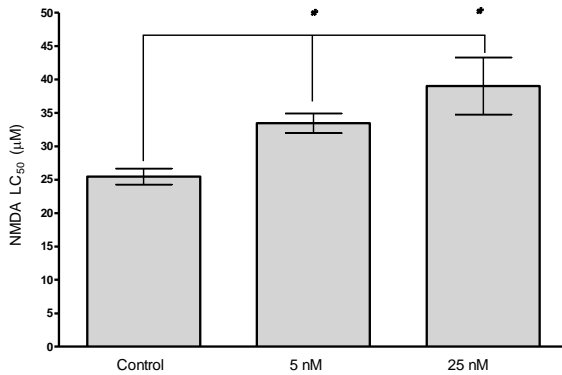
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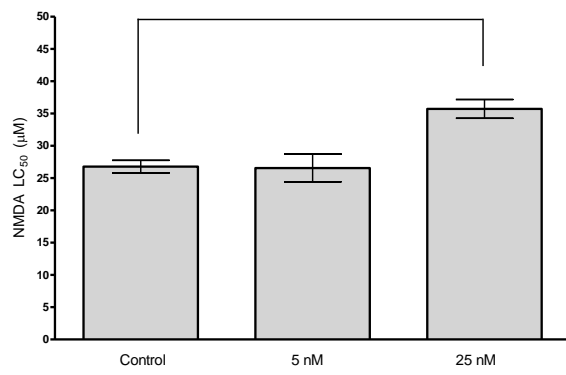
Figure 1f. SAG-1 in SOD1 [G93A] Motor Neurons

Figure 1a-f. Administration of rhShh or Shh Agonists increases resistance to excitotoxic challenge. Wild-type (1a,c,e) and SOD1^{G93A} (1b,d,f) primary mixed spinal cord cultures were treated for 24hrs with 3 doses of either recombinant hShh protein (1a,b), Shh agonist purmorphamine (1c,d), or SAG-1 (1e,f) followed by a 24hr NMDA challenge at a dose range of 8 NMDA concentrations. Values represent the calculated LC$_{50}$ of NMDA (mean ± SEM) for each condition and control. Significance was determined by a one-way ANOVA with a Dunnett's multiple comparison post-hoc test (*$p<0.05$, **$p<0.001$).
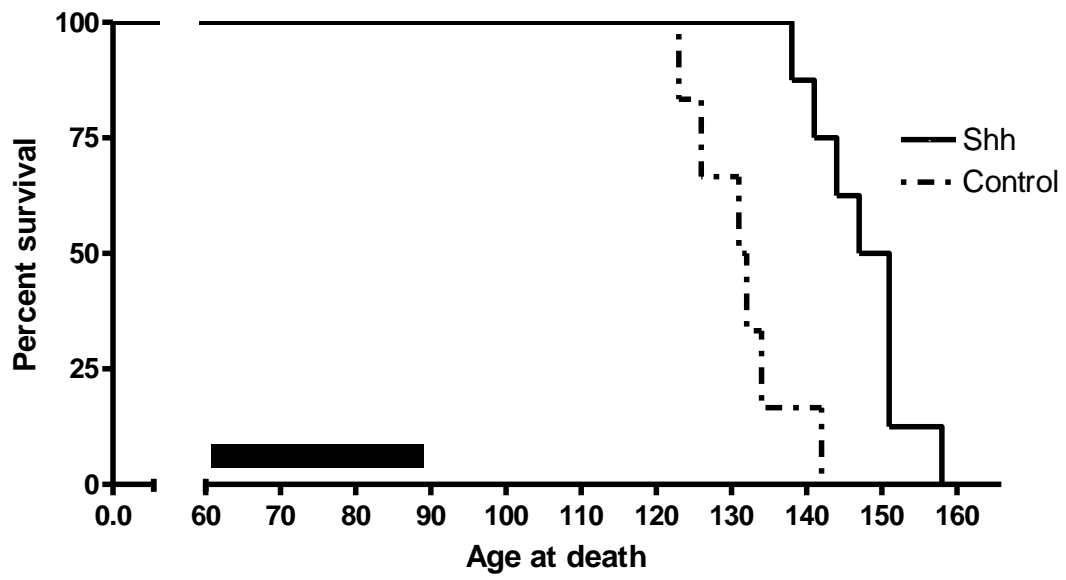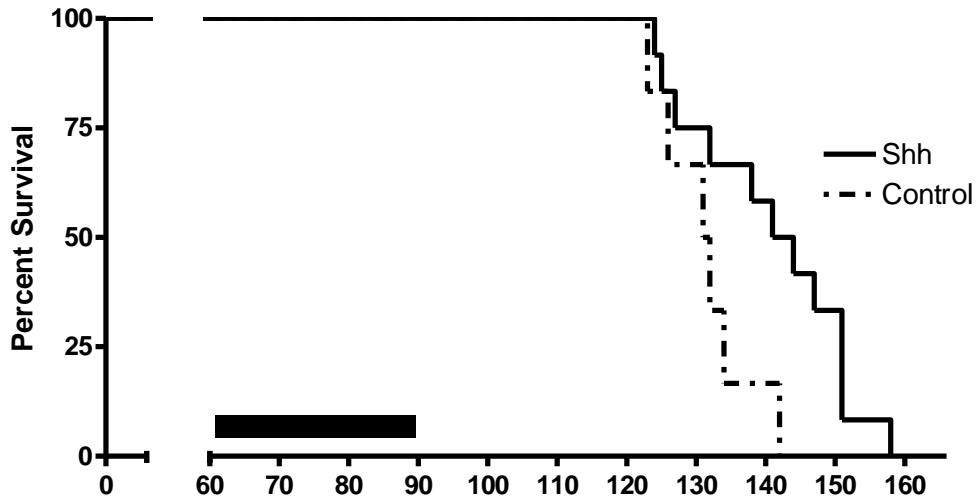
Figure 2 a,b. **Kaplan Meier plots of survival of Shh treated and control mice.** The upper graph shows survival in the intention to treat analysis, and the lower graph the per protocol analysis. The black bar represents the time Shh or medium was infused via the osmotic minipump. Both curves are significant when analysed alone, and when the PTCH1 1267 polymorphism was entered as a covariate.

ACC/ACC   ACC/AAC   AAC/AAC
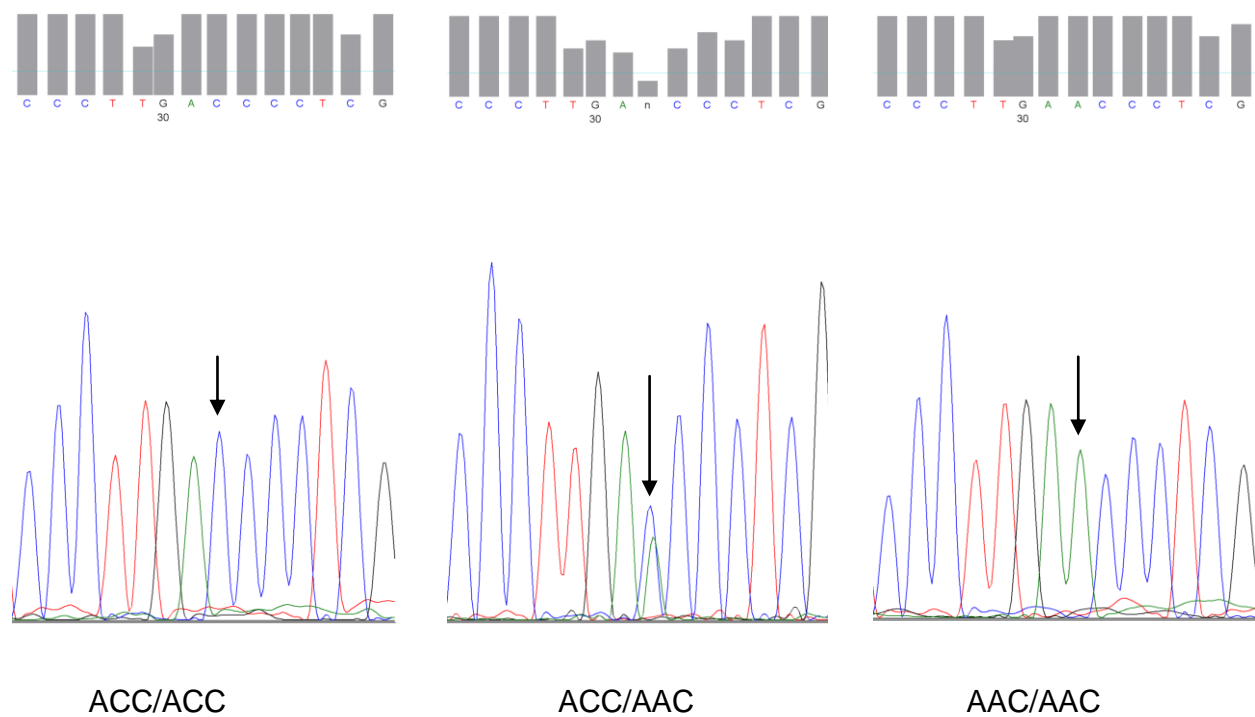
Figure 3. Sequencing electropherograms showing the T/N polymorphism at 1267 in the PTCH1 gene. Most mice (74%) were ACC/ACC (T/T) (left); some (24%) were ACC/AAC (middle), while a single mouse was AAC/AAC (N/N).
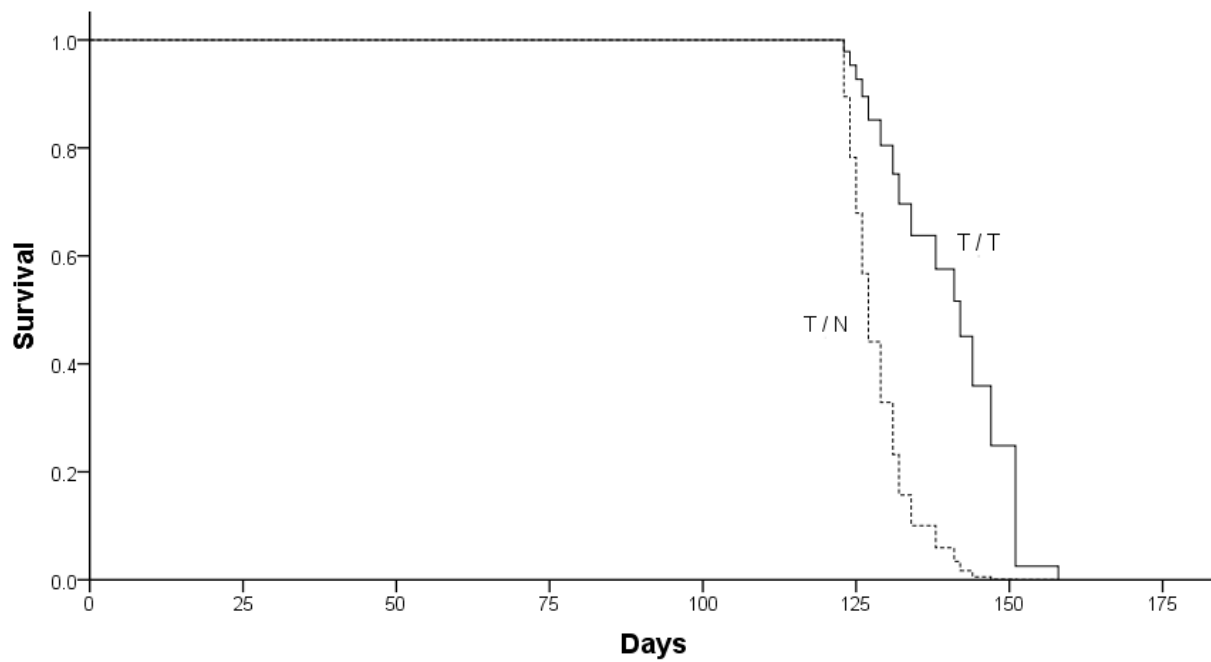
Figure 4. Survival at mean of covariates (ie Shh/Control) plotted for Patched

polymorphism. Animals with PTCH 1267 T/N did less well than animals with PTCH 1267 T/T.

------CHAPTER 5------

DISCUSSION

5.0 Discussion

5.1 Context

The investigations presented in this dissertation are the first of their kind in the field of ALS research and represent our efforts to demonstrate the utility of Shh and its agonists for the treatment of motor neuron disease. This includes preliminary proof-of-concept studies in a defined cell-culture system, which were followed by investigations in a primary cell-culture model of ALS and culminated in an animal study focusing on survival. To avoid repeating material presented in the introduction and in the discussion sections of Chapter 2-4, the following text emphasizes the integration of the findings into a broader context. A discussion of outstanding issues is also provided, along with suggestions for future experiments for elucidating the role of Shh as a therapeutic agent.

5.2 Shh increases resistance to oxidative stress

Historically the importance of Shh during neurodevelopment is relatively well defined. A recent study, that demonstrated the activation of the Shh pathway in primary rodent cortical neurons under oxidative stress [1], led us to hypothesize that the Shh signalling cascade may be an important modulator of free radical damage in the context of transient expression of mutant SOD1. The results of our first study are presented in Chapter 2. They illustrate that treating transiently transfected cells with either recombinant human Shh protein, or the Shh agonist purmorphamine, conferred some protection against a subsequent oxidative challenge. Downregulation of the Shh signalling cascade by pharmacological means (cyclopamine), or genetic manipulation (miRNA 324-5p), had the opposite effect of sensitizing these cells to oxidative challenge. A closer inspection of the individual members of the Shh pathway, at the level of relative mRNA expression, revealed that mutant SOD1 expression had a negative impact

on the transcript levels of crucial effectors of Shh signalling. This involved Gli2 and Gli3, and resulted in a decrease in output from a Gli mediated transcriptional reporter.

These results constitute the first of their kind examining how mutant SOD1 could impact the Shh pathway. We built on published reports which indicated that Shh mediated neuroprotection by regulating key intracellular stress factors, and also a consideration of the synergistic cooperation between Shh and BDNF [1-4]. Specifically, Schwann cells demonstrated increased expression of Shh directly adjacent to the crush site in a rodent nerve-crush model, and this elevated expression was directly correlated with an increase in BDNF expression [4]. A second study using a primary rat cortical neuron culture model went on to demonstrate that the elevated expression of Shh was induced by BDNF, and that Shh pre-conditioning resulted in neuroprotection against 3-nitropropinonic acid toxicity in a model of Huntington's disease [2].

A different group verified the importance of Shh as a neuroprotective agent utilizing the same experimental system, but focused mainly on oxidative stress *via* exposure to hydrogen peroxide as the challenge medium [1]. This study also showed that exogenous administration of Shh increased both the activity and transcript levels of SOD1 and glutathione peroxidase (GSH-PX), implying a direct relationship between Shh and SOD1 functioning. Shh also promoted expression of the anti-apoptotic Bcl-2 protein, with a concurrent inhibition of the pro-apoptotic protein Bax--both of which have been shown to be deregulated in ALS-affected tissues [5]. This study also confirmed the upregulation of BDNF and vascular endothelial growth factor (VEGF).

Neurotrophic factors have been known to modulate expression from Gli responsive promoters. Endothelial Growth Factor (EGF) was shown to act synergistically with Gli proteins, leading to transcription of growth regulating factors such as CyclinD1, S100A7, S1009a, and also the

cytokine interleukin (IL) antagonist IL1R2 [6]. Conversely, Fibroblast Growth Factor (FGF) blocks the proliferative response elicited by Shh/Gli induction upstream of Gli activation. This anti-proliferative action of FGF was dependent upon FGF receptor engagement along with the concurrent action of both ERK and JNK kinases [7].

The Shh/Gli axis has also been shown to upregulate a cadre of different mitogenic factors (such as IGF2, N-Myc, TIMP3, Bim1, Snail, FOXF1, FOXM1, FOXE1, NR2F2, FGF10, and PKCε), which are responsible for regulating cell growth and homeostasis [8]. Interestingly, the inflammatory cytokine Interferon Gamma (IFNγ) was shown to block Gli expression in the presence of abundant Shh in a rodent model of multiple sclerosis [9], thereby demonstrating a link between neuroinflammation and attenuation of the Shh pathway.

Taken together, the cumulative results of the investigations reviewed frame the underlying biochemical mechanism of Shh mediated neuroprotection. A general model can thus be put forth whereby, upon sensing of stress, Shh expression is upregulated. This in turn triggers Gli mediated transcription *via* the canonical pathway resulting in increased activity of SOD1 and GSH-PX, a concurrent upregulation of the anti-apoptotic protein Bcl-2, downregulation of the pro-apoptotic Bax, and increased expression of a number of neurotrophic and mitogenic factors. The effectiveness of these responses may be reduced in SOD1^{G93A} models, and by extension, may be reduced in other ALS and neurodegenerative disease models. As such, our findings suggest that exogenous Shh treatment might be a viable therapeutic option to increase cellular resistance to oxidative stress.

Although these initial findings were novel and represented the establishment of the potential role of Shh in neuroprotection, they nevertheless focused on cortical neuronal cell-lines which do not

accurately portray the cellular or physiological environment of the spinal cord. A more applicable experimental model was needed to further extend these investigations and make them more pertinent to ALS. The observations presented in Chapters 3 and 4 are based upon findings from studies involving the use of the SOD1^{G93A} mouse model.

5.3 Cilial profile in the SOD1^{G93A} mouse model of ALS

Shh is a secreted extracellular signalling morphogen which binds its cognate receptor Patched located on the surface of target cells. Although research into Shh signalling has advanced in the past 30 years the preponderance of Shh signal transduction was discovered only recently to be restricted to one particular organelle; specifically, the primary cilium (reviewed in detail in Chapter 1). The latter has been hypothesized to exist on the vast majority of cell types, but has only been demonstrated to exist on certain cell lineages [10]. In Chapter 3, we present the first qualitative phenotypic assessment of primary cilia in the spinal cord. Overall, it revealed a differential cilial profile between wild type (WT) and SOD1^{G93A} mice. The decrease in the proportion of ciliated motor neurons in culture and in the ventral horn of SOD1^{G93A} mice might indicate that a deficiency in Shh signalling exists in these cells. This could also indicate aberrant Shh signalling in ALS in general. Although a conclusion on whether or not ALS can now be classified as a ciliopathy cannot be made at this point, it is nonetheless a noteworthy observation which begs further study.

The term 'ciliopathy' describes a category of ailments which until a few years ago, were thought to constitute syndromes that were rare and isolated. Currently, this new classification of diseases encompasses a number of different illnesses with new discoveries being added to the list frequently. Our research suggests that ciliopathies might include neurodegenerative diseases.

Indeed in a very recent discovery in the field of Huntington's disease research has implicated abnormal ciliogenesis and morphology as key pathological features of the disease [11]. This study demonstrated that the Huntington's gene (Htt) and its protein product were involved in the normal assembly of the cilia, and that deletion or mutation of the gene led to either complete cilial ablation or abnormal cilial morphology. The functional outcome of Htt mutation was aberrant cilial orientation in cerebral ventricles of Htt transgenic mice, which resulted in abnormal flow of cerebral spinal fluid (CSF). The abnormal flow of CSF maybe especially deleterious because it results in the impairment of neuronal migration to the olfactory bulb [12], and may also alter homeostatic control of brain functioning and result in to a build-up of toxic metabolic-waste products. Aberrant CSF movement may also interfere with the normal Shh delivery to more distal targets. Within the brain, Shh production is restricted to cells in the subventricular zone (SVZ) of the lateral ventricles (LV) and the subgranular zone of the dentate gyrus [13, 14]. Disruption of typical CSF flow would presumably attenuate paracrine induction of distal cellular targets.

These investigations mark the starting point for the delineation of the role of cilia in neurodegeneration and provides the ground work for further research into cilial involvement in other neurological diseases such as Parkinson's and Alzheimer's.

5.4 Effect of Shh on primary mixed spinal cord cultures and the SOD1^{G93A} mouse model.

The research summarized in Chapter 4 showed that administration of recombinant human Shh (or its agonist) elicited neuroprotection in a primary cell culture model of ALS, and that chronic Shh administration prolonged survival of SOD1^{G93A} mice. Shh, purmorphamine, and SAG-1 all had neuroprotective effects on mixed spinal cord cultures of both WT and SOD1^{G93A} cultures. These observations complimented our initial findings in HT22 cells (Chapter 2). Pre-treatment of these cultures with these compounds resulted in an increase in the relative lethal concentration (LC$_{50}$) of NMDA necessary to kill 50% of the motor neurons (i.e., increased resistance to excitotoxic damage).

The most significant increase in resistance occurred when cultures were treated with the Shh agonist purmorphamine, which exerts its action downstream of Patched receptor binding by Shh at the level of Smoothened activation (reviewed in Chapter 1). Purmorphamine pre-treatment seemed to have the greatest effect on neuronal survival in the mutant mice, with even the lowest concentration conferring protective effects. Similar results were produced with SAG-1 which is another Shh agonist that directly binds to and activates Smoothened. Given the demonstrated beneficial agonism of Smoothened it can be concluded that the beneficial effect of activation of the Shh pathway occurs *via* the traditional canonical pathway.

While this work does not explore the underlying biochemical mechanism by which Shh and its agonist elicits neuroprotection, it would not be far-fetched to surmise similar molecular interplay between BDNF, SOD1, GSH and other members (discussed above). Such activity might not be limited to motor neurons but also occur in other glial lineage cells of the embryonic spinal cord. In addition, one cannot discount the possible effect of Shh treatment on the endogenous action of

the primary cilia. Our primary culture model allowed for closer approximation of the cellular niche in which the treatment might be administered in humans.

In the second part of this investigation, chronic intrathecal delivery of the recombinant human Shh protein to SOD1^{G93A} mice over a period of one month resulted in extension of survival of SOD1^{G93A} mice. Analysis of survival was based upon both intention-to-treat (ITT) and per-protocol (PP) analysis. Although, the results were significant in both cases, the PP results were more striking. Shh treatment of SOD1^{G93A} mice in the ITT group resulted in a life-extension of 11 days over the control group (Control=131.5 days, SOD1^{G93A}=142.5 days). By contrast, the increase in life-span was 17.5 days in the PP group (Control=131.5 days, SOD1^{G93A} =149.0 days).

It is noteworthy to mention that the recombinant human Shh which has been used throughout these investigations did not possess the post-translational modifications inherent to *in vivo* produced Shh protein. The C-termini of Shh is coupled to cholesterol, while the N terminal portion is palmitoylated, and these lipid modifications enhance the ability of Shh to interact with membrane surfaces, and also potentiate its secretion. Likewise, these modifications can direct the dynamic action of Shh to be more locally active or to be transported to more distal regions [15]. As described in the materials and methods sections of Chapters 2-4, the recombinant Shh protein used was the N-terminal portion which did not possess lipid modification. The lack of these alterations may have aided the diffusability of the protein within the spinal cord of treated animals by allowing the protein to move freely within the CSF, instead of being locally sequestered by binding to available cellular membranes.

The observations presented in Chapter 4 demonstrated that Shh agonism does indeed confer neuroprotection in a primary culture model of ALS. More importantly, Shh therapy can extend survival in the animal model. Having been proven effective, speaking generally the Shh signalling pathway and more specifically the compounds tested have the potential of becoming candidates in human therapeutic development trials.

5.5 Effect of Ptch1 polymorphism on primary culture results and survival of SOD1^{G93A} mouse model

The SOD1^{G93A} mouse model is maintained on a mixed B6SJL genetic background. Along with mice used in the survival study, primary mixed spinal-cord cultures were obtained from transgenic embryos from the successful crossing of wild-type dams with transgenic SOD1^{G93A} males. Recently a report has indicated that a unique polymorphism within the Ptch1 gene, at position 1267, rendered FVB/N mice more susceptible to K5Hras-induced cutaneous squamous cell carcinomas [16]. To elucidate whether our mouse model possessed this same polymorphism, we sequenced the Ptch1 gene of animals within the survival study. Approximately 25% of the transgenic animals were positive for the 1267N/T polymorphism. The effect of this polymorphism with respect to the applied Shh treatment *in vitro* could have resulted in altered basal activity of the Shh signalling cascade thereby leading to attenuation of the true magnitude of Shh mediated resistance to excitotoxicity.

Survival of SOD1^{G93A} transgenic animals has been reported to be affected by the genetic background onto which the transgene is being expressed [17]. Animals which expressed the transgene on a pure B6 background had a more significant extension of survival than those who were bred on a pure SJL background. A possible contributing factor to this apparent differential survival could be due to the polymorphism in Ptch1. Although this observation cannot account

for the entire variance in survival between different strains of mice, it does however remain a very significant finding. For the first time, polymorphism in Ptch1 is suggested to be associated with altered survival in a neurodegenerative disease model.

5.6 Future studies

Several questions remain unanswered which need to be addressed in relation to Shh-mediated neuroprotection before the potential of this approach can be fully realized. An evaluation of the contribution of other cell-types (i.e., astrocytes, glia, etc.) to the survival of motor neurons when Shh/agonist is administered needs further investigation. In addition, the mechanisms of Shh neuroprotection outlined earlier in the papers and discussion need further clarification. Also a more detailed examination of the effects of both chronic and acute Shh/agonist treatment on the cilial profile of motor neurons would be beneficial, along with a more detailed understanding of whether the cilial profile is a good indicator of neuronal survival when Shh is administered. In addition, a more detailed survey is warranted of the cilial profile of the cerebrum of $SOD1^{G93A}$ mice, paying particular attention to the ventricles. Such investigation would explore whether aberrant cilial morphology or orientation is apparent as observed in Huntington's disease.

As previously mentioned, the downstream effectors of Shh signalling are the GLI family of transcriptional activators. It would be interesting to examine the expression profile of gene(s) activated by these factors in motor neurons, and whether inactivation (if any) of these targets occurs in the disease state. Given the success of the *in vivo* Shh treatment, further investigations testing the efficacy of purmorphamine and SAG-1 would be the logical next step in animal studies. They are chemically more stable, readily pass the blood-brain barrier, have very good bioavailability, and also possess much lower effective doses than the recombinant Shh protein

(reviewed in Chapter 1). They can also be injected intraperitoneally (IP) or intramuscularly (IM), avoiding the invasiveness of intrathecal pumps.

The effectiveness of Shh in embryogenesis has previously been proven to be augmented when combined with retinoic acid [18]. Retinoic acid (RA) is a derivative of vitamin A, and has been demonstrated to possess a variety of metabolic functions as an anti-oxidant, and also as a cellular differentiation factor [19]. Similar to Shh, RA has also been shown to possess neuroprotective capabilities against oxidative stress damage [20, 21]. Thus far, the focus of the investigations into the synergistic capabilities of Shh and RA have only been directed to their role in developmental differentiation of neural stem cells [22-25]. Our findings would suggest that inclusion of RA into future experimental studies might aid in Shh-directed neuroprotection.

Indeed, the idea of combination therapies for the treatment of ALS in not a new one (reviewed in Chapter 1). In terms of our new research perspective, the potential for novel permutations of synthetic molecules (such as purmorphamine, and SAG-1) with the recombinant Shh protein and/or RA represents a new frontier in ALS therapeutics. In addition, these therapies can be used in conjunction with other trialled drugs to determine if they alter their already reported efficacy on disease. The potential for new treatments using Shh/agonist, along with RA represents a new and exciting avenue of research which holds great promise; not only in the field of ALS, but also for the understanding and treatment of other neurodegenerative diseases.

A further extension of this work would include detailed surveys of the cilial profile in spinal cords and brains of ALS patients who have succumbed to the disease. Analysis of the CSF for both bioactivity and endogenous levels of Shh would aid in the understanding of the role this morphogen plays during disease progression. This would also indicate if there was any loss of

activity of Shh attributable to the disease. Determination of the pre-existing levels of Shh in the CSF of ALS patients would help elucidate whether the mode of action of Shh was either pharmacological or physiological. An *a priori* low level of Shh in the CSF might indicate that any beneficial effect of Shh treatment is due to physiologic replacement/augmentation back to basal levels. By contrast, normal levels of Shh would indicate that the positive effect of Shh was associated with a pharmacological mode of action.

Genetic screening of blood or tissue samples from ALS patients for polymorphisms in genes within the Shh signalling cascade seems warranted. The identification of genes associated with decreased or uncharacteristic activity would help elucidate the efficacy of any applied Shh based treatment and would also assist in tailoring better therapeutic strategies. It seems not unreasonable to suggest that in the foreseeable future Shh levels or activity in the CSF, along with the detection of polymorphisms may become reliable tools for the prognostication of ALS and aid in its diagnosis.

Even though he importance of the Shh signalling pathway is no doubt a key player in the health and maintenance of spinal cord motor neurons, by extension of the advancements reported here might have applicability to research of other neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's diseases. Shh therapeutics may also have potential in the treatment of spinal cord injuries and diabetic neuropathies.

References

[1]   Dai RL, Zhu SY, Xia YP, Mao L, Mei YW, Yao YF, et al. Sonic hedgehog protects cortical neurons against oxidative stress. Neurochemical research. Jan;36(1):67-75.

[2]   Wu CL, Chen SD, Hwang CS, Yang DI. Sonic hedgehog mediates BDNF-induced neuroprotection against mitochondrial inhibitor 3-nitropropionic acid. Biochemical and biophysical research communications. 2009 Jul 17;385(1):112-7.

[3]   Wu CL, Chen SD, Yin JH, Hwang CS, Yang DI. Erythropoietin and sonic hedgehog mediate the neuroprotective effects of brain-derived neurotrophic factor against mitochondrial inhibition. Neurobiology of disease. Oct;40(1):146-54.

[4]   Hashimoto M, Ishii K, Nakamura Y, Watabe K, Kohsaka S, Akazawa C. Neuroprotective effect of sonic hedgehog up-regulated in Schwann cells following sciatic nerve injury. Journal of neurochemistry. 2008 Nov;107(4):918-27.

[5]   Mu X, He J, Anderson DW, Trojanowski JQ, Springer JE. Altered expression of bcl-2 and bax mRNA in amyotrophic lateral sclerosis spinal cord motor neurons. Annals of neurology. 1996 Sep;40(3):379-86.

[6]   Kasper M, Schnidar H, Neill GW, Hanneder M, Klingler S, Blaas L, et al. Selective modulation of Hedgehog/GLI target gene expression by epidermal growth factor signaling in human keratinocytes. Molecular and cellular biology. 2006 Aug;26(16):6283-98.

[7]   Fogarty MP, Emmenegger BA, Grasfeder LL, Oliver TG, Wechsler-Reya RJ. Fibroblast growth factor blocks Sonic hedgehog signaling in neuronal precursors and tumor cells. Proceedings of the National Academy of Sciences of the United States of America. 2007 Feb 20;104(8):2973-8.

[8]   Chari NS, McDonnell TJ. The sonic hedgehog signaling network in development and neoplasia. Advances in anatomic pathology. 2007 Sep;14(5):344-52.

[9]   Wang Y, Imitola J, Rasmussen S, O'Connor KC, Khoury SJ. Paradoxical dysregulation of the neural stem cell pathway sonic hedgehog-Gli1 in autoimmune encephalomyelitis and multiple sclerosis. Annals of neurology. 2008 Oct;64(4):417-27.

[10]   Louvi A, Grove EA. Cilia in the CNS: the quiet organelle claims center stage. Neuron. Mar 24;69(6):1046-60.

[11]   Keryer G, Pineda JR, Liot G, Kim J, Dietrich P, Benstaali C, et al. Ciliogenesis is regulated by a huntingtin-HAP1-PCM1 pathway and is altered in Huntington disease. The Journal of clinical investigation. Nov 1;121(11):4372-82.

[12]   Sawamoto K, Wichterle H, Gonzalez-Perez O, Cholfin JA, Yamada M, Spassky N, et al. New neurons follow the flow of cerebrospinal fluid in the adult brain. Science (New York, NY. 2006 Feb 3;311(5761):629-32.

[13]   Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, et al. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron. 2003 Sep 11;39(6):937-50.

[14]   Palma V, Ruiz i Altaba A. Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. Development (Cambridge, England). 2004 Jan;131(2):337-45.

[15]   Peters C, Wolf A, Wagner M, Kuhlmann J, Waldmann H. The cholesterol membrane anchor of the Hedgehog protein confers stable membrane association to lipid-modified proteins. Proceedings of the National Academy of Sciences of the United States of America. 2004 Jun 8;101(23):8531-6.

[16]   Wakabayashi Y, Mao JH, Brown K, Girardi M, Balmain A. Promotion of Hras-induced squamous carcinomas by a polymorphic variant of the Patched gene in FVB mice. Nature. 2007 Feb 15;445(7129):761-5.

[17]   Heiman-Patterson TD, Deitch JS, Blankenhorn EP, Erwin KL, Perreault MJ, Alexander BK, et al. Background and gender effects on survival in the TgN(SOD1-G93A)1Gur mouse model of ALS. Journal of the neurological sciences. 2005 Sep 15;236(1-2):1-7.

[18]   Appel B, Eisen JS. Retinoids run rampant: multiple roles during spinal cord and motor neuron development. Neuron. 2003 Oct 30;40(3):461-4.

[19]   Lee HP, Casadesus G, Zhu X, Lee HG, Perry G, Smith MA, et al. All-trans retinoic acid as a novel therapeutic strategy for Alzheimer's disease. Expert review of neurotherapeutics. 2009 Nov;9(11):1615-21.

[20]   Katsuki H, Kurimoto E, Takemori S, Kurauchi Y, Hisatsune A, Isohama Y, et al. Retinoic acid receptor stimulation protects midbrain dopaminergic neurons from inflammatory degeneration via BDNF-mediated signaling. Journal of neurochemistry. 2009 Jul;110(2):707-18.

[21]   Wang S, Hu CP, Jiang DJ, Peng J, Zhou Z, Yuan Q, et al. All-trans retinoic acid inhibits cobalt chloride-induced apoptosis in PC12 cells: role of the dimethylarginine dimethylaminohydrolase/asymmetric dimethylarginine pathway. Journal of neuroscience research. 2009 Jun;87(8):1938-46.

[22]   Wang ZD, Xue Y, Shan ZY, Zheng Z, Li X, Wu YS, et al. [Generation of mouse parthenogenetic embryonic stem cells and preliminary study of the differentiation ability to motor neurons]. Yi chuan = Hereditas / Zhongguo yi chuan xue hui bian ji. Nov;33(11):1231-8.

[23]   Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, et al. Specification of motoneurons from human embryonic stem cells. Nature biotechnology. 2005 Feb;23(2):215-21.

[24]   Murashov AK, Pak ES, Hendricks WA, Owensby JP, Sierpinski PL, Tatko LM, et al. Directed differentiation of embryonic stem cells into dorsal interneurons. Faseb J. 2005 Feb;19(2):252-4.

[25]   Miles GB, Yohn DC, Wichterle H, Jessell TM, Rafuse VF, Brownstone RM. Functional properties of motoneurons derived from mouse embryonic stem cells. J Neurosci. 2004 Sep 8;24(36):7848-58.

6.0 Concluding Remarks

Taken together the work presented in this thesis constitutes the first investigation into the utility of Shh and its agonists as possible therapeutic agents for the treatment of ALS. As well, there may be implications for other neurodegenerative conditions. One of the key findings of this work was the extension of survival of treated transgenic mice. However, our enthusiasm may need to be tempered since there is a long list of compounds and therapies which were successful in prolonging the lifespan of transgenic mice, which subsequently failed to demonstrate efficacy in human patients. Furthermore, even if beneficial, consideration must also be given to the possible carcinogenic side effects which may be associated with increasing Shh signalling. Nonetheless, given the fatal outcome of ALS and the paucity of available effective treatments, the inherent chance of inducing cancer in patients may be acceptable depending on the magnitude of such risk.

We hope that our work will introduce a new research pathway in the field of neurodegeneration, and that investigators will broaden their scope to include the study of this and other morphogens in the pathogenesis of not only ALS but other neurodegenerative diseases.

------Appendix I------

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