ABNORMAL PROTEIN PHOSPHORYLATION IN HUMAN AMYOTROPHIC LATERAL SCLEROSIS

by

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ABNORMAL PROTEIN PHOSPHORYLATION IN HUMAN AMYOTROPHIC LATERAL SCLEROSIS (ALS)

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by loss of neurons in cortex, brain stem and spinal cord. Currently, ALS is believed to be triggered by a number of distinct factors including glutamate excitotoxicity and mutations in the *sod1* gene (mSOD).

To explore the role of protein kinase C (PKC) in N-methyl-D-aspartate (NMDA)-mediated cytotoxicity, cell death assays were performed in NR1A/NR2A-transfected human embryonic kidney (HEK) cells. NMDA-mediated cell death was potentiated by the activation of Ca^{2+}- and lipid-dependent isoforms of PKC, specifically PKCβ1. A discrete segment of the C-terminus of NR2A subunit contributed to this potentiation by PKC. These data demonstrate that the elevation of PKC activity increases cell death induced by NMDA receptor activation.

To examine the involvement of abnormal protein phosphorylation in ALS, the expression of over 130 proteins were evaluated in postmortem spinal cord tissues from patients having sporadic ALS and controls. There was increased expression of protein kinases and phosphoproteins in ALS tissue such as PKCα/β, protein kinase B α (PKBα) and phospho-PKCα/β. This suggests that both pro- and anti-apoptotic signaling pathways are up-regulated in ALS. It is possible that the final outcome for each individual cell is determined by which pathway dominates over the other.

Transgenic mice over-expressing mSOD have been used extensively as a model of familial ALS. Comparative studies have revealed a striking similarity in pathology between mSOD mouse and human ALS. The morphological analogy between these two

was investigated and we found that in G93A mSOD mice, the spinal nucleus of bulbocavernosus (SNB) is spared from degeneration, paralleling the survival of its functional and anatomical homologue, Onuf's nucleus. This provides evidence that mSOD mice may suitably models ALS. However, a distinct profile of changes in protein expression were observed in the CNS tissues of G93A mSOD mice compared with their control littermates, which was dissimilar to that between ALS patients and controls. This observation indicates that the activation of protein kinases is different with neuron loss in mSOD mouse compared with that seen in patients with sporadic ALS.

These findings suggest an important role for abnormal protein phosphorylation in ALS.

DEDICATION

This thesis is dedicated to my beloved parents, Yue Ping Hu and Yue Zhu Shi, and to my dear husband, Biao Kuang, with thanks for all their love, support, encouragement and understanding.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
aPKC	atypical PKC
BC	bulbocavernosus
BDNF	brain-derived neurotrophic factor
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
CaMKK	Ca^{2+}/calmodulin-dependent protein kinase kinase
CDK	Cyclin-dependent kinase
CK	casein kinase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COT	cancer Osaka thyroid oncogene
cPKC	conventional or classical PKC
CREB	cAMP regulatory element binding protein
Cy3	cyanine 3
DAB	diaminobenzidine tetrahydrochloride
DAG	diacylglycerol
DF	death-to-freezing interval
dichloro	5-7 dichlorokynurenic acid
DIV	days *in vitro*

DLN	dorsolateral nucleus
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
DTT	dithiolthreitol
EAA	excitatory amino acid
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinases
FALS	familial form of amyotrophic lateral sclerosis
FBS	fetal bovine serum
FDA	Food and Drug Administration
FPG	fast disease progression group
Gab1	Grb2-associated binder-1
GAPs	GTPase-activating proteins
GDNF	glial-derived neurotrophic factor
GFP	green fluorescent protein
GK	guanylate kinase
GKAP	guanylate kinase-associated protein
GRK2	G protein-coupled receptor kinase 2
GSK3α/β	glycogen synthase kinase 3 α/β
HEK	human embryonic kidney
HPK1	hematopoietic progenitor kinase 1
HRP	horseradish peroxidase

HSP	heat shock protein
ICC	immunocytochemistry
IF	intermediate filament
Ig	immunoglobulin
IGF	insulin-like growth factor
IHC	immunohistochemistry
IHI	intracytoplasmic hyaline inclusion
IPC	insoluble protein complex
IRS	insulin receptor substrate
JAK3	Janus kinase-3
JNK	c-Jun N-terminal kinase
KA	kainite
KAP	Cyclin-dependent kinase-associated phosphatase
LA	levator ani
LDH	lactate dehydrogenase
LI	Lewy body-like inclusions
L-NAME	N(omega)-nitro-L-arginine methyl ester
LRR	leucine-rich region
LTP	long term potentiation
MAGUK	membrane-associated guanylate kinase
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C kinase substrate

MEK	mitogen-activated protein kinase kinase
MEM	minimum essential medium
MND	motoneuron disease
MOPS	3-(N-morpholino) propane sulfonic acid
mPSDp	major PSD protein
mSOD	mutant superoxide dismutase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NF	neurofilament
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
nPKC	novel PKC
NT	neurotrophin
NTR	neurotrophin receptor
OD	optical density
ON	Onuf's nucleus
PAK	p21-activated kinase
PBS	phosphate buffered saline
PBST	0.3% Trition X-100 in PBS
PCR	polymerase chain reaction
PDK	phosphoinositide-dependent kinase
PFA	paraformaldehyde

PI	phosphotyrosine interaction
PI3-K	phosphatidylinositol 3-kinase
PK	protein kinase
PKA	cAMP-dependent protein kinase
PKB	protein kinase B
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PKR	dsRNA-dependent protein kinase
PMA	phorbol 12-myristate 13-acetate
pmn	progressive neuronopathy mutation
PMSF	phenylmethylsulfonyl fluoride
PP	protein phosphatase
PP2B	protein phosphatase 2B
PP2C	protein phosphatase 2C
PS	phosphatidylserine
PSD	postsynaptic density
PSD-95	postsynaptic density protein 95
PSS	physiological salt solution
PTB	phosphotyrosine-binding
PTP1δ	protein-tyrosine phosphatase 1 δ
PVDF	polyvinylidene difluoride
RB	retinoblastoma
RDLN	retrodorsolateral nucleus

RO	RO 32-0432
ROS	reactive oxygen species
rpm	revolutions per minute
RSK	ribosomal S6 Kinase
S6K	S6 kinase
SALS	sporadic form of amyotrophic lateral sclerosis
SAPAPs	SAP50/PSD-95-associated proteins
SAPKβ	stress-activated protein kinase β
SDS	sodium dodecylsulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SH2/3	Src-homology 2/3
SNB	spinal nucleus of the bulbocavernosus
SOD1	Cu/Zn superoxide dismutase
Sos	son-of-sevenless
SPG	slower disease progression group
SRE	serum response element
SRF	serum response factor
S-SCAM	synaptic scaffolding molecule
SynGAP	synaptic GTPase-activating protein for Ras
TE	Tris-EDTA buffer
Trk	tyrosine receptor kinase
wt	wild type
ZIPK	ZIP kinase

X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

LIST OF AMINO ACID CODES

Name	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glycine	Gly	G
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Histidie	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CHAPTER 1
GENERAL INTRODUCTION

1.1 Amyotrophic lateral sclerosis (ALS)

The disorder amyotrophic lateral sclerosis (ALS), also known as motoneuron disease (MND) or Lou Gehrig's disease, is a neurodegenerative disease of humans where there is death of motoneurons in spinal cord and brain stem, as well as degeneration on the corticospinal tract and other descending tracts (Eisen and Krieger, 1998). ALS has a prevalence of approximately 5 per 100,000 people (Scarlato, 1997; Julien, 2001). It usually begins in mid-life and happens more frequently in males (Eisen and Krieger, 1998). This disease manifests as progressive decline in muscular function resulting in eventual paralysis, speech deficits and, ultimately, death due to respiratory failure, generally within 2–5 years of onset (see Julien, 2001).

There are three forms of ALS: the most frequent form is sporadic ALS (SALS, not apparently inherited); another form is familial ALS (FALS), generally, but not exclusively having autosomal dominant inheritance (see Strong, 2001), with exceptions that are autosomal recessive or X-linked (Ben Hamida et al., 1990; La Spada et al., 1991; Hentati et al., 1994; 1998); the third form of ALS is found on the islands of Guam and Rota and maybe linked to potential neurotoxin(s) in the local environment (Guamanian ALS-parkinsonism-dementia complex). Clinically and pathologically, FALS is very similar to SALS. FALS constitutes about 10% of all ALS cases, and approximately 20% of FALS patients have mutations in the gene for Zn/Cu superoxidase dismutase (SOD1)

that is mapped to chromosome 21q22 (Rosen et al., 1993). In some patients with FALS and rarely those with SALS, other gene mutations are found less commonly, such as mutations in the *als2* gene, a putative GTPase regulator (Hadano et al., 2001), the heavy neurofilament (NF-H) gene (Figlewicz et al., 1994; Al-Chalabi et al., 1999), or other genes (see Hand and Rouleau, 2002).

1.1.1 Pathogenesis of ALS

ALS is characterized by selective cell loss that is confined to the nervous system (or musculoskeletal system secondary to the loss of neurons), but it seems likely that cells outside the nervous system are spared (see Eisen and Krieger, 1998; Strong, 2003). This observation suggests that the disease targets neuron-specific gene(s), or protein(s). Within the nervous system as well, there appear to be neurons with a higher probability of being involved in the disease and those with a lower probability of involvement. For instance, motoneurons innervating skeletal muscle appear to be universally involved in ALS. Yet, motoneurons that supply extraocular (intrinsic eye) muscles are often spared. Furthermore, motoneurons that innervate the external anal sphincter, which has somewhat distinctive types of skeletal muscle, are often spared (Bergmann et al., 1995; Pullen, 1996). In the spinal cord, interneurons can show extensive loss, yet dorsal root ganglion neurons rarely die. This puzzling pattern of cell loss is made more confusing as the neurons do not have to be lost in anatomically adjacent regions (Brooks et al., 1998). Clinically, the onset of ALS often is seen in one or other hand, or in the muscles of phonation, maybe because there are a relatively large number of motoneurons innervating these structures (Eisen and Krieger, 1998). ALS affects corticospinal tract neurons prominently, but also neurons in brain areas that would be thought of as cognitive, or

motor planning regions (Strong, 2001). Involvement in these brain regions demonstrates that ALS is not restricted to motor system neurons and likely can affect other brain regions, but with a lower probability. This belief is supported by observation of ALS patients who are maintained for long periods of time on assisted ventilation. In some of these patients, involvement of motoneurons innervating extraocular muscles is seen, suggesting that the disease can involve these structures, but with a much lower probability than somatic motor neurons (Eisen and Krieger, 1998).

The basis of the selective vulnerability of motoneurons and other motor system cells in ALS is now a topic of intensive study. What are the factors that could explain this selective vulnerability of motoneurons and other motor cells to die? There are numerous possibilities. The currently favoured hypotheses include a role for N-methyl-D-aspartate (NMDA), or α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor mediated excitotoxicity, oxidative damage by free radicals, mutant superoxide dismutase (mSOD) protein aggregates, mitochondrial defects and microglial-mediated neurotoxicity, etc. (see review: Julien et al., 2001; Shaw et al., 2001; Patel and Maragakis, 2002; Strong, 2003), but there is no guarantee that any of these proposed mechanisms will be relevant for ALS. Many of these theories have been proposed for ALS on the basis of having some experimental support, or because the theory has been proposed for somewhat analogous problems (such as Parkinson's Disease or Alzheimer's Disease). However, the data supporting any of these theories are fragmentary.

The following review will focus on the potential role of NMDA receptor neurotoxicity in ALS and the regulation of NMDA receptor channel activities by protein phosphorylation and on abnormal protein phosphorylation in ALS more generally, both

in human and in the mSOD transgenic mouse model.

1.2 N-methyl-D-aspartate (NMDA) receptor neurotoxicity

NMDA receptors are a class of postsynaptic receptors in the central nervous system (CNS). They have been extensively studied and considerable evidence suggests these receptors have an important role in some types of neurotoxicity such as excitotoxicity and oxidative damage (Olney, 1969; Choi, 1988; Meldrum and Garthwaite, 1990; Dawson et al., 1991; Choi, 1992; Dawson et al., 1993). In ALS, several studies have suggested that NMDA-mediated toxicity may be very important in producing motoneuron death (see Plaitakis, 1990; Eisen and Krieger, 1998). The NMDA receptor produces excitotoxicity following its activation by excessive concentrations of glutamate, with the subsequent influx of Ca^{2+} through the receptor channel. The influx of Ca^{2+} induces an increase of cytosolic free calcium and results in the activation of important calcium-dependent signaling processes, which include calcium-dependent protein kinases (PKs), protein phosphatases (PPs), phospholipases, proteases and endonucleases (Mayer et al., 1992; McBain and Mayer, 1994). The NMDA receptor is also likely involved in oxidative toxicity. Oxidative damage is produced by reactive oxygen species (ROS) and the vast majority of ROS are produced in the mitochondria under physiological conditions (Krieger and Duchen, 2002). Following NMDA receptor activation, several events likely contribute to an increase in ROS formation. Receptor generated Ca^{2+} influx leads to elevations in mitochondrial Ca^{2+} concentration, as mitochondria are an important buffer of increased intracellular Ca^{2+} ions. The rise in intramitochondrial Ca^{2+} concentration will drive respiration and increase production of oxidative phosphorylation, a major source for unpaired electrons. Possibly, there is also uncoupling of the respiratory

chain. Additionally, Ca^{2+} influx through activated NMDA receptor channels activates Ca^{2+}-sensitive neuronal nitric oxide synthase (nNOS), which catalyzes the formation of the gaseous messenger molecule nitric oxide (NO) from arginine (Bredt and Snyder, 1989; Garthwaite et al., 1989; Dawson et al., 1993). Evidence for the involvement of nNOS in glutamate neurotoxicity includes the finding that inhibitors of nNOS reduce glutamate toxicity in primary cultures of rat cerebellum neurons (Felipo et al., 1998). NO may cause destruction of neurons because of the ability of NO to react with superoxide ion yielding a potent oxidant, peroxynitrite (Beckman et al., 1990; Dawson et al., 1991). Peroxynitrite can diffuse for several micrometers before decomposing to form the cytotoxic oxidants, hydroxyl radical and nitrogen dioxide, which oxidize and damage most cellular constituents including DNA (Beckman, 1991). Thus, excitotoxicity and oxidative toxicity are influenced by NMDA receptor activation. Moreover, NMDA receptors can complex with several signaling molecules either by direct association, or via anchoring proteins. These interactions modulate NMDA receptor function and couple the NMDA receptor to various signaling pathways. Furthermore, the channel activities of NMDA receptor can be regulated through phosphorylation by a number of PKs and PPs.

1.2.1 NMDA receptor

The NMDA receptor is one of three major classes of ionotropic glutamate receptors, which constitute the major excitatory neurotransmitter system in the mammalian CNS. The other two classes are AMPA receptors (GluR1-GluR4; alternatively GluRA-GluRD) and kainate receptors (GluR5-GluR7, KA-1, KA-2), which are together called non-NMDA receptors (Hollmann and Heinemann, 1994). Although NMDA receptors play a mojor role in neurotoxicity, AMPA and KA receptors also

attribute to excitotoxicity in neurons that is mainly Ca^{2+}-dependent (for review, see Sattler and Tymianski, 2001; Heath and Shaw, 2002).

NMDA receptors can be encoded by *nr1* (Moriyoshi et al., 1991; Nakanishi et al., 1992), *nr2* (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992), and the more recently identified *nr3* genes (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998). The *nr1* gene undergoes alternative RNA splicing to give eight NR1 splice variants (NR1A-G subunits) (see Zukin and Bennett, 1995); the *nr2* gene family has four separate genes which encode NR2A-D subunits, respectively (see Hollmann and Heinemann, 1994); and the *nr3* gene encodes NR3A and NR3B two subunits (Das et al., 1998; Nishi et al., 2001). Functional NMDA receptors are heteromeric proteins composed of NR1 subunits (ζ1), along with one or more subunit, such as NR2A (ε1), NR2B (ε2) NR2C (ε3), or NR2D (ε4) (Sucher et al., 1996).

The NR1 sununit exhibits the typical structure of a ligand-gated ion channel (Hollmann and Heinemann, 1994). It has two extracellular binding domains for its agonist glutamate and co-agonist glycine (O'Hara et al., 1993; Kuryatov et al., 1994; Stern-Bach et al., 1994); three transmembrane-spanning domains (TM1, TM3 and TM4); a fourth hydrophobic segment (TM2) that is thought to make a hairpin turn within the membrane and line the ion channel (Stevens, 1991; Zukin and Bennett, 1995); and an intracellular carboxy-terminus domain holding a C1 and/or a C2 region that contains protein phosphorylaton residues. Although there is only 26–27% homology between NR1 and NR2 subunits (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992), NR2 subunits have the same basic structure as NR1 subunits, but are considerably larger because of their extensive C-terminal domains (Hollmann and Heinemann, 1994; Monyer

et al., 1992). The large C-termini of NR2 subunits could be the target domains of modulatory or accessory proteins, might promote receptor assembly, or be involved in the sorting or targeting of NMDA receptor channels. Alternatively, different C-termini of NR2 subunits might produce different conformations of the intracellular domain between transmembrane domains (TMD) III and IV and thereby modulate receptor function (Kutsuwada et al., 1992; Monyer et al., 1992). The NR3A and NR3B subunits have a high similarity of 51% (Nishi et al., 2001). NR3B was reported to have a typical structure of glutamate receptor channel with a signal peptide and four membrane-associated regions (Nishi et al., 2001). This NR3 class of NMDA receptors acts as a dominant-negative in the NMDA receptor complex since both NR3A and NR3A were found to suppress glutamate-induced current when they were co-expressed with NR1 and NR2A subunits (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998; Nishi et al., 2001). When NR3A and NR3B are co-assembled with NR1 subunits, they form excitatory glycine receptors that are unaffected by glutamate or NMDA (Chatterton et al., 2002).

1.2.2 NMDA receptor interacting proteins

Recent studies have discovered a number of postsynaptic proteins that play important roles in NMDAR signaling (Fig. 1). These proteins include postsynaptic density protein 95 (PSD-95) (Kornau et al., 1995), nNOS (Sattler et al., 1999), GTPase-activating proteins (GAPs) (Chen et al., 1998; Kim et al., 1998; Pak et al., 2001), guanylate kinase-associated protein (GKAP) (Yamada et al., 1999a), Shank (Naisbitt et al., 1999), Homer (Tu et al., 1999), SAP50/PSD-95-associated proteins (SAPAPs) (Takeuchi et al., 1997), synaptic scaffolding molecule (S-SCAM) (Hirao et al., 2000), alpha-A-actinin-2 (Wyszynski, 1997; 1998), CRIPT (Passafaro et al., 1999), yotiao (Lin

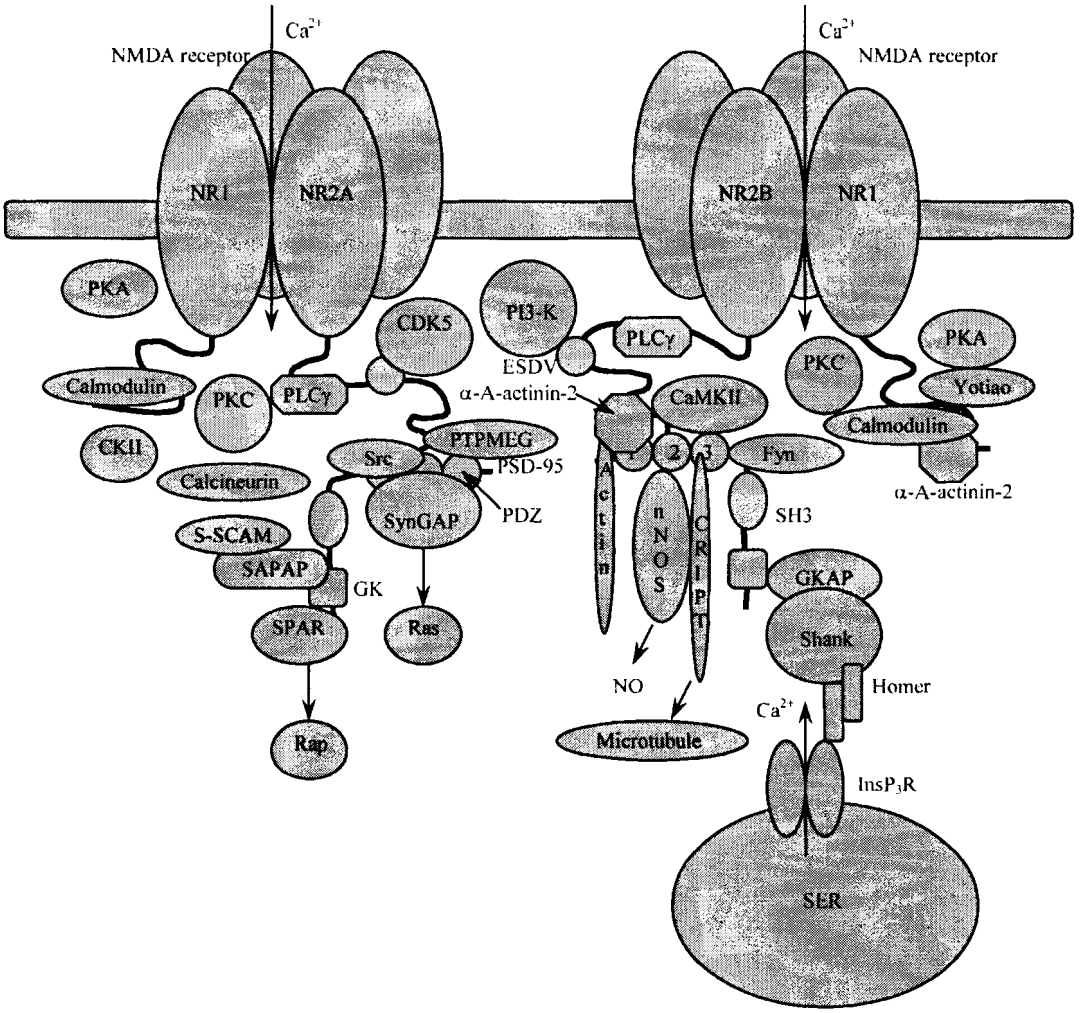

Figure 1: Molecular organization of PSD proteins.
PSD proteins include PSD-95, nNOS, SynGAP, GKAP, Shank, Homer, SPAR, S-SCAM, α-A-actinin-2, CRIPT, yotiao and calmodulin. Among them, PSD-95 plays a central role in coupling NMDA receptor to diverse signaling molecules. The protein kinases and phosphatases which modulate NMDA receptor function are also shown, including PKC, Src, Fyn, CDK5, CaMKII, PKA, CKII, Calcineurin, PTPMEG and PTP1D.

et al., 1998), and calcium/calmodulin (Ehlers et al., 1996; Krupp et al., 1996). Among the proteins described above, PSD-95, nNOS have been most extensively studied.

1.2.2.1 Postsynaptic density (PSD) protein PSD-95

The PSD-95 protein is a member of the membrane-associated guanylate kinase (MAGUK) family, forming a subfamily with the other mammalian homologs of Dlg: PSD-93/chapsyn-1,10, SAP97/dlg, and SAP102 (Cho et al., 1992; Craven and Bredt, 1998). All of the proteins except SAP97 are components of the PSD and are associated with NMDA receptors. The PSD-95 family is characterized by 3 PDZ domains, a Src-homology 3 (SH3) domain, and a C-terminal catalytically-inactive guanylate kinase (GK) domain (Cho et al., 1992). Each of these regions has been shown to be a site for protein-protein interactions and PSD-95/SAP90 binds NMDA receptors through the PDZ domains.

NR2A-D subunits have long intracellular cytoplasmic tails, the C-termini of which end in the conserved sequences –E-S-D-V (NR2A, NR2B) or –E-S-E-V (NR2C, NR2D). This short peptide motif directs the binding of NR2 subunits to PSD-95/SAP90 (Kornau et al., 1995; Niethammer et al., 1996). However, NR1C and NR1D subunits do not display any interaction with PSD-95 although they also have a consensus C-terminal -T/S-X-V motif (Bassand et al., 1999). This is because the C-terminal T/S-X-V motif is mandatory but not sufficient for efficient interaction with PSD-95. An, as yet unidentified, upstream sequence on the receptor subunits determine whether the -T/S-X-V motifs will bind to PSD-95 (Bassand et al., 1999). Also, Migaud et al. (1998) found that PSD-95 deletion did not affect NMDA receptor currents, subunit expression and localization.

1.2.2.2 Neuronal nitric oxide synthase (nNOS)

The potential scaffolding functions of PSD-95 in NMDA receptor signaling have been suggested by the identification of other proteins that interact with PSD-95. One of these proteins is nNOS, which catalyzes the formation of the gaseous messenger molecule NO from arginine. Neuronal NOS has been shown to bind to PDZ2 of PSD-95 via a PDZ-PDZ interaction (Brenman et al., 1996; Hillier et al., 1999). Christopherson et al. (1999) further explored this PDZ-PDZ interaction between PSD-95 and nNOS and found that PSD-95 was actually recognizing an internal motif adjacent to the consensus nNOS PDZ domain, which is a structured "pseudo-peptide" extention of the nNOS PDZ. This asymmetric interaction between this internal motif and the peptide-binding pocket of PSD-95 PDZ2 leaves the nNOS PDZ domain available to associate with additional COOH-terminal PDZ ligands (Christopherson et al., 1999). By interacting with both nNOS and NMDA receptors, PSD-95 may bring NMDA receptors, which allow calcium influx, into close proximity of nNOS, which is regulated by calcium/calmodulin. Thus, binding to PSD-95 may be involved in the coupling of NMDA-mediated calcium influx to nNOS activation.

Several lines of evidence support this hypothesis. Suppressing the expression of PSD-95 in cultured cortical neurons using anti-sense techniques blocks calcium-activated nitric oxide production by NMDA receptors selectively, without affecting the expression or function of NMDA receptors or nNOS (Sattler et al., 1999). Furthermore, internalization of a peptide which disrupts PSD-95 interactions in cerebellar granule cells prevents NMDA-receptor mediated stimulation of nNOS, but does not affect calcium influx through the NMDA receptor (Craven and Bredt, 1998). Thus, the NMDA

receptor/PSD-95/nNOS complex represents a molecular signaling microdomain for calcium regulation of NMDA-induced neurotoxicity by nNOS.

1.2.3 Protein phosphorylation of NMDA receptor

Phosphorylation of a channel protein can alter its physical conformation leading to a variety of changes in channel behavior. Many PKs phosphorylate NMDA receptors. These include protein kinase C (PKC), members of the Src family of non-receptor tyrosine kinases such as Src and Fyn, Cyclin-dependent kinase 5 (CDK5) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII), as well as other kinases (Fig. 1). Also, several protein phosphatases such as calcineurin and PTPMEG regulate the phosphorylation state of the NMDA receptor channel. Here, I review only the PKs that are closely related to my study.

1.2.3.1 Protein kinase C (PKC)

The family of Ca^{2+}- and lipid-dependent PKCs comprise at least twelve structurally related phospholipid-dependent, serine/threonine kinases which catalyze the covalent transfer of phosphate from ATP to serine and threonine residues on proteins (see reviews: Casabona, 1997; Parekh et al., 2000). PKC isozymes have been grouped into three subclasses according to their regulatory properties, which are conferred by specific domains in the proteins. The 'conventional' or 'classical' PKCs (cPKCs), including PKCα, βI, βII, and γ, can be activated by Ca^{2+} and/or by diacylglycerol (DAG) and phorbol esters. The 'novel' PKCs (nPKCs) δ, ε, θ, η and μ can also be activated by DAG and phorbol esters but are Ca^{2+} independent. Finally, the atypical PKCs (aPKCs), which include PKCζ and PKCι (its mouse homologue has been named PKCλ), are unresponsive

to Ca^{2+} and DAG/phorbol esters. PKCs possess regions that are highly conserved between different isozymes (regions C1 to C4) and variable regions (regions V1 to V5). Among these regions, C1 has its distinct feature that it has the cysteine-rich phorbol ester binding motif(s).

PKC has a critical role in NMDA receptor signaling. So far there is no direct evidence showing that PKC physically binds to the NMDA receptor. However, Suen and colleagues (Suen et al., 1998) preliminarily disclosed the interaction of the NMDA receptor with PKC by an observation of the co-immunoprecipitation of NR1 with PKCγ and a major PSD protein (mPSDp). PKC is reported to potentiate the NMDA response by increasing the probability of channel opening and by reducing the voltage-dependent Mg^{2+} block of the NMDA receptor channels (Chen and Huang, 1992; Xiong et al., 1998). PKC activation modulates NMDA receptor-mediated currents (Ben-Ari et al., 1992; Kelso et al., 1992; Markram and Segal, 1992; Urushihara et al., 1992; Mori et al., 1993; Raymond et al., 1994; Zukin and Bennett, 1995; MacDonald et al., 1998; Xiong et al., 1998; Logan et al., 1999; Zheng et al., 1999) and Ca^{2+} influx through NMDA receptor channels (Grant et al., 1998; Murphy et al., 1994). Also, Ca^{2+} influx through PKC-potentiated NMDA receptors can further increase the NMDA response (Ca^{2+} amplification) (Zheng et al., 1997). However, activation of PKC in some hippocampal neurons suppresses responses to NMDA (Markram and Segal, 1992). The effect of PKC on NMDA responses depends on the specific NR2 subunit found in the receptor (Grant et al., 2001).

The molecular mechanism underlying PKC-induced NMDA receptor potentiation still remains unclear. Most of the potential sites of phosphorylation by PKC are located in

the large C-terminus of the NR1 subunit and this is the region that is subject to extensive alternative splicing. The C1 (amino acids 864–890) and C2 domains contain four serines (Ser889, 890, 896, 897) each of which can be phosphorylated by exposure to a phorbol ester and activation of an endogenous PKC (Tingley et al., 1997). Mutation of each of the serines to alanines dramatically reduces phosphorylation of the subunit suggesting that most of the PKC-dependent phosphorylation occurs at the C-terminus. There is some evidence showing that NMDA receptor potentiation can be mediated by direct phosphorylation (Leonard and Hell, 1997). However, Zheng and colleagues (1999) revealed the unexpected finding that PKC still potentiated NMDA receptor function even when NMDA receptors were assembled from subunits lacking all known sites of PKC phosphorylation. Another study showed that the most active PKC phosphorylation site, the NR1 subunit C1 region, is mainly responsible for PKC modulation (Logan et al., 1999), with the absence of the C1 region leading to a higher level of potentiation. Furthermore, recent studies also suggest that PKC-induced potentiation involves an increase in the opening rate of NMDA receptor channel, the delivery of new channel molecules to the cell surface via exocytosis, and the insertion of new channels to the plasma membrane (Zheng et al., 1999; Lan et al., 2001). Neither of these two mechanisms might require direct phosphorylation of the NMDA receptor protein. These conflicting findings do not explain whether PKC-induced potentiation of NMDA receptors occurs via direct phosphorylation of the receptor or not.

PKC activation has been thought to be responsible for modulating excitotoxic neuronal death. However, the evidence is contradictory (Favaron et al., 1990; Durkin et al., 1997; Noh et al., 2000). For example, Felipo et al. (1993) claimed that inhibitors of

PKC were able to protect cultured cerebellar neurons from excitotoxic death. Riluzole, an inhibitor of glutamate neurotransmission with neuroprotective properties and which is used as a therapy in ALS, was demonstrated to both block the PMA-induced increase of PKC activity and attenuate PMA-induced oxidative neuronal death in cortical culture (Noh et al., 2000). In contrast, Durkin and coworkers (1997) reported that cerebral cortical neurons which had been in culture for 8 days became highly vulnerable to neuron death when exposed to excitatory amino acids (EAAs), including NMDA, in the presence of PKC inhibitors. In the absence of PKC inhibitors the neurons were relatively resistant to neurotoxicity.

1.2.3.2 Cyclin-dependent kinase 5 (CDK5)

CDK5 is a proline-directed, serine-threonine PK that belongs to the cyclin-dependent kinase (CDK) family. Since it was originally isolated through its structural homology to human CDC2 (Meyerson et al., 1992), CDK5 has been extensively studied because of its involvement in a number of physiological processes in nerve and muscle cells, including neurogenesis, neuritic outgrowth, axonal transport of membrane-bound organelles and myogenesis (Bajaj, 2000).

Most of the CDKs are key regulators of the cell cycle and they require subunit association for their activation (Nurse, 1990). For example, CDK2 association with cyclin A and E regulates the G1 to S transition and S phase progression (Sherr, 1994); CDK4 and CDK6 associate with cyclin D and are essential for G1 phase progression (Sherr, 1994); CDK5 protein has a ubiquitous distribution but with the highest level in brain, also brain is the only tissue that shows CDK5 histone H1 kinase activity (Tsai et al., 1994). Although CDK5 does not control cell cycle progression like its CDK family homologues,

its activation also needs the association with cyclin-related proteins. Neuron-specific p35 and p39 proteins are two CDK5 regulators that have similar folding to cyclins (Tsai et al., 1994; Poon et al., 1997; Tang et al., 1997; Humbert et al., 2000; Patzke and Tsai, 2002). Both the p35 and p39 proteins deregulate the activity of CDK5; however, their hydrolyzed products by calpain, p25 and p35, activate CDK5 (Lew et al., 1994; Patrick et al., 1998; 1999; Ahlijanian et al., 2000; Lee et al., 2000; Patzke and Tsai, 2002).

A recent study has found that CDK5 co-localizes with the NMDA receptor NR2A subunit at postsynaptic sites and phosphorylates a serine residue, Ser1232, in the CDK5-recognizing RSPFK motif which is present in the NR2A subunit (Li et al., 2001). Also this investigation shows that roscovitine, an inhibitor of CDK5, blocks both NMDA-evoked currents through the NMDA receptor and long term potentiation (LTP) in rat CA1 hippocampal neurons. Since the NMDA receptor is likely involved in excitotoxicity in ALS, CDK5 phosphorylation of the NR2A subunit may contribute to ALS pathology, directly or indirectly.

1.2.3.3 Ca^{2+}/calmodulin-dependent kinase II (CaMKII)

CaM kinase is a family of protein serine/threonine kinases that require the binding of Ca^{2+}/calmodulin in order to be activated. CaMKII is an abundant PSD protein, and can be activated by NMDA receptor stimulation. The influx of Ca^{2+} through the NMDA receptor channel upon receptor activation indicates that CaMKII may be involved in NMDAR-mediated function. Kitamura et al. (1993) first suggested that CaMKII appeared to induce the phosphorylation of the channel domain of the NMDA receptor channel and then enhanced Ca^{2+} influx through the channel. Lickteig and coworkers (1995) demonstrated that both NR2A and NR2B subunits of the NMDA receptor were

phosphorylated by CaMKII in the hippocampus. Also, the NR2B subunit is a high affinity substrate for CaMKII-induced phosphorylation at ser1303 (Omkumar et al., 1996; Kennedy, 1997). The synaptic localization n of CaMKII appears to be independent of PSD-95. However, the cytoplasmic tails of NMDA receptor subunits interact directly with CaMKII (Gardoni et al., 1998; Strack and Colbran, 1998; Leonard et al., 1999; Shen and Meyer, 1999; Gardoni et al., 2001a; 2001b) (see Fig. 1).

1.2.4 A signaling model of NMDA receptor neurotoxicity

A signaling model of NMDA receptor neurotoxicity is shown in Figure 2. NMDA receptor neurotoxicity is initiated by the binding of excessive amounts of glutamate. The activation of the NMDA receptor channel by glutamate produces channel opening and an influx of Ca^{2+}, so as to cause the activation of Ca^{2+}-dependent PKs, PPs and other enzymes. As indicated above, elevated intracellular Ca^{2+} has numerous other effects, such as elevating mitochondrial Ca^{2+}, increasing the production of free radicals and collapsing the mitochondrial potential gradient. Not surprisingly, there are various interactions between the PKs and PPs activated by NMDA and elevated cytosolic free Ca^{2+}concentrations. PKC activation is of importance in producing neuron death and evidence that PKC is stimulated by NMDA receptor activation is discussed above in section 1.2.3. Activation of PKC augments cell death in some models (Favaron et al., 1990; Felipo et al., 1993; Noh et al., 2000), but not in others (Durkin et al., 1997). However, whether PKC exerts its potentiation through direct phosphorylation of NMDA receptor subunits remains unclear.

It is possible that the effects of PKC may be indirect and mediated by Src. The Src family of non-receptor tyrosine kinases associates with and phosphorylates the

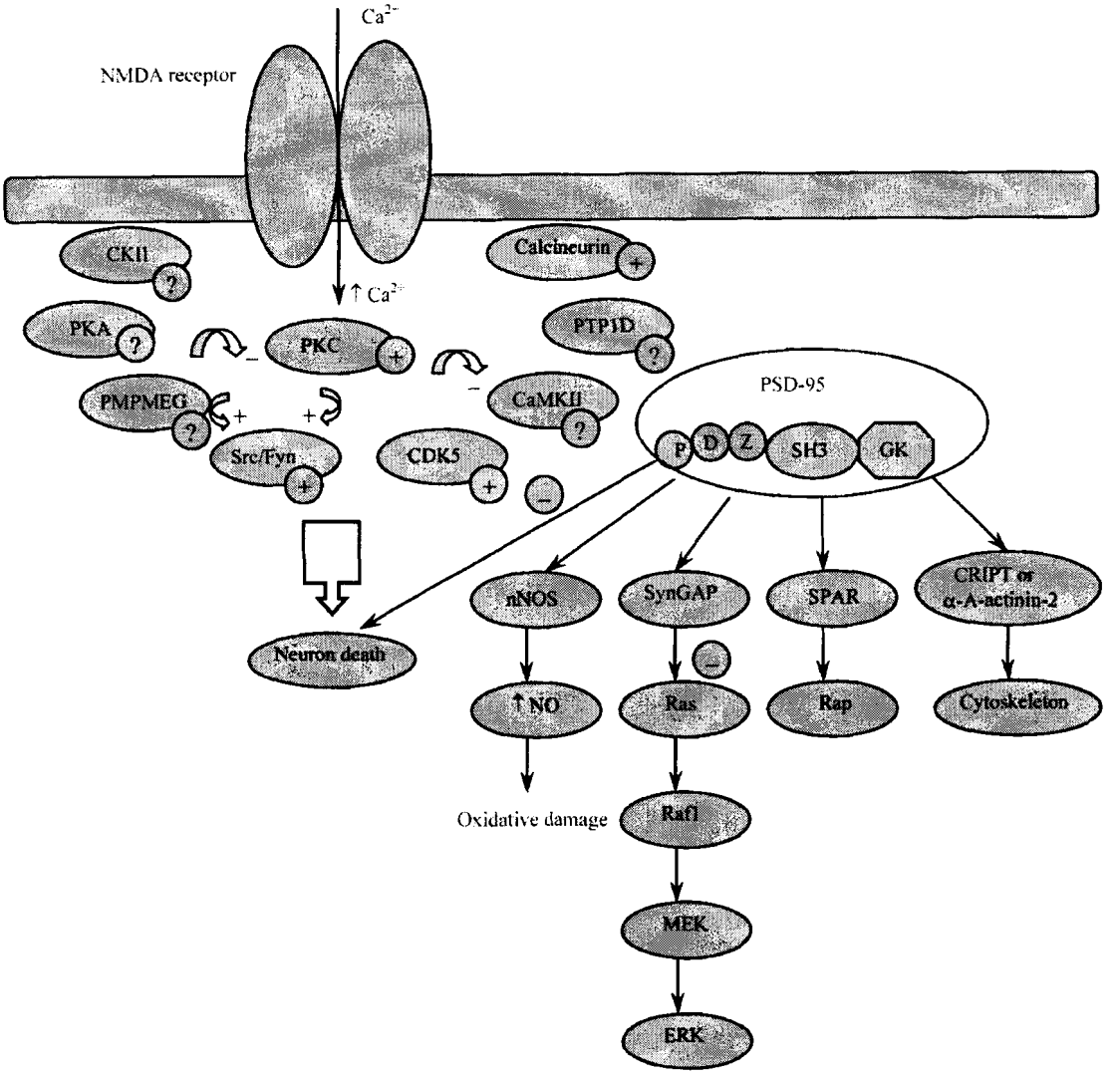

Figure 2: A possible signaling model of NMDA receptor neurotoxocity.
A few of protein kinases and phosphatases that regulate NMDA receptor neurotoxicity and their effects are indicated: the phosphorylation of the NMDA receptor by PKC, Src/Fyn and CDK5 increases NMDA receptor neurotoxocity. Calcineurin also contributes to neuron death. The functions of PKA, CKII, PMPMEG and PTP1D are not clear yet. There are also interactions among these kinases and phosphatases. The role of PSD-95 in neuron death is complicated: PSD-95 can inactivate the Ras/Raf1/MEK/ERK survival pathway by associating with SynGAP; it can also promote the Ca^{2+}-activation nNOS and thereby lead to neuron oxidative damage; however PSD-95 itself seems to be neuroprotective. PSD-95 can also couple NMDA receptor to Rap signaling and to cytoskeletal proteins but whether they are related to neurotoxicity is uncertain.

NMDA receptors. Src has also been found to up-regulate NMDA receptor channel activity and cytotoxicity (Chen et al., 1996; Manzerra et al., 2001). There is a recent study suggesting that PKC potentiation of NMDA receptor function may be mediated via tyrosine phosphorylation of NR2A and/or NR2B subunits. The PKC activator, PMA, was shown to increase NR2A and NR2B tyrosine phosphorylation in rat hippocampal CA1 mini-slices and this increase was blocked by pretreatment with the selective PKC inhibitor, chelerythrine, the tyrosine kinase inhibitor, Lavendustin A, or the Src family tyrosine kinase inhibitor, PP2 (Grosshans and Browning, 2001). CaMKII is another Ca^{2+}-dependent protein kinase found in the PSD that could interact with PKC. However, many other protein kinases and protein phosphatases may interact with PKC, modifying NMDA receptor toxicity.

In addition to the importance of protein phosphorylation in NMDA receptor neurotoxicity, PSD-95 may play a dual role in neurotoxicity. PSD-95 serves as a docking site by anchoring signaling molecules close to NMDA receptors. The interaction between PSD-95 with both NMDA receptors and nNOS may bring nNOS close to the NMDA receptor channel so that the interaction allows Ca^{2+} to activate nNOS and then produce $NO^{\cdot}$, which plays an important role in oxidative damage of neurons. However, PSD-95 has a protective role of against neuronal excitotoxicity by decreasing the glutamate sensitivity of the channels and by inhibiting the protein kinase C-mediated potentiation of the channels (Yamada et al., 1999b). Rutter and Stephenson (2000) demonstrated that co-expression of PSD-95 with NR1A/NR2A in HEK 293 cells resulted in a decreased sensitivity to L-glutamate and this decrease was mediated by the interaction between the NMDA receptor and PSD-95. However, the expression of NR2A and NR2B subunits

were enhanced in this case. Furthermore, coexpression of PSD-95 with mouse NR1/NR2A receptor subunits in Xenopus oocytes disclosed that PSD-95 coexpression eliminated PKC potentiation of NMDA currents while the presence of PSD-95 was required for Src potentiation of mouse NR1/NR2A receptor channel currents (Liao et al., 2000). Taken together, PSD-95 plays its dual roles in NMDA receptor neurotoxicity by promoting NO production and by decreasing the receptor sensitivity to its ligand.

Signaling cascades activated by the NMDA receptor are related to those activated by small G proteins since both Ras- and Rap-GTPase activating proteins bind to the PSD-95 protein. Also, the Rho effector, Citron, interacts with PSD-95 so that there may be links between NMDA receptor and Rho signaling. Whether Ras and Rho signaling are related to NMDA receptor neurotoxicity needs to be explored further. Also, the sequential interaction of the NMDA receptor, PSD-95, GKAP, Shank and Homer can couple NMDA receptor function to mGluRs, and to the $InsP_3Rs$ found in the SER membrane through a Homer-Homer multimerization. Also, α-A-actinin-a and CRIPT link NMDAR-PSD95 activation to cytoskeletal proteins.

1.3 Neurotrophin-mediated cell survival signaling pathways in nervous system

Neurotrophins are key regulators of cell fate in the vertebrate NS. They can cause diverse signaling events which may promote neuronal survival or lead to apoptosis and cell death, by binding to different kinds of receptors. There is growing evidence that reduced neurotrophic support is a significant factor in the pathogenesis of neurodegenerative diseases such as ALS. Elevated expressions of neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-

3 (NT-3) and altered phosphorylated levels of their receptors have been found in ALS, either in the early stage of the disease or during the course of the disorder (Stuerenburg and Kunze, 1998; Mutoh et al., 2000; Kust et al., 2002). Therefore, the changes of signaling pathways that are triggered by a variety of neurotrophins in ALS are of great interest in this study. The following is a brief summary of neurotrophin signaling with emphasis on the cell survival signaling pathways that are activated by neurotrophins.

In the nervous system, neurotrophins regulate the survival and apoptosis of neurons by binding to two types of cell membrane receptors, the tyrosine receptor kinase (Trk) and p75 neurotrophin receptor (p75NTR). The family of neurotrophins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (for review, see Davies, 1994; Bibel and Barde, 2000). Specifically these neurotrophins bind to Trk receptors with a preference of NGF to TrkA, BDNF and NT4/5 to TrkB, and NT3 for TrkC (Barbacid, 1994), although some of them, such as NT3, can activate more than one isoform of Trk receptors (Sofroniew et al., 2001). All of these neurotrophins can bind to p75NTR with a low affinity of ~10^{-9} M (Rodriguez-Tebar et al., 1990).

There are distinct signaling pathways being activated downstream of Trk and p75NTR receptors. It is generally believed that activated Trks initiates cell survival signaling to prevent programmed cell death, while p75NTR mediates apoptosis following injury and during development. However, this is not always true and these two types of neurotrophin receptors can interact with each other, which determines the eventual fate of the cell.

1.3.1 Trk receptor signaling

All three isoforms of Trk receptors belong to the family of receptor tyrosine kinases (Bibel and Barde, 2000). They are transmembrane glycoproteins containing an extracellular region comprised of an array of three tandem repeats of leucine-rich regions (LRR1-3), two distinct cysteine clusters flanking the LRRs (C1 and C2), and two immunoglobulin-like domains (Ig1 and Ig2) (Schneider and Schweiger, 1991). The binding of neurotrophins to Trks initiates a sequential series of events which include receptor dimerization, trans-autophosphorylation of specific tyrosine residues residing in the activation loop of the tyrosine kinase domain, activation of tyrosine kinase, and another autophosphorylation of tyrosines outside the activation loop (Cunningham et al., 1997; Cunningham and Greene, 1998). These autophosphorylated tyrosine residues residing outside the activation loop initiate downstream pathways by serving as docking sites for signaling proteins. Specifically, phosphorylated-Y490 is the target that recruits adapter molecules such as Shc and FRS-2, etc., which can in turn stimulate the mitogen-activated protein kinase kinase/extracellular signal-regulated kinases (MEK/ERK) and the phosphatidylinositol 3-kinase/protein kinase B (PI3-K/PKB) survival promoting pathways (Dikic et al., 1995; Kouhara et al., 1997).

1.3.2 The mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) survival pathway

The Trk-mediated MEK/ERK survival pathway is initiated from the binding of an adaptor protein, Shc, to the phosphorylated Y490 residue in the intracellular region of Trk via a phosphotyrosine interaction (PI) domain (Fig. 3; Basu et al., 1994; Dikic et al., 1995). Shc is phosphorylated by Trk receptor intrinsic tyrosine kinase and then interacts

with another adaptor protein Grb2 via the binding of an SH2 domain of Grb2 with the phosphotyrosine residue of Shc (Rozakis-Adcock et al., 1992). In addition, Grb2 associates with a protein named son-of-sevenless (Sos) (Rozakis-Adcock et al., 1993; Hallberg et al., 1998), which is a guanine nucleotide exchange factor and functions as an activator of Ras (Bonfini et al., 1992; Bowtell et al., 1992). Therefore, this Shc-Grb2-Sos complex brings Sos into proximity to membrane-associated GTPase Ras so as to activate the Ras signaling pathway (Thomas et al., 1992; Wood et al., 1992).

Similarily, another lipid-anchored docking protein FRS2 can also target Grb2/Sos complex to the plasma membrane in response to NGF stimulation (Kouhara et al., 1997; Meakin et al., 1999; Ong et al., 2000) FRS2 is myristylated and localized to the membrane (Kouhara et al., 1997). It binds to phospho-Y490 residue of Trk directly via its phosphotyrosine-binding (PTB) domain and can be phosphorylated by receptor tyrosine kinase (Meakin et al., 1999). Through the C-terminal binding sites for the SH2 domains, FRS2 recruits adapter protein Grb2 and intracellular protein tyrosine phosphatase Shp2 and activates Ras (Wright et al., 1997; Hadari et al., 1998; Ong et al., 2000).

The activated Ras directly binds to and activates serine/threonine kinase Raf, possibly through triggering Raf dimerization or oligomerization (Barnard et al., 1995; Farrar et al., 1996; Luo et al., 1996; 1997). Once activated, Raf phosphorylates and thereby stimulates the activation of the dual-specificity mitogen-activated protein (MAP) kinase kinase MEK1 at Ser217 and 221 (Vaillancourt et al., 1994). MEK1 activation leads to the selective phosphorylation of MAP kinase (MAPK) on Thr202 and Tyr204, which results in the activation of two members of the MAPK family, ERK1/2 (Payne et al., 1991). ERK1/2 are proline-directed serine/threonine kinases that regulate the

phosphorylation and activation of ribosomal S6 Kinase (RSK) (Zhao et al., 1996). Phosphorylation of Rsk leads to its translocation into the nucleus and consequent activation of cAMP regulatory element binding protein (CREB), by phosphorylating its Ser133 residue (Xing et al., 1996). Phosphorylation of Ser133 stimulates the ability of CREB to trigger the transcription of early gene c-fos (De Cesare et al., 1998). Activation of ERK1/2 can also induce nuclear translocation (Chen et al., 1992), where they phosphorylate the transcription factor Elk-1 and eventually initiate c-fos transcription by activating the serum response factor (SRF) and its binding to serum response element (SRE) within the c-fos promoter region (Miranti et al., 1995). The expression of immediate-early genes such as c-fos can regulate the transcription of other genes such as NGF that could have important functional consequences on neuronal survival. Also, MEK/ERK signaling may promote cell survival by stimulating CREB-mediated expression of anti-apoptotic proteins such as Bcl-2 (Riccio et al., 1999).

1.3.3 The phosphatidylinositol 3-kinase/protein kinase B (PI3-K/PKB) survival pathway

The PI3-K/PKB signaling pathway plays a very important role in neurotrophin-mediated cell survival, mainly by inhibiting the activity of apoptotic proteins. Adaptor docking proteins such as Grb2-associated binder-1 (Gab1) and insulin receptor substrates (IRS) IRS-1 and IRS-2 have been found to regulate the activity of PI-3K and potentiate neurotrophin-induced cell survival (Holgado-Madruga et al., 1997; Yamada et al., 1997; Korhonen et al., 1999). PI3-K is a lipid kinase possessing an intrinsic protein-serine/threonine kinase activity, with an 85-kDa regulatory subunit whose SH2 domains bind to phosphotyrosine, and a 110-kDa catalytic subunit (Dhand et al., 1994). The

interaction of PI-3K with adaptor proteins and/or Trk causes a translocation of PI3K to the plasma membrane where it is close to its lipid substrate (Fig. 3).

PI3K catalyzes membrane phosphoinositides to produce different phosphorylated isomers of phosphotidylinositol such as PI-3,4-P2 and PI-3,4,5-P3. The 3-phosphoinositide-dependent kinase-1 (PDK1) has a COOH-terminal pleckstrin homology (PH) domain that interacts with PI-3,4-P2 and PI-3,4,5-P3, and this interaction results in the activation of PDK1 (Alessi et al., 1997). Activation of PKB is believed to be PI3-K-dependent. A model of the regulation of PKB activation by Downward (1998) is as follows: when inactivated, the PH domain of PKB masks its Thr308 site for PKD1 phosphorylation. Upon the binding of PtdIns(3,4,5)P_3 with the PH domain of PKB, PKB translocates to the plasma membrane and exposes its Thr308 site. Then PKB is sequentially phosphorylated at Thr308 by PDK1 and at Ser473 by PDK2, to yield a fully activated kinase. The Ser473 phosphorylation of PKB by PDK2 is probably also regulated by PIP3. However, the debates on whether PDK1 and PDK2 are the same enzyme and what kinase exactly phosphorylates PKB at Ser473 remains open (Delcommenne et al., 1998; Balendran et al., 1999; Toker and Newton, 2000; Hill et al., 2002).

After being activated, PKB is believed to remove from the plasma membrane and translocate to the cytosol and nucleus. PKB suppresses apoptosis by phosphorylating three possible target substrates: Bad, pro-caspase 9 and Forkhead. Bad is a pro-apoptotic protein of the Bcl-2 family. The phosphorylation of Bad at Ser136 by PKB stimulates the binding of Bad to a regulatory protein 14-3-3, therefore prevents its association with and its inactivation of the two anti-apoptotic proteins of the same Bcl-2 family (Datta et al.,

1997; del Peso et al., 1997). Pro-caspase 9 is proposed to be a target of PKB is based on the observation that PKB phosphorylation of caspase 9 on Ser196 reduces its protease activity (Cardone et al., 1998). However, this phosphorylation site of human caspase 9 by PKB is not found in mouse caspase 9, suggesting that the inactivation of pro-caspase 9 by PKB is not generally the case happening in PKB- promoted survival (Fujita et al., 1999). Forkhead is a transcription factor that induces apoptosis by up-regulating the expression level of Fas ligand (Brunet et al., 1999; Suhara et al., 2002). In a similar way to Bad, the PKB-phosphorylated-Forkhead 1 (FKHRL1) associates with 14-3-3 and so that is detained in the cytoplasm (Brunet et al., 1999).

1.4 Mutant superoxide dismutase (mSOD) transgenic mouse model of familial ALS (FALS)

1.4.1 SOD mutation and gain of toxic function

Since 1993, over 100 mutations have been identified in the *sod1* gene in 20% patients with FALS, spanning all five exons (Rosen et al., 1993; Cudkowicz et al., 1997). These mutations include A4V, D90A, G37R, G85R, G86R and G93A, etc. Among all these mutations, A4V is the most commonly detected in FALS and is associated with an aggressive, rapidly progressive disease, lasting only an average of 1.2 years after the onset of symptoms until death (Deng et al., 1993; Rosen et al., 1994).

The function of wild type (wt) Cu/Zn superoxide dismutase (SOD1) is to catalyze the dismutation of superoxide radical (O2-) into hydrogen peroxide (H_2O_2) (McCord and Fridovich, 1969; Brunori and Rotilio, 1984). The exact mechanisms underlying neuronal death induced by mSOD are still under investigation. The initial belief that lack of dismutase activity of mSOD underlies the toxic effect of this enzyme has not been

substantiated (Yim et al., 1996; 1997). Furthermore, SOD1 null mice do not develop weakness or histological abnormalities (Reaume et al., 1996). Expression of FALS-associated A4V and G37R mSOD in mammalian neural cells enhanced cell death and these two mSOD enzymes had nearly the same activities as wt SOD1 (Rabizadeh et al., 1995). G37R and G93A mSOD transgenic mice developed progressive motoneuron disease despite the fact that both G37R and G93A mSODs almost retained full specific activity (Borchelt et al., 1994; Gurney et al., 1994).

Currently it is believed that mSOD gains a toxic function. One hypothesis is that the toxic mSOD has aberrant copper-mediated catalysis. In addition to catalyzing the dismutation of O2- into H_2O_2 and oxygen molecules, SOD1 also has a peroxidatic action that utilizes its own product H_2O_2 as a substrate to generate free radicals (McCord and Fridovich, 1969). A number of *in vivo* and *in vitro* studies have shown that the hydroxyl radical generating ability of some of the mSOD enzymes, such as A4V and G93A, is enhanced relative to the wt enzyme, resulting in an increase of free radical generation (Wiedau-Pazos et al., 1996; Yim et al., 1996, 1997; Bogdanov et al., 1998). These observations are consistent with the findings of an augmented oxidative damage of cellular targets including DNA, protein and lipid, in both SALS and FALS, as well as in the mSOD mouse model of FALS (Ferrante et al., 1997a; 1997b). Also, this hydroxyl radical induced oxidation was found to be inhibited by copper chelators (Wiedau-Pazos et al., 1996).

A second possibility is that imperfectly folded mSOD would allow the access of abnormal substrate such as NO to the catalytic site so as to produce peroxynitrite leading to the nitration of tyrosine residues (Beckman et al., 1993). In support of this hypothesis

are the findings of increased levels of free 3-nitrotyrosine in the motoneurons of both SALS and FALS patients, as well as in the spinal cord of two different transgenic mouse models expressing G37R and G93A mSOD enzymes (Beal et al., 1997; Bruijn et al., 1997a; Ferrante et al., 1997b). However, no increases in protein-bound nitrotyrosine were found in ALS patients or in mSOD mice as compared to controls (Bruijn et al., 1997a). Another piece of evidence opposing this hypothesis is the failure to delay the death of motoenurons expressing G93A mSOD by preventing the formation of nitrotyrosine (Doroudchi et al., 2001).

A third hypothesis is that mSOD gains its toxic function via its capacity to form aggregates. The mSOD proteins are observed to aggregate into distinct, high molecular weight, insoluble protein complexes (IPCs), which are then sequestered into cytoplasmic inclusion bodies in motoneurons from mSOD transgenic mice or cultured motoneurons expressing mSOD (Tu et al., 1996; Bruijn et al., 1997b; Durham et al., 1997; Bruijn et al., 1998; Johnston et al., 2000). Also, there is intense SOD1 immunoreactivity present in the intracytoplasmic hyaline inclusions (IHIs) of the anterior horn cells from ALS patients at autopsy (Shibata et al., 1996). The aggregation of mSOD may cause stress to motoneurons since the stress-inducible chaperones such as 70-kDa heat shock protein (HSP70) can interact directly with mSODs, reduce formation of mSOD aggregates, and prolong the viability of cultured motoneurons expressing mSOD (Bruening et al., 1999; Shinder et al., 2001). Also, a decrease in overall chaperoning activity was observed in G93A mSOD mouse, together with the absence of HSP70 in spinal cords from ALS patients and in primary culture of motoneuron that was gene-transferred with mSOD (Bruening et al., 1999; Batulan et al., 2003). It has been suggested that motoneurons with

mSOD aggregates have an impaired ability to activate a main heat shock-stress sensor, HSF1, so that they are unable to induce the heat shock-stress response which could pretect themselves against further damage (Batulan et al., 2003).

1.4.2 mSOD mouse model of ALS

Transgenic mouse models of FALS have been extensively used to study the role of mSOD in motoneuron death. To date, there are 7 mouse models with different mutations which include A4V (Gurney et al., 1994), D90A (Brannstrom et al., 1997), G37R (Wong et al., 1995), G85R (Bruijn et al., 1997b), G86R (Ripps et al., 1995), G93A (Gurney et al., 1994) and G93R (Friedlander et al., 1997). It is well established that in mSOD transgenic mice, the age of onset of the disease, the severity of pathological abnormalities and clinical features are crucially determined by the copy numbers of the mutant transgene, or the expression level of the mutant enzymes (Wong et al., 1995; Dal Canto et al., 1996; see Pramatarova et al., 2001).

Comparative studies reveal a striking similarity in pathology between the mSOD mouse and human ALS, which include the loss of motoneurons, muscle atrophy, and the presence of large amount of vacuoles and proteineous aggregates in the axons and perikaryal regions of motoneurons. Affected mSOD mice initially showed hind limb weakness; when lifted by tail, they had difficulty extending and moving their hind limbs (Wong et al., 1995; Gurney et al., 1994). With time, they exhibited fine axial tremors, muscle wasting along the flanks and a rough coat appearance suggestive of impaired grooming (Gurney et al., 1994; Wong et al., 1995). Eventually their hind limbs were completely paralyzed and within weeks the mice became unable to eat and drink because of the degeneration of motoneurons in brainstem leading to the deinnervation of related

muscles (Wong et al., 1995; see Shibata et al., 2000).

In mSOD transgenic mice with the mutations of G37R and G93A, extensive vacuolation has been found in motoneurons at the regions such as dendrites, axons and perikarya (Wong et al. 1995; Mourelatos et al., 1996). Light microscopic and ultrastructural studies have revealed that the vacuoles originate from dilated endoplasmic reticulum and degenerating mitochondria (Dal Canto and Gurney, 1994). However, Kong and Xu (1998) observed that massive vacuolation was not derived from dilated endoplasmic reticulum, but rather abundant abnormal mitochondria. They also suggested that the toxicity of mSOD damaged mitochondria, which in turn triggered the decline of motoneuron function and the onset of clinical disease (Kong and Xu, 1998).

The abnormal accumulation of cytoskeletal proteins such as the NF, in the perikaryon and axon of spinal cord neurons, is a common pathological hallmark in both SALS and FALS (Hirano et al., 1984; Rouleau et al., 1996). NF is a major type of intermediate filaments (IFs) and is co-polymerized by high-, medium- and low-molecular weight subunits (NF-H, NF-M and NF-L). The NF aggregates within motoneurons in ALS were intensely immunolabelled with anti-SOD1 antibody (Chou et al., 1996; Shibata et al., 1996). Similarly, Tu and colleagues (1996) detected the NF inclusions (i.e., spheroids, Lewy body-like inclusions) in the spinal cord motoneurons of G93A mSOD mice at about the time these mice first showed clinical evidence of disease. It was suggested that the aggregates of mSOD are sequestered into the NF enriched inclusions and this process requires retrograde transport on micotubules (Johnston et al., 1998, 2000). Also, deletion/insertion mutations in the gene encoding NF-H are present in a few patients with SALS and FALS (Figlewicz et al., 1994; Al-Chalabi et al., 1999). These

observations led to the proposal that an impairment of NF assembly caused by mSOD or by mutations in NF itself is directly related to motoneuron death in ALS. A number of studies using transgenic mouse provide supporting evidences to this hypothesis. For example, transgenic mice overexpressing human NF-L or NF-H have neurofilamentous swellings and show symptoms related to neurodegeneration such as axonopathy, muscle atrophy, and altered axonal conductances (Cote et al., 1993; Xu et al., 1993; Kriz et al., 2000). In G85R mSOD mice, a lack of NF-L delayed both disease onset and progression (Williamson et al., 1998). However, the role of neurofilament disorganization in motoneuron death is not fully understood. Some recent observations provide new insight into the relation between neurofilament abnormalities and motoneuron degeneration. Unexpectedly, the overexpression of human NF-L in G37R mSOD mice did not expedite the disease progression and the expression of NF-H were seen to prolong the lifespan of G37R mSOD mice in a mSOD/NF-H-double transgenic (Couillard-Despres et al., 1998; 2000). Also, Kong and Xu (2002) had similar observations that both NF-L/G93A mSOD and NF-H/G93A mSOD transgenic mice had a later onset of disease and they lived longer than G93A mSOD mice. Furthermore, the perikaryal inclusion of NF proteins was suggested to confer protection by act as a sink for abnormal protein phosphorylation (Nguyen et al., 2001b). Combining all these findings, a possible explaination is proposed that a disruption of axon transport by aberrant NF assembly is pathogenic in ALS , while the perikaryal NF accumulatios have a beneficial role in motoneuron survival (See review: Julien, 2001; Al-Chalabi and Miller, 2003).

Other intermediate filaments such as peripherin are also involved in neurodegeneration. Peripherin is associated with IF accumulations occuring in

motoneurons of ALS patients and mSOD transgenic mouse (Corbo and Hays, 1992; Migheli et al, 1993; Tu et al., 1996; Beaulieu et al., 1999). Experiments using transgenic mouse models showed that peripherin overexpression induced the formation of aggregates and had a detrimental effect on neuron survival (Beaulieu et al.; 1999; 2000). The same observations were obtained in cultured motoneurons overexpressing peripherin (Robertson et al., 2001), and the Per61 splice variant of peripherin was found to be neurotoxic in motoneurons in culture (Robertson et al., 2003). However, the excess or lack of peripherin expression in G37R mSOD mice does not have any effect on disease onset and life span, indicating that peripherin may not be a key component of mSOD-associated motoneuron degeneration (Lariviere et al., 2003).

It is reasonable to postulate that except for the abnormality in intermediate neurofilaments, alterations in the integrity of other cytoskeletal proteins such as microtubule-associated proteins (MAPs) and membrane-associated proteins like Adducin, are potential causes of motor neurodegeneration. MAPs regulate the organization and stabilization of microtubules and the disruption of Tau or MAP1B were found to cause defects in axonal elongation and neuronal migration (Harada et al., 1994; Takei et al., 2000). Decreases in the protein levels of MAPs such as MAP1A, MAP2 and different tau species were observed in the spinal cord of G37R mSOD mice as early as 5 months before onset of symptoms (Farah et al., 2003). A possible involvement of Adducin in the pathogenesis of ALS will be discussed in Chapter 4 and in the general discussion.

1.4.3 Alteration of protein phosphorylation in nervous tissues of ALS and mSOD mouse

There are only a few reports about altered protein phosphorylation in postmortem

tissue from ALS patients. Lanius et al. (1995) showed an increase of Ca^{2+}-activated phospholipid-dependent PKC phosphotransferase activity in both cytosol and membrane fractions of cervical spinal cord tissue from ALS patients, compared with controls. This increase in activity is partially due to an increase of PKC protein expression. Wagey et al. (1998) confirmed this abnormality in both the expression level and activity of PKC in ALS spinal cord tissues. These observations suggest that PKC dysfunction could be involved in the pathogenesis of this disease. Wagey et al. (1998) also demonstrated increased activity and protein level of PI3-K in particulate fraction of spinal cord tissues from patients with SALS, compared with control subjects. Furthermore, this study measured the activities and protein levels of the possible downstream PKs of PI3-K, which are PKB and S6 kinase (S6K). There were significant elevations of PKB and S6K expression in tissue from ALS patients, but no changes in the activities of these PKs were found in ALS (Wagey et al., 1998). Another group demonstrated that both Cdk5 and accumulation of phosphorylated NFs were present in affected motoneurons of ALS tissue, together with the finding that CDK5 could induce cellular phosphorylation of NF-H side-arms (Bajaj et al., 1999).

The observations of abnormal protein phosphorylation in mSOD mice are also limited. An investigation of G93A mSOD transgenic mice showed decreased PI3-K and PKB immunoreactivity in spinal motoneurons at the early and presymptomatic stage (Warita et al., 2001). Janus kinase-3 (JAK3) is likely one of the contributors leading to neuron death since a specific inhibitor of JAK3, WHI-P131, delayed diasease onset and increased survival in G93A mSOD mouse (Trieu et al., 2000). Consistent with its presence in ALS tissue, CDK5 is also found to be distributed in spinal motoneurons of

G37R mSOD mice (Nguyen et al., 2001b). Increased immunoreactivity of both CDK5 and its activator, p25, were observed in G37R mSOD mouse, concomitant with the hyperphosphorylation of NF-M (Nguyen et al., 2001). This up-regulation of CDK5 causes the hyperphosphorylation of NF-H and NF-M proteins, as well as the microtubule-associated protein, tau (Nguyen et al., 2001). This same group of investigators has recently demonstrated that another member of the CDK family, CDK4, is also abnormally activated in G37R mSOD mice (Nguyen et al., 2003). Both an increase in activity and nuclear distribution of CDK4, as well as an elevated level of the CDK4 activator, cyclin D1, were seen in spinal cords of this transgenic mouse and it was suggested that the up-regulation of CDK4 leads to the hyperphosphorylation of the retinoblastoma (RB) protein (Nguyen et al., 2003).

1.4.4 Hypothesized protein phosphorylation networks in motoneuron death in ALS

A variety of PKs, PPs and and signaling molecules are involved in signaling pathways either promoting cell survival or leading to cell death. These numerous signaling pathways have interactions with each other because of the multiple-substrate properties of PKs and PPs, and/or the direct association between signaling molecules. Therefore, all these signaling pathways constitute an extensive protein phosphorylation networks that underlie the mechanisms of cell death.

The possible protein phosphorylation networks in motor neuron death in ALS are presented in figure 3. The two important survival pathways are the Ras/Raf1/MEK/ERK and the PI3-K/PKB pathways. They both can induce the phosphorylation of transcription factors or transcription factor-binding proteins and further increase the translation of antiapoptotic proteins that promote neuron survival. Furthermore, the PI3-K/PKB

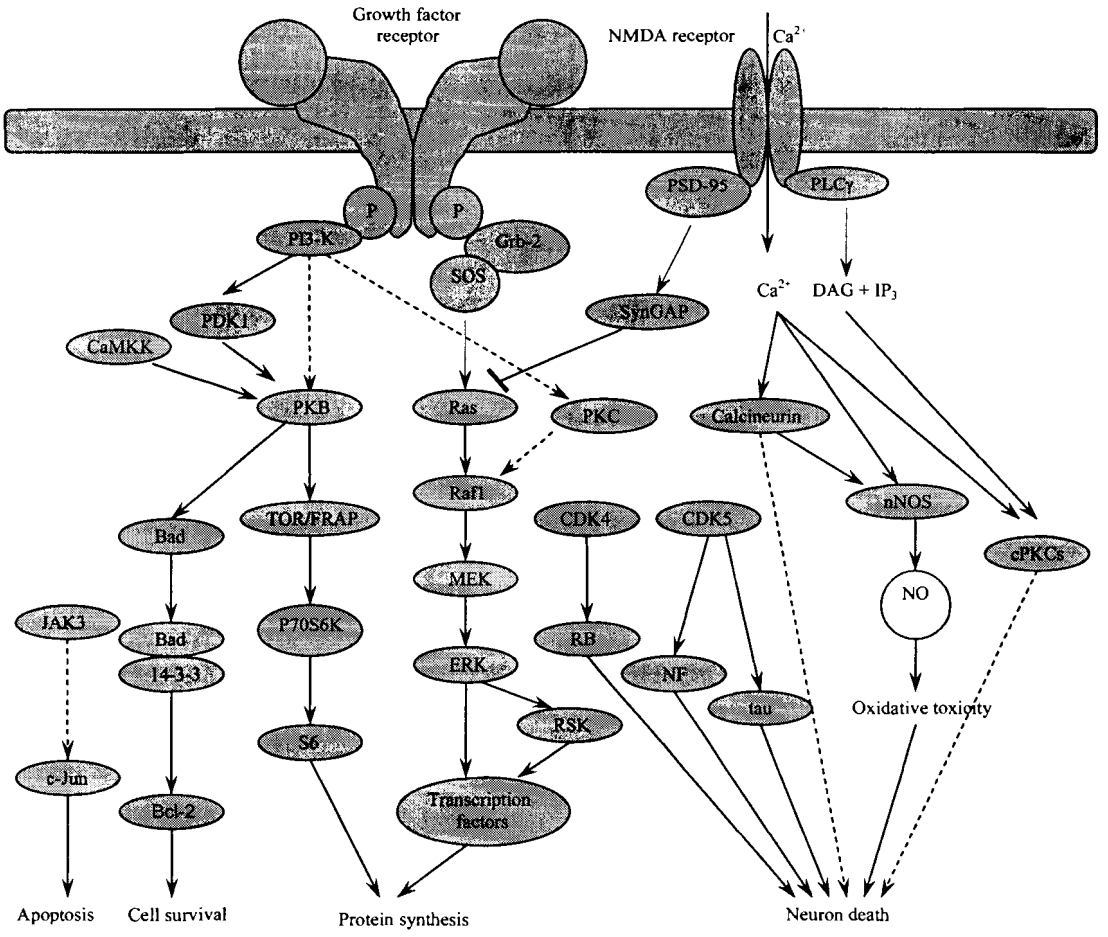

Figure 3: Possible protein phosphorylation networks in motoneuron death in ALS.
The impairment (block or down-regulation) of the PI3-K/PKB and Ras/Raf1/MEK/ERK survival pathways can result in motoneuron death. NMDA receptor neurotoxicity may play an important role in neuron death by the activation of Ca^{2+}-dependent enzymes including cPKCs, calcineurin and nNOS. Also, the NMDA receptor/PSD-95 complex inhibits the Ras/Raf1/MEK/ERK pathway by interacting with the regulator of Ras, SynGAP. CaMKK is likely to be neuroprotective by activating the PI3-K/PKB signaling pathway. However, JAK3 is believed to be involved in neuron apoptosis with the underlying mechanisms unclear. The deregulation of the Cyclin-dependent protein kinases CDK4 and CDK5 can cause the hyperphosphorylation of RB and cytoskeletal proteins such as NF and tau, wich leads to neuron death. Different isofoms of PKC may have opposite roles in neuron death since some isoforms of PKC can phosphorylate Raf1 and up-regulate the Raf1/MEK/ERK survival pathway.

pathway protects neurons by phosphorylating the pro-apoptotic protein Bad, pro-caspase 9 and the transcription factor Forkhead, thereby inhibiting their proapoptotic activity (see section 1.3.3). The Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) was reported to potentiate the role of PKB in preventing cells from undergoing apoptosis, via a direct phosphorylation and activation of PKB (Yano et al., 1998). And the NMDA receptor and PSD-95 possibly down-regulates the Ras/Raf1/MEK/ERK pathway, given the evidence that the NMDA receptor/PSD-95 complex associates with the regulator of Ras, SynGAP, so as to promote the inactivation of Ras.

NMDA receptor neurotoxicity may have an important role in motoneuron death in ALS. Excessive concentrations of glutamate activate NMDA receptors leading to a subsequent influx of Ca^{2+}, and this influx of Ca^{2+} can stimulate Ca^{2+} sensitive proteins such as PKC, calcineurin and nNOS. The Ca^{2+}-dependent PKC is proposed to be neurotoxic but its role in neuron death is still unclear. However, there is also evidence suggesting a neuroprotective role for PKC. These contradictory findings may arise from differences in the function or distribution of various isoforms of PKCs when using different experimental models. Several isoforms of PKC are believed to activate the Ras/Raf1/MEK/ERK survival pathway and may couple PKB/PI3-K signaling to the Ras/Raf/MEK/ERK cascade. Akimoto and colleagues (1996) demonstrated a novel signaling pathway from EGF or PDGF receptor tyrosine kinases to PKCλ through PI3-K. An *in vitro* study showed that cPKCs and nPKCs can phosphorylate and activate Raf1, while all the three isoforms of PKCs are capable of activating ERK as well as its immediate upstream activator, MEK1 (Schonwasser et al., 1998). Also, treatment of mouse 3T3 cells with a PKC activator, phorbol 12-myristate 13-acetate (PMA), leads to a

potent phosphorylation of Raf1 (Morrison et al., 1988). Furthermore, wortmannin, one of the most commonly used PI3-K inhibitors, blocks the activation of Raf1 and /or ERK triggered in L6 cells (Cross et al., 1994).

CDK4 and CDK5 are noteworthy because of the aberrant regulation of these two PKs in ALS patients and/or in the mSOD mice. They may be involved in motoneuron death since they can hyperphosphorylate NFs, tau, and possibly other cytoskeletal proteins, which finally contribute to neuron death, although the underlying mechanisms are not clear yet. Also, JAK3 may be involved in neuron apoptosis by inducing the phosphorylation of c-Jun (Virgo and Belleroche, 1995; Jaarsma et al., 1996; Goodman et al., 1998; Trieu et al., 2000).

In summary, two important signaling pathways that need to be explored for their involvement in motoneuron death in ALS include the PI3-K/PKB and the Ras/Raf1/MEK/ERK pathways. Also worthy of study are the consequences of activation of proapoptotic and oxidative damage pathways. The intricate protein phosphorylation networks described above will likely contribute to insights into the treatment of ALS.

1.5 Rationale and objective

1.5.1 Rationale

PKs regulate important cellular functions such as ion channel activity, neurotransmitter receptor properties, axon transport and cell survival. Human embryonic kidney (HEK)-293 cells transfected with NMDA receptor subunits have been used extensively as an *in vitro* model to study the mechanism of the modulation of NMDA receptor currents and NMDA-mediated cytotoxicity. Recent studies have reported that PKC activation modulates NMDA receptor-mediated currents and excitotoxic neuronal

death, however, the evidence is contradictory. The NMDA-mediated excitotoxicity has been identified as one of the several likely underlying mechanisms of ALS. Therefore, to establish if the activation of PKC is involved in NMDA-induced cell death and how PKC executes its role will help to elucidate the pathogenesis of ALS. Moreover, the imbalance between the actions of protein kinases and protein phosphatases could lead to impairment in biological function of a cell. Perturbations in the activities or expression of protein and lipid kinases have been reported in a variety of human neurodegenerative disorders such as ALS. Abnormalities in the activities or expression of PKC, PI 3-K and CDK5 have been reported in CNS tissue from patients who died with ALS, compared with control patients. Interestingly, the alteration of expression and activity of PKC in ALS spinal cord tissue coincides with its active role in modulating NMDA receptor activity and cytotoxicity. Also it is possible that, except for the reported PKs, other PKs and their downstream kinases are regulated inappropriately, and that the PI3-K/PKB and MAPK/ERK cell survival signaling pathways are impaired. This hypothesis provided a motive to screen and evaluate a total of over 130 PKs, PPs and phosphoproteins in spinal cord tissues of patients with sporadic ALS and control subjects. Mice overexpressing mSOD have been used as a murine model of ALS to investigate the cause of neuron loss in this neurodegenerative disorder of brain and spinal cord. The G93A mSOD transgenic mouse model of ALS that will be employed in my study is among the most extensively used models of ALS. Motoneurons of the medial sacral spinal cord within Onuf's nucleus (ON) are often spared in the neurodegenerative disorder ALS. To explore the fate of motoneurons in the spinal nucleus of the bulbocavernosus (SNB), the homologue to ON, as well as in the dorsolateral nucleus (DLN) and the retrodorsolateral nucleus (RDLN) of

severely affected G93A mSOD mice and controls will be studied. Furthermore, the expression profiles of various protein kinases, phosphoproteins, protein phosphatases will also be examined in order to find out whether the molecular mechanism producing neuron loss in ALS patients and in mSOD mice are similar.

1.5.2 Objectives

The objectives of the experiments in this thesis include:

1. To evaluate the role of PKC activation in NMDA-mediated toxicity and to elucidate how PKC executes its role;
2. To examine whether the impairment in cell survival signaling pathways could lead to cell death in ALS and which PKs and PPs are involved in the pathogenesis of ALS;
3. To investigate the fate of motoneurons in SNB, DLN and RDLN in lumbosacral spinal cord from mice over-expressing G93A mSOD, a murine model of familial ALS;
4. To explore the mechanisms underlying motoneuron death in mSOD mice and to compare whether these mechanisms are similar to those underlying neuron loss in patients with sporadic ALS.

CHAPTER 2
MATERIALS AND METHODS

2.1 General Materials

2.1.1 Chemical Reagents

ABC kit	Vector Laboratories
Acrylamide	Sigma
Adenosine 5'-triphosphate disodium salt (ATP)	Sigma
Agar	VWR
Ampicillin (D[-]-α-aminobenzylpenicillin)	Sigma
Aprotinin	Sigma
[γ-^{32}P] ATP	Amersham
Bactotryptone	Fisher
Bactoyeast extract	Fisher
Benzamidine	Sigma
Bis-acrylamide	Sigma
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	ICN
Calcium chloride ($CaCl_2$)	BDH
Chelex	Bio-Rad
Cyanine 3 (Cy3)	Jackson Laboratories
Diacylglycerol (DAG)	Sigma
Diaminobenzidine tetrahydrochloride (DAB)	Vector Laboratories
5,7-dichlorokynurenic acid (dichloro)	Sigma
Dimethyl sulfoxide (DMSO)	BDH
Dithiothreitol (DTT)	BDH
Enhanced chemiluminescence solution (ECL)	Amersham
Ethanol	Fisher
Ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA)	Fisher/ICN
Ethylene diamine tetraacetate disodium salt (EDTA)	Fisher/ICN
Fetal bovine serum	Gibco
Formaldehyde	Gibco
Glucose	BDH
Glutaraldehyde	BDH
Glycerol	Sigma
β-glycerophosphate	Sigma
Glycine	Sigma
Histone H1	Sigma
Hydrochloric acid (HCl)	Fisher

Hydrogen peroxide (H_2O_2)	BDH
Leupeptin	Sigma
L-glutamine	Gibco
Lipofectamine	Gibco
L-NAME	Sigma
LY 294002	Calbiochem
Magnesium chloride ($MgCl_2$)	Fisher
Magnesium sulfate ($MgSO_4$)	Fisher
β-mercaptoethanol	Fisher
Methanol	Fisher
Minimum essential medium (MEM)	Gibco
MOPS	Sigma
N-methyl-D-aspartate (NMDA)	Sigma
Nonidet P-40 (NP-40)	BDH
Opti-MEM	Gibco
Paraformaldehyde (PFA)	ACROS
PCR kit	Invitrogen
PD 98059	Gibco
Penicillin-streptomycin	Gibco
Pepstatin	Sigma
Permount	Fisher
Phenol:chloroform:isoamyl alcohol (25:24:1)	Ivitrogen
Phenylmethylsulphonylfluoride (PMSF)	Sigma
Phorol 12-myristate 13-acetate (PMA)	Sigma
Phosphate buffered saline (PBS)	Gibco
Phosphatidylserine (PS)	Sigma
Poly-D-lysine	Sigma
Poly-L-lysine	Sigma
Ponceau S concentrate	Sigma
Potassium chloride (KCl)	Fisher
Potassium ferricyanide	Sigma
Potassium ferrocyanide	Sigma
Protamine	Sigma
Protein A-Sepharose (PAS)	Pharma Biotech
Protein assay reagent	Bio-Rad
Proteinase K	Invitrogen
Pseudo A	Quality Controlled Biochemicals
Pseudo Z	Quality Controlled Biochemicals
Qiagen plasmid mega kit	Qiagen
RNase	Sigma
RO 32-0432	Calbiochem
Sodium azide (NaN_3)	Fisher
Sodium chloride (NaCl)	Fisher
Sodium dihydrogen phosphate (NaH_2PO_4)	BDH

Sodium dodecylsulphate (SDS)	Sigma
Sodium fluoride (NaF)	Sigma
Sodium hydrogen carbonate ($NaHCO_3$)	BDH
Sodium hydroxide (NaOH)	Fisher
Sodium orthovanadate (Na_3VO_4)	Sigma
Sodium pyrophosphate ($Na_4P_2O_7$)	Sigma
Sodium pyruvate	Gibco
Sucrose	BDH
Thionin	Sigma

2.1.2 Laboratory supplies

Nitrocellulose	Gelman Sciences/VWR
Polyvinylidene difluoride (PVDF) membrane	Millipore Corp.
3MM filter paper	VWR
Fluor-S MultiImager	Bio-Rad

2.1.3 Plasmid, bacterial strain and cell line

cDNA NR1A, NR1C	Gift from S. Nakanishi
cDNA NR2A	Gift from M. Mishina
cDNA $NR2A_{\Delta 1,267\text{-}1,458}$ (NR2A')	Gift from D.R. Lynch
cDNA pCMVβ	Clontech Laboratories
DH5α bacteria	Pharmacia
HEK-293 cell line	American Type Culture Collection

2.1.4 Antibody Reagents

The anti-PKC antibody (PKC-αβ, monoclonal) was a gift of Dr. S. Jaken. It is directed to Ca^{2+}-dependent PKC isoforms including PKCα and PKCβ, but not PKCμ or PKCζ. The anti-ERK1-CT antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA). It is directed to the C-terminal domain of ERK1, but will cross react with ERK2 (ERK1/2). The anti-phospho-ERK, anti-phospho-PKC αβ II and anti-phospho-Adducin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-phospho-Adducin antibody reacts with Ser662 phosphorylated Adducin γ, Ser724 phosphorylated Adducin α and Ser13 phosphorylated Adducin β.

The primary antibodies used for Kinetworks™ KPKS 1.0 Protein kinase, KPPS 1.1 Protein Phosphatase and KPSS 1.1 Phosphoprotein Screens and individual Western blot analysis of human postmortem samples were principally from Santa Cruz Biotechnology (Santa Cruz, CA, USA), BD Biosciences (Lexington, KY, USA), Upstate Biotechnology (Lake Placid, NY, USA), Biosource International (Camarillo, CA, USA), and Exalpha Biopharmaceuticals (Boston, MA, USA).

The anti-rabbit and anti-mouse second antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.1.5 Cell lysates

Detergent-solubilized extracts from HEK-293 were prepared as described below.

2.1.6 Rat tissues

Tissue from rat brain, thymus and skeletal muscle were used to examine the presence of various isoforms of PKC. Tissues were removed after Sprague-Dawley rats (male, 50 g) were euthanized with halothane.

2.1.7 Human tissues

For the study of the presence of PKC isoforms, human postmortem spinal cord tissue was obtained from eight patients without evidence for neurological disease [mean age ± SE, 73.1 ± 5.1 years; mean death-to-freezing interval (DF) ± SE, 10.1 ± 2.0 h].

For the study of PK and PP expression, human postmortem spinal cord tissues (restricted to thoracic levels) were collected from patients with ALS [n = 7; mean age ± SE, 69.1 ± 0.9 year (n = 6, age of one patient not known); mean DF interval ± SE, 6.7 ± 2.5 h] and control subjects [n = 5; mean age ± SE, 67.5 ± 7.8 year (n = 4, age of one

patient not known); mean DF interval ± SE, 14.0 ± 6.0 h] and stored at –80 °C before analysis.

2.1.8 Mouse tissues

Hemizygous B6SJL-TgN(SOD1-G93A)1Gur^{dl} transgenic mice which overexpress human SOD1 containing the G93A mutation found in FALS and wt controls were obtained from Jackson Laboratory (Bar Harbor, MA), or locally bred from such progenitor stock. Identification of the genotype of mSOD mice was confirmed using PCR protocols to amplify the 236 bp product from exon 4 of the human *sod1* gene using protocols described below.

For the study of lumbar motoneuron fate, mSOD mice were killed at approximately 4–5 months of age, with a pre-determined end-point based on the appearance of a set of behavioral markers (including hind limb ataxia, and an inability to forage due to paralysis of the hindlimbs). Age-matched male littermates served as controls. Mean (± SD) survival after disease onset was 19.6 ± 9.4 days (n = 12), but a small cohort displayed a variable disease progression.

For the study of PK and PP expression, all transgenic animals were clinically affected when sacrificed, and demonstrated moderate hind limb weakness resulting in a slow, unstable gait. However, animals were still able to ambulate, to feed and to right themselves. Sacrifice occurred around day 123 ± 2.2 days (mean ± SE). Mice over-expressing mSOD to this extent (~20-fold), have been reported to die at around 130 days of age, following rapidly progressive weakness lasting about 2 weeks (Gurney et al., 1994). The time of death was chosen so as to be similar to the severity of involvement of some ALS patients at the time of death. Controls comprised age-matched littermates that

were phenotypically normal as well as were PCR negative for human *sod1* gene and were sacrificed at the same age as mSOD mice. Controls (n = 5) and mSOD mice (n = 5) were killed using blended CO_2 and O_2 and their brains and spinal cords were removed and stored at –80 °C.

The use of human postmortem tissue and rodent tissue was approved by the ethical review panels at the University of British Columbia and Simon Fraser University, and meets the Canadian tri-council guidelines. The use of mouse tissue was in accordance with the requirements of the SFU Animal Care Committee and the Canadian Council for Animal Care. Efforts were made to minimize the number of animal used.

2.2 General methods

2.2.1 Plasmid transformation and purification

Competent cells (DH5α) were thawed on ice, mixed and aliquoted into microcentrifuge tubes. As a control for transformation efficiency, 0.5 ng control pUC 19 was added to one vial of competent cells. DNA (1−10 ng) of intereste was added to one of the microtubes containing competent cells, incubated on ice for 30 min. Cells were then heatshocked at 42 °C for 20 seconds then placed on ice. SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) medium was added to the cell-DNA mixture and shaken for 1 h at 37 °C for expression. After expression, the mixture containing the control pUC19 was diluted into medium (1:10). Then 100 μl of the diluted and the undiluted mixture were spread onto LB plates with 100 μg/mL ampicilin and 50 μg/ml X-gal. For the experimental mixture, after expression the mixture was diluted and spread (150 μL) onto LB plates and

incubated overnight at 37 °C.

After transformation of the required plasmid, a bacteria *E coli* culture containing the plasmid was grown overnight at 37 °C. A toothpick was immersed in the culture and streak onto an agar plate (LB, agar and ampicilin) which was incubated at 37 °C ovenight. The next morning a colony was picked from the agar plate and a starter culture of 3 ml medium containing ampicilin (100 μg/mL) was inoculated and left in the shaker for about 8 h. The starter culture was diluted 1 ml into 400 ml of LB medium (+Ampicilin). The culture was grown at 37 °C for 16 h, shaking at 225 revolutions per minute (rpm) for a preparation of high copy plasmids.

Plasmid purification was done using Qiagen plasmid mega kit (Qiagen Inc.) which is a purification procedure based on a modified alkaline lysis followed by binding of plasmid DNA to the anion-exchange resin. Other impurities such as RNA and proteins are removed through washes with a medium-salt buffer. The DNA is eluted in a high-salt buffer and concentrated further through isopropanol precipitation.

2.2.2 DNA yield

The yield of DNA concentration obtained from plasmid DNA purification was determined by UV spectrophometry. The DNA plasmid was diluted (1:5) with ddH_2O and measured at optical density (OD) 260 nm to determine the DNA concentration. The ratio of OD 260 nm over 280 nm was used to determine the purity of the obtained plasmid DNA.

2.2.3 Cell culture

HEK-293 cells were cultured in minimum essential medium (MEM) containing

10% fetal bovine serum (FBS), penicillin/streptomycin (100 U/mL), L-glutamine (2 mM) and sodium pyruvate (1 mM) as described previously by Chen et al. (1997). Cells were plated at a density of 1 x 10^6 cells /1.5 mL MEM medium onto poly-D-lysine pre-coated tissue culture plates and incubated in a 5 % CO_2 incubator at 37 °C for 24 h.

2.2.4 Transfection

Cells were transiently transfected using lipofectamine or Lipofectamine™ 2000. A total of 1.5 μg of cDNA was added to each 35 mm plate in 150 μL of Opti-MEM. In experiments that used two different plasmids, equal amounts of the plasmids were added to give a total of 1.5 μg. With co-transfection of lacZ, lacZ cDNA comprised 20% of total cDNA. Transfection employed 7.5 μL lipofectamine/plate (5 μL lipofectamine for 1 μg cDNA). Cells were left for 5 h at 37 °C in a 5% CO_2 incubator. The transfection was terminated by adding serum-containing MEM and the NMDA receptor antagonist, 5-7 dichlorokynurenic acid (dichloro; 250 μM).

2.2.5 β-Gal staining

To determine transfection efficiency, β-Gal staining was performed on the plates transfected with the NMDA receptor subunits. Briefly, in addition to the NMDA subunits, the cells were co-transfected with a plasmid containing *E. coli* β-Gal gene. After transfection and treatment, cells were washed once with PBS (37 °C), fixed at room temperature for 5 min with a fixative solution [37% formaldehyde, 25% glutaraldehyde and phosphate buffered saline (PBS), pH 7.3] and washed again with PBS (3 times). Cells were then stained with a freshly made solution of 1.2 mM 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 5 mM potassium ferrocyanide, 5 mM potassium

ferricyanide and PBS, pH 7.3, then incubated at 37 °C for about 16 h. The expression of β-Gal was assessed under the microscope by counting the ratio of cells that incorporated β-Gal (blue) versus cells that did not incorporate β-Gal (white). β-Gal expression was calculated as the percentage of cells that incorporated β-Gal. Plates with transfection efficiencies ranging from 60–80% were used for further experiments.

2.2.6 Preparation of inhibitors and activators

The inhibitors and activators of PKs that have been used in this study include RO 32-0432, PMA, Psuedo A, Pseudo Z, LY 294002 and PD 98059. RO 32-0432 (RO), PMA and PD 98059, were initially dissolved in 100% dimethyl sulfoxide (DMSO). PseudoA and PseudoZ were in H_2O. LY-294002 was solubilized in 100% ethanol. The nonselective inhibitor of NOS, N(omega)-nitro-L-arginine methyl ester (L-NAME) was dissolved in H_2O. Due to the instability of some substances, solutions having color changes or precipitate were discarded.

2.2.7 Treatment protocols

Cells were exposed to various treatments 22 h after the start of the transfection. Two 35-mm plates were prepared for each treatment. Control cells were incubated in a physiological salt solution (PSS; 140 mM, NaCl; 1.4 mM $CaCl_2$; 5.4 mM KCl; 1.2 mM NaH_2PO_4; 21 mM glucose; 26 mM $NaHCO_3$; pH 7.4) containing dichloro 250 μM for 10 min in a 5 % CO_2 incubator at 37 °C. Activation of NMDA receptors was performed by incubating the cells in PSS containing NMDA (1 mM) and glycine (50 μM) for 10 min. Experiments using the PKC inhibitor, RO (1 μM), a bisindolymaleimide derivative of staurosporine, were performed in the presence and absence of NMDA and glycine. In the

presence of the NMDA receptor agonists, RO treatment was begun 10 min prior to incubation with NMDA and glycine. In the absence of NMDA receptor agonists, RO was applied in PSS with dichloro for 10 min. After the 10 min treatments at 5% CO_2, and 37 ^{o}C, cells were placed in fresh media containing dichloro for 6 h before assessment of cell death.

Cells were exposed to NMDA receptor agonists in the presence and absence of phorbol PMA (85 nM), a PKC agonist. As a control, transfected cells were incubated in PSS containing dichloro for 10 min. Treatment with NMDA receptor agonists was performed as described above. Treatment with PMA was performed by adding the compound directly to the media 10 min before replacing the media with PSS and dichloro. In the presence of the NMDA receptor agonist, cells were exposed to PMA 10 min prior to incubation in PSS containing NMDA and glycine. Following treatment with the NMDA receptor agonists, cells were incubated in fresh media containing dichloro for 6 h prior to cell death assessment.

To determine the effect of the PKC inhibitor RO on PMA and NMDA-mediated cytotoxicity, this compound was added 10 min prior to adding PMA, as described above. Cells were then incubated for 10 min in PSS containing NMDA, glycine and RO before replacing PSS with fresh media containing dichloro and RO for 6 h. These same procedures were used for cells transfected with NR1C and NR2A subunits. RO was used at a concentration of 1 μM which is similar to that employed by other investigators to produce PKC antagonism in cultured cells [see: Li et al., 1999 (10 μM); Huang et al., 2000 (200 μM)].

To evaluate the role of different PKC isoforms in cytotoxicity, isoform-specific

PKC inhbitors were used such as pseudo A (40 μM), which inhibits the action of Ca^{2+}-dependent PKC isoforms, and pseudo Z (40 μM) which inhibits activation of PKCζ, an atypical PKC isoform. These two inhibitors were added separately 30 min prior to exposure of cultures to PMA. Cells were further incubated in PSS in the presence of NMDA receptor agonists and pseudo A, or pseudo Z for 10 min before replacing PSS with media containing dichloro in the presence of pseudo A or pseudo Z.

2.2.8 Trypan blue assay

Cell death was determined by trypan blue exclusion by live cells (see Cik et al., 1993). Two plates of HEK-293 cells were prepared for each treatment. Six h following treatment, cell death was assessed both in attached and floating cells by trypan blue exclusion. To determine the percentage of dead cells in the media, media from each plate (1.5 mL) was removed and trypan blue added. Cell counts were performed on a hemocytometer in 10 counting areas and the average number of live and dead cells was calculated as a percent cell death. Death of attached cells was determined by trypsinizing cells and assessing the percent cell death in a similar manner as for floating cells. All data points correspond to the mean ± SE of values performed in duplicate from at least 3 separate experiments.

2.2.9 Preparation of cell lysates and mouse, rat and human tissues

The whole lysates of HEK 293 cells, the brain and spinal cord tissues of control and mSOD mice, as well as the brain, thymus and skeletal muscle tissues of rats, were obtained by homogenizing tissue in a lysis buffer [150 mM NaCl, 20 mM Tris pH 8.0, 0.5% (w/v) Nonidet P-40, 1 mM dithiolthreitol (DTT), 20 mM β–glycerolphosphate, 1

mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/mL aprotinin and 10 mg/mL leupeptin] and sonicated for 30 s. Cell or tissue debris was removed by centrifugation at 109,000 g for 30 min at 4°C.

Human spinal cord sections were weighed and lysed in homogenization buffer [20 mM 3-(N-morpholino) propane sulfonic acid (MOPS), 2 mM ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA), 5 mM ethylene diamine tetraacetate disodium salt (EDTA), 30 mM sodium fluoride, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM PMSF, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin, pH 7.2] (4 mL homogenization buffer/1 g tissue). Tissues were then sonicated and ultracentrifuged (100,000 g, 30 min, 4 °C). The supernatant (cytosolic fraction) was stored at –80 °C. The particulate pellet was resuspended in homogenization buffer containing 0.5% NP-40 by sonicating and ultracentrifuged under the same condition as above. The supernatant, which is the solubilized particulate fraction, was stored at –80 °C. Protein concentration was assessed by Bradford assay (1976).

2.2.10 Assessment of protein concentration

The Bradford method (1976) was used to assess the protein concentration. A series of protein standards randing from 0−12 μg bovine serum albumin (BSA) were prepared. The Bio-Rad protein assay reagent (Bio-Rad) was added to each standard. The samples to be quantitated were diluted with dH_2O to within 5−20 μg/10 μl, 200 μl of the protein assay reagent was added to each sample and mixed by gentle vortexing. After 10 min incubation, the absorbance of the solutions was measured at 595 nm in a spectrophotometer and the concentrations of the samples calculated through linear

regression plotting of the standards.

2.2.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

2.2.11.1 SDS-PAGE

Protein concentrations cell or tissue samples were determined as described (Bradford, 1976), and adjusted to 1 mg/ml in sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2.5% SDS, 10% glycerol, 50 mM Tris-HCl, pH 6.8, 0.5 M β-mercaptoethanol and 0.01% bromophenol blue) before use.

Protein samples were then diluted with 4x concentrated Laemmli sample buffer [120 mM Tris-HCl, pH 6.8; 4% SDS (w/v); 20% glycerol (v/v), 10% β-mercaptoethanol, 0.01% bromophenol blue (w/v)], boiled for 5 min then loaded and resolved on SDS-PAGE gels (4% stacking gels and 8%, 10% or 12% separating gels). The gels were electrophoresed for 1 h at constant voltage of 200V in running buffer [25 mM Tris, 192 mM glycine, 0.1% SDS (w/v), pH 8.3]. After electrophoresis, the separating gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol (v/v), pH 8.6), and transfer to nitrocellulose or polyvinylidene difluoride (PVDF) membranes was performed for 1 h at constant current of 300 mA.

2.2.11.2 Multi-immunoblotting screens

The kinetworks^{TM} KPKS 1.0 Protein Kinase, KPPS 1.1 Protein Phosphatase and KPSS 1.1 Phosphoprotein Screens were performed as described (Pelech and Zhang, 2002; Zhang et al., 2002; or view the Kinexus Bioinformatics Corp. website, http://www.kinexus.ca); each screen requires 600 μg, 350 μg and 300 μg proteins,

respectively. The kinetworks™ analysis involves resolution of a single lysate sample by SDS-PAGE and subsequent immunoblotting with panels of up to three primary antibodies per channel in a 20-lane Immunetics multiblotter. The antibody mixtures were carefully selected to avoid overlapping cross-reactivity with target proteins.

2.2.11.3 Western blot analysis

The nitrocellulose or PVDF membranes were blocked in 5% skim milk (w/v) or 3% BSA in TBST buffer (50 mM Tris base, 150 mM NaCl, 0.5% Triton x-100 (v/v), pH 7.4). After blocking, membranes were rinsed with TBST and then incubated overnight at 4 °C with selected primary antibodies. The membranes were then rinsed with TBST and followed by an incubation with the relevant horseradish peroxidase (HRP) conjugated secondary antibodies for 45 min at room temperature. The blots were developed with enhanced chemiluminescence (ECL) reagent, signals were captured by fluor-S MultiImager and quantified using Quantity One software (Bio-Rad, Hercules, CA, USA). Background was less than 500 for these analyses. The reproducibility of these signal transduction protein screens was typically within 15%.

2.2.11.4 Stripping and reprobing of western blotting

To reuse a nitrocellulose or PVDF membrane for the detection of another antibody, the membranes were stripped by incubation in a stripping solution [100 mM β-mercaptoethanol, 2% SDS (w/v), 62.5 mM Tris-HCl (pH 6.7)] for 30 min at 55 °C with occasional agitation. The membranes were rinsed with TBST several times to wash out the β-mercaptoethanol. The membranes were then reblocked in 5% skim milk or 3% BSA in TBS for 1 h and the Western blot analysis procedure was used as described in

2.2.11.3.

2.2.12 Immunoprecipitation and in vitro kinase assay

For measurement of PKC activity, PKC was immunoprecipitated with an anti-PKC αβ antibody (0.5 mg/mL) using methods as described previously (Lanius et al., 1995; Wagey et al., 1998). Immunoprecipitated PKC was then used for an *in vitro* kinase assay by adding KII buffer [12.5 mM β-glycerophosphate, 12.5 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM $MgCl_2$, 50 mM NaF and 0.25 mM DTT, pH 7.2], histone H1 (1 mg/mL), or protamine (1 mg/mL) and 10 μL [$\gamma^{32}P$] ATP (specific activity 1250 cpm/ pmol), as described previously (Wagey et al., 1998). To assess lipid-dependent activity, additions of Mg^{2+}, Ca^{2+} and lipid [6 μg/mL of DAG and 60 μg/ml of phosphatidylserine (PS)] were made; Mg^{2+} alone was added for lipid-independent assays.

2.2.13 Polymerase chain reaction (PCR) test of mouse genotype

The genotype of mSOD mouse was confirmed via PCR test using mouse genomic DNA as template. Mouse genomic DNA was extracted by incubating mouse blood or ear tissue in a mixture of Chelex (5% w/v), Proteinase K (2 mg/mL) and RNase (1 mg/mL), followed by extracting with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitating with ethanol. The genomic DNA pellet was air dried, then dissolved in Tris-EDTA buffer (TE, pH 8.0) and stored at –20 °C before using as template.

PCR was done undergoing the following cycles: once cycle of 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s; and a final extention at 72 °C for 10 min. The forward and reverse primers used are CATCAGCCCTAATCCATCTGA and CGCGACTAACAATCAAAGTGA, respectively. The detection of PCR product was

performed by running a 1.2% agarose gel electrophoresis.

2.2.14 Histology of mSOD mouse lumbaosacral spinal cord

Experimental animals (n = 9) and controls (n = 12) were killed using blended CO_2 and O_2, and rapidly perfused transcardially with phosphate buffered saline (0.1M PBS, pH 7.4) followed by buffered 10% paraformaldehyde (PFA; pH 7.4). Lumbosacral spinal cords were dissected out, post-fixed in 10% PFA for 24 hours, and then transferred to a 20% sucrose/PBS solution overnight for cryoprotection. Specimens were blocked at the lumbar enlargement, frozen, and sectioned in the transverse plane at 50 μm on a sliding microtome. After mounting every third section on gelatin-subbed slides, the tissue was Nissl-stained using 8% thionin, dehydrated in graded alcohol solutions, cleared in xylene, and cover-slipped with Permount for analysis.

2.2.15 Analysis of motoneuron loss and muscle atrophy

Two raters blind to experimental conditions examined the thionin stained tissue at 100X using a Nikon Eclipse E600 light microscope. The mean number of motoneurons per section for the three motoneuron pools was calculated for each animal. On average, about 6 slices per animal were analyzed. All large, multipolar motoneurons in the SNB, DLN, and RDLN darkly stained for Nissl substance, and displaying apparent nuclei and nucleoli, were included in the analysis.

Bulbocavernosus muscles were dissected out, trimmed of fat, cleaned in de-ionized water, blotted dry, and weighed. We also noted the condition of the testes and the degree of atrophy in the thigh muscles of the affected animals upon dissection.

2.2.16 Immunohistochemistry (IHC)/Immunocytochemistry (ICC)

The frozen sections of mouse spinal cord (50 μm) were obtained as described in 2.2.14. The immunolabelling of phospho-Adducin in spinal cord is performed as following, with a 3-time wash using 0.3% Triton X-100 in 0.1 M PBS (PBST, pH 7.4) between any two incubations. To abolish endogenous peroxidase activity, the free-floating sections were firstly incubated with 0.3% H_2O_2 in 10% methanol for 30 min. After a blocking with 10% serum in PBST for 30 min, sections were incubated with anti-phospho-Adducin antibody at a dilution of 1:1000 in PBST containing 10% serum, for 48 h at 4 °C. Then sections were incubated with biotinylated anti-rabbit secondary antibody (1:500) for 1 h at room temperature, followed by an incubation with ABC reagent (1:1,000) in PBST for 90 min. The ABC regeant contains Avidin DH (A) and biotinylated horseradish peroxidase H (B), which was pre-mixed 1 h before use to allow the formation of AB complex. Color development was done by using diaminobenzidine tetrahydrochloride (DAB) as a substrate of peroxidase and the reaction was stopped by flood with dH_2O. In the end free-floating sections were mounted on slides coated with poly D-lysine, air dried and coverslipped with Permount media.

The dorsal root ganglia (DRGs) were isolated from mSOD and control mice perfused with 4% PFA, post-fixed overnight and dehydrated with ethanol. After they were embedded in paraffin, 5 μm sections were cut and mounted on poly-L-lysine-coated glass slides. After de-waxing in xylene, sections were fixed with 2% PFA for 5 min. Then sections were permeabilized and blocked using 0.2% Triton X-100 in PBS with 10% serum, for 10 min, follwed by an incubation with a rabbit polyclonal antibody to phospho-Adducin at a dilution of 1:200 in PBS with 1% serum for 1 h. After that, cells

were incubated with a donkey anti-rabbit antibody tagged with cyanine 3 (Cy3) at a dilution of 1:500 in PBS with 1% serum for 45 min at room temperature in the dark. Finally sections were washed in PBS, dehydrated in graded ethanol and coverslipped using Permount.

The culture, transfection and treatment of HEK 293 cells was described in 2.2.3, 2.2.4 and 2.2.7, respectively, with the only difference that cells for immunostaining of phospho-adducin were initially plated onto glass coverslips. The staining procedure started with a fixation of cells with 4% PFA in PBS for 10 min. The following steps were the same as described above using DRG tissue, except that the anti-phospho-Adducin primary antibody was used at a dilution of 1:1000 and that in the end cells were mounted on slides using Vectashield mounting media.

2.3 Statistical analysis

For the trypan blue cell death in HEK cells, statistical comparisons of cell death between groups were performed using ANOVA to determine a group effect (p-value $\leq$ 0.001). A student's *t*-test with Bonferroni correction was then used to determine which groups were statistically different from each other.

For the Kinetworks screens of PKs, PPs and phosphoproteins in tissues from human and mouse, results are expressed as mean ± SD and were evaluated using Student's *t*-test with $p < 0.05$ being considered as statistically significant. No correction was made for multiple tests.

The expression levels of selected proteins in human spinal cord tissues were measured using individual Western blot analysis. The difference in expression of each protein between patients with ALS and control subjects was analyzed using Student's *t*-

test.

The overall effects of motoneuron loss were analyzed using a 2 X 3 ANOVA, treating genotype (mSOD vs control) as a between-subjects factor, and cell type (SNB, DLN, or RDLN) as a within-subjects factor. *A priori* planned comparisons of means were tested using independent-samples *t*-tests. For analysis of the distribution of motoneuron death as a function of the length of survival after disease onset, animals were divided into two groups based upon the duration of survival after the onset of limb weakness; the mean number of motoneurons per section in the SNB, DLN, and RDLN were compared between these groups. One group consisted of animals that had a rapid progression of weakness and were killed, on average, 11 days after the onset of symptoms [the fast disease progression group (FPG); n = 4] and the other group consisted of animals killed approximately 18 days after the onset of signs [the slower disease progression group (SPG); n = 3].

CHAPTER 3
MODULATION OF NMDA-MEDIATED EXCITOTOXICITY BY PROTEIN KINASE C*

3.1 Summary

Excessive activation of NMDA receptors leads to cell death in HEK cells which have been transfected with recombinant NMDA receptors. To evaluate the role of PKC activation in NMDA-mediated toxicity, we have analyzed the survival of transfected HEK cells using trypan blue exclusion. We found that NMDA-mediated death of HEK cells transfected with NR1/NR2A subunits was increased by exposure to phorbol esters and reduced by inhibitors of PKC activation, or PKC down-regulation. The region of NR2A that provides the PKC-induced enhancement of cell death was localized to a discrete segment of the C-terminus. Use of isoform-specific PKC inhibitors showed that Ca^{2+}- and lipid-dependent PKC isoforms (cPKCs), specifically PKCβ1, was responsible for the increase in cell death when phorbol esters were applied prior to NMDA in these cells. PKC activity measured by an *in vitro* kinase assay was also increased in NR1A/NR2A transfected HEK cells following NMDA stimulation. These results suggest that PKC acts on the C-terminus of NR2A to accentuate cell death in NR1/NR2A transfected cells and demonstrate that this effect is mediated by cPKC isoforms. These data indicate that elevation of cellular PKC activity can increase neurotoxicity mediated by NMDA receptor activation.

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3.2 Introduction

NMDA receptors are heteromeric proteins composed of NR1 subunits (ζ1), along with one or more subunit, such as NR2A (ε1), NR2B (ε2) NR2C (ε3), or NR2D (ε4) (Sucher et al., 1996). The regulation of NMDA receptor function has been extensively studied using recombinant NMDA receptors expressed heterologously in HEK-293 cells, or other cells. A proportion of HEK cells transfected with NMDA receptor subunits die following exposure to NMDA receptor agonists, depending on the NMDA receptor subunit composition (Cik et al., 1994; Anegawa et al., 1995; Raymond e t al., 1996). For instance, transfection of HEK-293 cells with cDNAs for the NR1 subunit of the NMDA receptor produces much less cell death than when cells are co-transfected with cDNAs for NR1 and NR2A subunits (Raymond et al., 1996). Analysis of NMDA-evoked currents has demonstrated that HEK cells transfected with NR1 subunits do not express functional NMDA receptor channels and that the increased cell death observed with the NR1/NR2A co-transfected cells is dependent on the expression of functional NMDA receptor channels having appreciable ionic flux to both Na^+ and Ca^{2+}. Experiments in HEK cells using mutant NMDARs have shown that cell death is more frequent in cells with NMDARs having high Ca^{2+} permeability (Raymond et al., 1996).

PKC activation modulates NMDA receptor-mediated currents (Ben-Ari et al., 1992; Kelso et al., 1992; Markram and Segal, 1992; Urushihara et al., 1992; Mori et al., 1993; Raymond et al., 1994; Zukin and Bennett, 1995; Xiong et al., 1998; Logan et al., 1999; Zheng et al., 1999; see also MacDonald et al., 1998 for review) and Ca^{2+} influx through NMDAR channels (Murphy et al., 1994; Grant et al., 1998). In neurons, PKC-mediated modulation of NMDA-mediated currents is complex. In some neurons, PKC

activation results in an increase in NMDA current (e.g. Ben-Ari et al., 1992), whereas activation of PKC in some hippocampal neurons suppresses responses to NMDA (Markram and Segal, 1992).

Although PKC activation has been thought to be responsible for modulating excitotoxic neuronal death, the evidence is contradictory (e.g. Favaron et al., 1990; Durkin et al., 1997). For example, Felipo and colleagues (1993) claimed that inhibitors of PKC are able to protect cultured cerebellar neurons from excitotoxic death. In contrast, Durkin et al. (1997) reported that cerebral cortical neurons which have been in culture for 8 days become highly vulnerable to neuron death when exposed to excitatory amino acids (EAAs), including NMDA, in the presence of PKC inhibitors. In the absence of PKC inhibitors the neurons are relatively resistant to neurotoxicity.

To establish if the activation of PKC is involved in NMDA-induced cell death we evaluated the extent of cell death in HEK cells transfected with NMDA receptor subunits.

3.3 Results

3.3.1 PKC protein and activity in transfected HEK 293 cells

To evaluate the presence of PKC isoforms in HEK-293 cells transfected with NR1A/NR2A subunits, Western blots were performed by Kinetworks™ KPKS-1.0 analysis, which employs nine PKC isoform-specific antibodies. As shown in Fig. 4a, HEK-293 cells highly expressed the μ and ζ calcium-independent isoforms of PKC, and had moderate levels of the β1 Ca^{2+}-dependent PKC isoform. This pattern of PKC isoform expression most closely resembled the results obtained from human spinal cord (Fig. 4b) when it was also compared with nine different rat tissues (data shown only for brain, thymus and skeletal muscle in Fig. 4c-e). A major difference from human spinal cord was

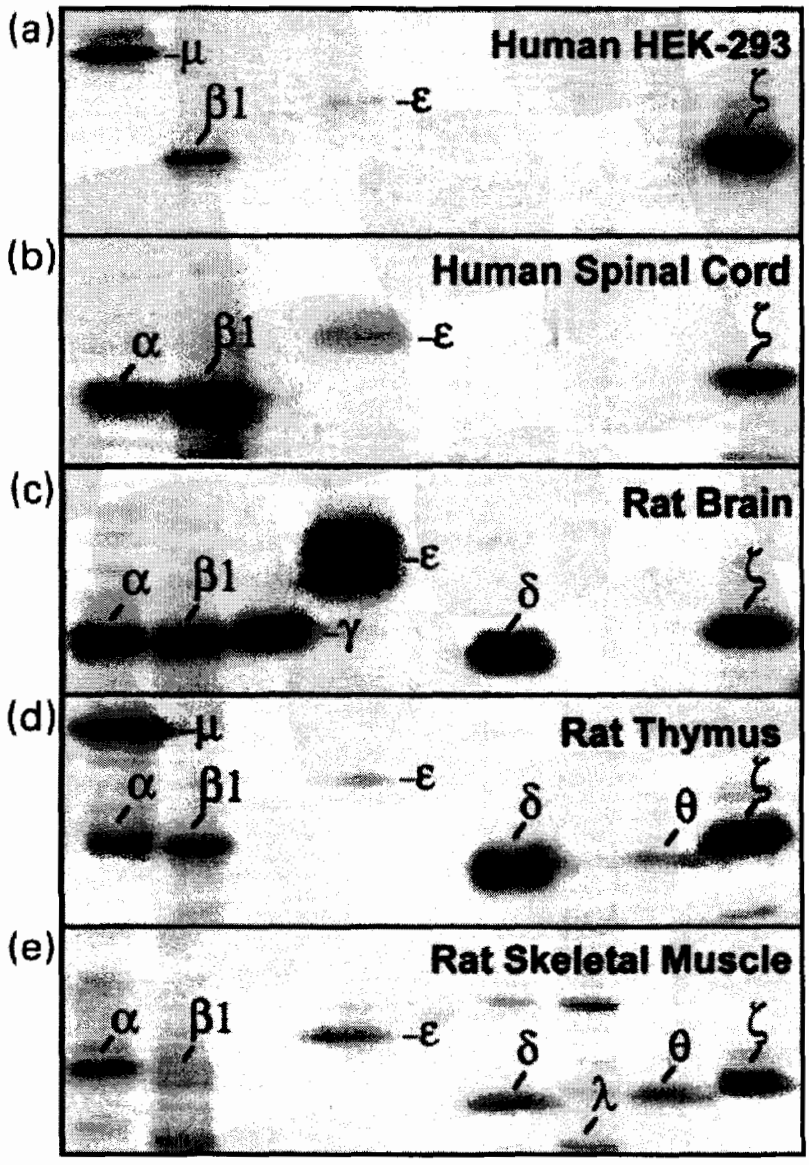


Figure 4: Immunoblotting analysis of PKC isoforms in HEK cells, human and rat tissues.
(a) Kinetworks™ immunoblotting analysis of PKC isoforms in HEK-293 cells. Enhanced chemiluminescent detection of the presence of immunoreactivity principally to the μ, β1 and ζ PKC isoforms is shown, as quantified with a Bio-Rad FluorS MAX scanner. Only the regions of the Kinetworks™ blot that demonstrate the various PKC isoforms are shown. (b) PKC isoform expression in adult human spinal cord tissue resembles that seen in HEK-293 cells, except for the added expression of the PKCα isoform. (c-e) PKC isoform expressions in adult rat tissue serve as positive controls and demonstrate the diversity of patterns of expression of PKC isoforms.

the absence of detectable PKC-α in the HEK-293 cells.

PKC was expressed in HEK cells transfected with NR1A, or NR1A/NR2A subunits, both in the presence and absence of NMDA. Western blotting was performed using a monoclonal antibody against PKCαβ (Fig. 5a). We assessed PKC activities in total lysates from HEK-293 cells transfected with NR1A/NR2A subunits prior to and following exposure to NMDA using histone H1 (Fig. 5b) or protamine (not shown) as a substrate for PKC. Ca^{2+}- and lipid-dependent PKC activity was evident in unstimulated cells (Fig. 5b). PKC activity increased 10 min following exposure to NMDA and glycine (NMDA) and declined by 30 min. Analysis of cellular fractions demonstrated that most of the PKC activity was cytosolic (Fig. 5c). Histone phosphotransferase activity was considerably greater in the presence of Ca^{2+}, indicating that this effect was mediated by Ca^{2+}-dependent PKCs. PKC activity towards histone H1 and protamine was increased by twofold (105 ± 28%; n = 3) compared with control cells following PMA (85 nM) in the absence of NMDA.

As an indirect measure of PKC activation, we evaluated the expression of ERK1, as ERK1 is activated by PKC, or PMA stimulation in many cell types (Fiore et al., 1993). The increased tyrosine phosphorylation of ERK1 and ERK2 produces a doublet due to a reduction in its mobility shift in SDS-PAGE (Fiore et al., 1993). Western blot analysis using an anti-ERK1 antibody demonstrated that PMA exposure led to time-dependent ERK1 activation in NR1A/2A-transfected HEK cells (Fig. 6a). Ten min following PMA (85 nM), a reduction in mobility (bandshift) was evident compared with unstimulated cells (pp44). The bandshift associated with PMA stimulation was maximal at 10–20 min and gradually decreased over 60 min. Further confirmation of ERK1 activation was

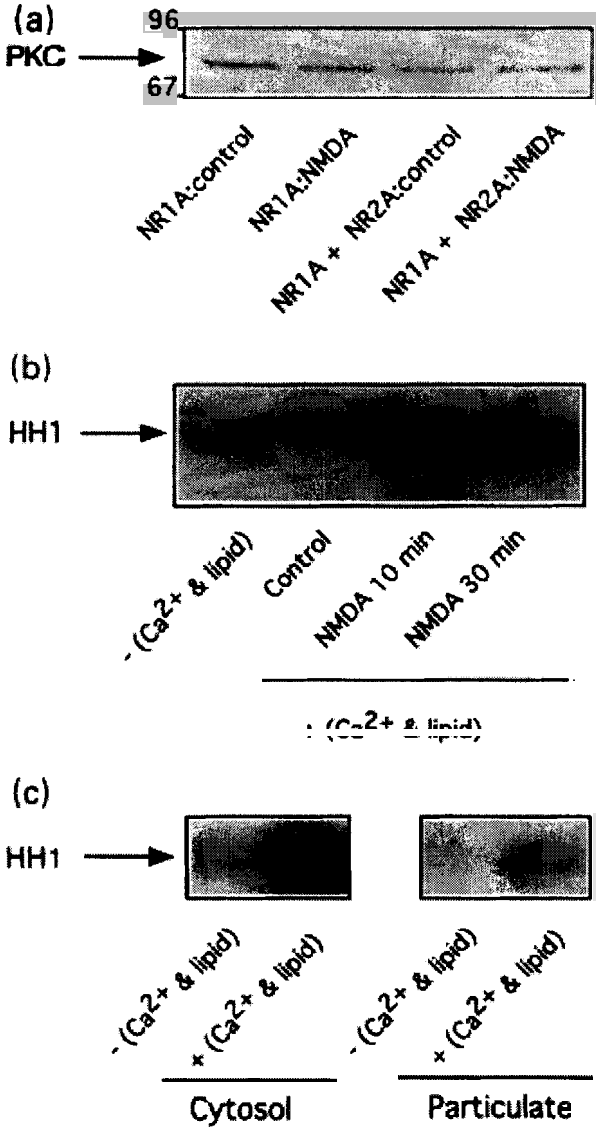


Figure 5: PKC activity and protein level in transfected HEK cell.
(a) Western blot analysis of PKCαβ. HEK-293 cells transfected in parallel with NR1A, or NR1A + NR2A subunits in the presence of NMDA and glycine (NMDA), or unstimulated (control) were immunoblotted with a monoclonal antibody against PKCαβ. Identical amounts of protein were loaded in each lane. The 80-kDa immunoreactive band corresponds to PKC (arrow). (b) Autoradiography of HH1 phosphotransferase activity of PKC in transfected cells. Total lysates of NR1A/NR2A-transfected HEK cells were used for an *in vitro* PKC assay using HH1 as a substrate. Samples were assayed for Ca^{2+} and lipid-dependent (control) and independent (no additions) activity. Parallel cultures were unstimulated and stimulated with NMDA and glycine (NMDA) and assayed at 10 min (NMDA 10 min) and 30 min (NMDA 30 min) following NMDA. The highest band density was 10 min following NMDA. (c) Autoradiography of HH1 phosphorylation from an *in vitro* assay using immunoprecipitated PKC from cytosolic and particulate fractions of NR1A/NR2A transfected cells under basal conditions (unstimulated). Autoradiography reveals increased phosphotransferase activity assayed with Ca^{2+} and lipid compared with without Ca^{2+} and lipid (no additions). The distribution of HH1 activity showed that most of the HH1 phosphorylation is present in the cytosol.

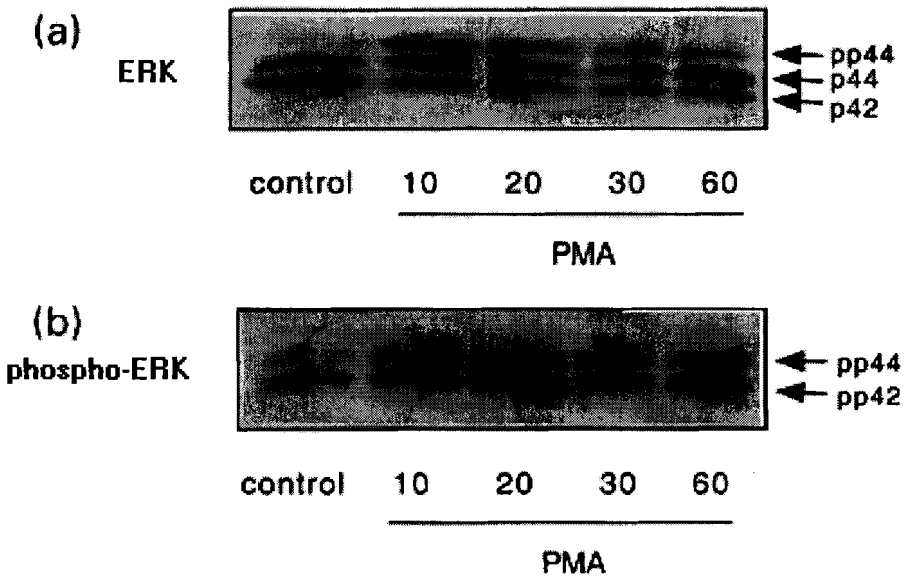


Figure 6: Western blot analysis of ERK following PMA stimulation in NR1A/NR2A-transfected HEK cells.

(a) Western blot analysis of ERK. Transfected HEK cells were treated with PMA for different time periods (10–60 min) and probed with anti-ERK1-CT antibody. The Western blot of ERK1 exhibited a reduced mobility band (pp44) which is attributed to the activation of ERK1 in cells pretreated with PMA. ERK1 activation is time-dependent and is maximal between 10 and 20 min. (b) Western blot of anti-phospho-ERK in transfected HEK cells following PMA treatment (10–60 min). The Western blot shows an increase in the density of the p44 ERK1 and p42 ERK2 bands in cells following PMA stimulation. Each lane in (a) and (b) represents the same amount of protein loaded from cell lysates of sister cultures.

obtained using a mouse monoclonal anti-phospho-ERK antibody which reacts specifically with Tyr204 of phosphorylated ERK1 and ERK2 (Crews and Erikson, 1992). Western blotting with the anti-phospho-Erk antibody demonstrated the presence of phosphorylated ERK1 and ERK2 following PMA stimulation, with the peak expression occurring at 10–20 min (Fig. 6b, pp44).

3.3.2 PKC activation potentiates NMDA-induced cell death

To assess cell death in HEK-293 cells, a trypan blue exclusion assay was performed 6 h following various treatments. Under our experimental conditions, control culture transfected with NR1A/NR2A subunits and maintained in a physiological saline solution (PSS) had a mean of 13.8 ± 1.4% cell death at 6 h (Fig. 7). We attribute this level of cell death to low levels of glutamate present in the cultures which stimulate functional NMDA receptors present in NR1A/NR2A transfected cells. In cultures that were transfected with NR1A subunits alone and maintained in PSS, cell death at 6 h was 3.3 ± 0.1% (data not shown; significantly lower than cultures transfected with both NR1A and NR2A subunits, $p< 0.005$, unpaired *t*-test).

To determine if stimulation of PKC has a cytotoxic effect on transfected HEK cells, PMA (85 nM) was applied for 10 min, either alone (PMA), or with NMDA and glycine (NMDA) and cell death was assessed 6 h following the treatments. As shown in Fig. 4, PMA alone did not produce a statistically significant change in the percent of dead cells compared with control cultures (PMA). Treatment with NMDA and glycine (NMDA) significantly enhanced cell death to 27.4 ± 1.3% ($p < 0.005$). Exposure of NR1A/NR2A transfected cultures to PMA 10 min prior to NMDA stimulation significantly potentiated the cytotoxic effect of NMDA and resulted in 37.6 ± 1.4% cell

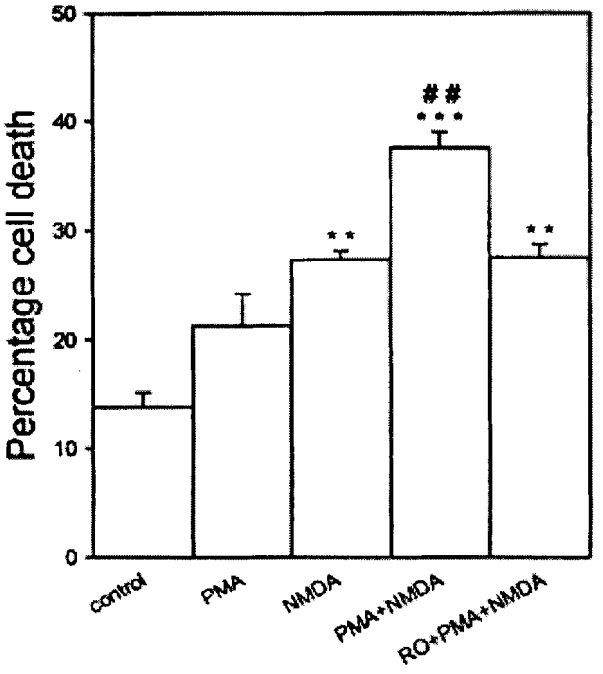


Figure 7: PMA potentiates NMDA-mediated cell death in NR1A/NR2A-transfected cells.
Cell death was assayed 6 h following treatment with NMDA, PMA and RO. Exposure to PMA alone was not significantly different from untreated controls, whereas NMDA-treated cells had a significantly higher percentage cell death compared with control ($p < 0.005$). A 10-min exposure to PMA prior to NMDA treatment potentiated the cytotoxic effect of NMDA and glycine (NMDA) and caused a significant increase in death compared with NMDA and glycine (NMDA) alone ($p < 0.05$). Preincubation with RO prior to exposure to PMA and NMDA led to a reduction in cell death compared with exposure to PMA and NMDA. Data derived from duplicate samples performed in at least 3 separate experiments. *** Significantly different from control ($p < 0.001$), and from PMA alone ($p < 0.001$); ** significantly different from control ($p < 0.005$); ## significantly different from NMDA and glycine alone (NMDA), and from pre-exposure to RO with PMA and NMDA ($p < 0.05$).

death (Fig. 7, PMA + NMDA). This result was statistically greater than the percentage of cells which died following exposure to NMDA alone ($p < 0.05$), PMA alone ($p < 0.001$) or under control conditions ($p < 0.001$). These data indicate that pre-exposure to PMA significantly enhanced the toxicity associated with NMDA receptor stimulation.

To confirm that the cytotoxic effect was mediated by PKC, a selective PKC inhibitor, RO was applied prior to treatment with PMA and NMDA. RO is a selective PKC inhibitor that interacts with the ATP binding site in the catalytic region of PKC (Birchall et al., 1994) and has the highest selectivity towards PKCα, followed by PKCβ (Wilkinson et al., 1993). Application of RO (1 μM) alone to NR1A/NR2A-transfected HEK cells did not change cell viability significantly (17.1 ± 3.1% dead cells; data not shown), compared with unexposed cultures (13.8 ± 1.4%), indicating that RO has no cytotoxicity at this concentration. When cultures were pretreated with RO for 10 min prior to exposure to PMA and NMDA (Fig. 7; RO + PMA + NMDA), the percentage of cell death was significantly reduced ($p < 0.05$) compared with PMA and NMDA exposure without RO pretreatment. The RO pretreatment appeared to block the augmentation of cell death produced by PMA exposure and produced a similar percentage of cell death as with NMDA treatment alone (Fig. 7; NMDA).

To further examine the potentiation effect of PMA on NMDA-mediated cell death in HEK cells transfected with NR1A/NR2A subunits, we performed Western blotting using antibodies against phosphorylated forms of PKCαβ (phospho-PKC) and ERK1 and ERK2 (phospho-ERK1/2). The expression of phosphorylated PKC protein did not change between control and treated cultures (Fig. 8a). In contrast, levels of phospho-ERK1 and phospho-ERK2 proteins were greater when cultures were exposed to PMA and had the

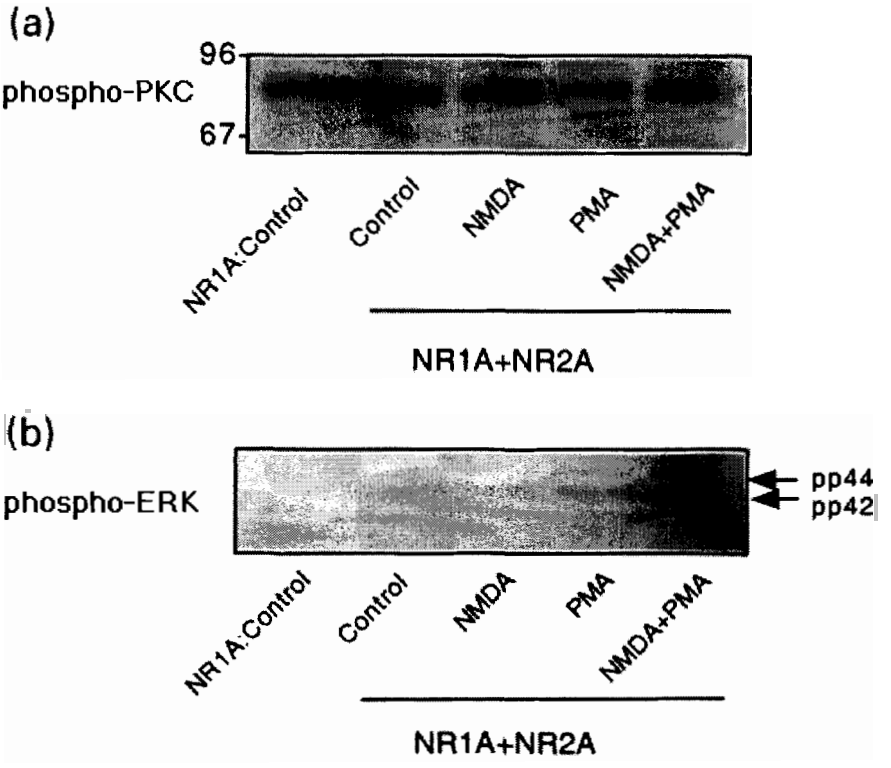


Figure 8: Western blot analysis of phospho-PKC and phospho-ERK in NR1A- and NR1A/2A-transfected HEK cells.

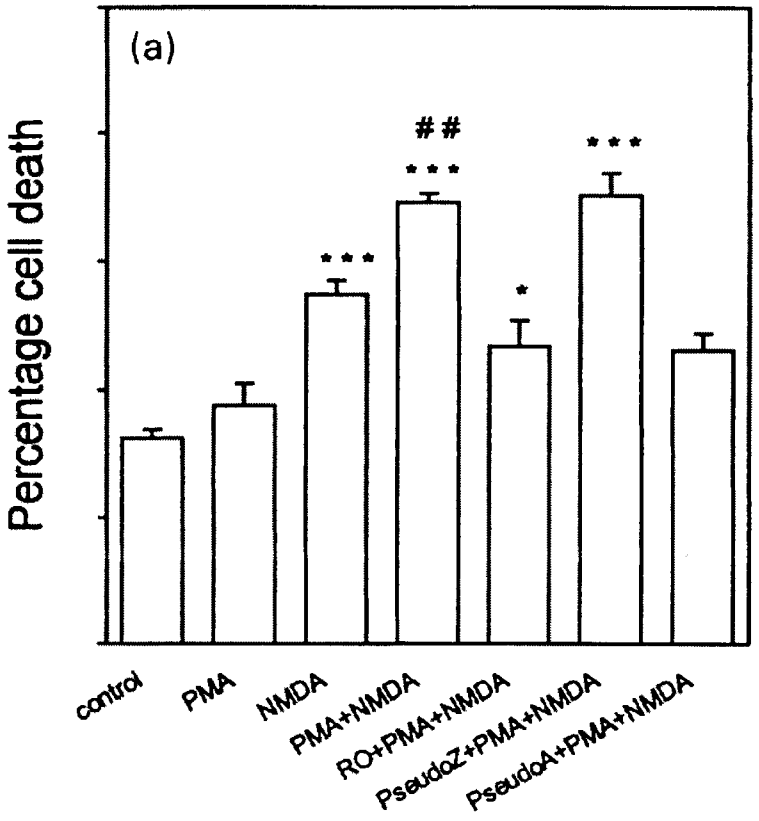
(a) Western blotting of PKC in NR1A- and NR1A/NR2A-transfected cells treated with PSS alone (control), NMDA, PMA or PMA and NMDA. The blot was probed with a phospho-PKCαβ antibody. No differences were apparent in the band density between the different treatments. (b) Western blotting analysis of phospho-ERK1/2 in NR1A and NR1A/NR2A-transfected cells. Cells were treated with PSS alone (control), NMDA and glycine (NMDA), PMA, or PMA and NMDA. The blot was probed with phospho-ERK1/2 antibody. The highest density band of the phosphorylated ERK was present in NR1A/NR2A-transfected cells which were exposed to PMA and NMDA (NMDA + PMA). Each lane in (a) and (b) represents the same amount of protein loaded from cell lysates of cultures transfected in parallel.

highest expression in cultures exposed to both PMA and NMDA (Fig. 8b). From this data it appeared that exposure to PMA and NMDA did not lead to changes in the phosphorylation of PKC itself. However we detected an increase in the phosphorylations of ERK1/ERK2, after exposure to cells to PMA, or to PMA with NMDA.

3.3.3 Calcium-dependent isoforms of PKC are involved in potentiating NMDA-mediated cell death

To examine which isoforms of PKC are responsible for the enhancement of NMDA-mediated cell death, NR1A/NR2A-transfected HEK cells were pretreated with specific PKC inhibitors. Pseudo A is a myristoylated peptide containing amino acids 19-27 of PKC which is cell permeable and binds to the pseudosubstrate sequence of PKC specifically inhibiting cPKC isoforms (α, β and γ). Pseudo Z is a myristoylated cell permeable compound which specifically inhibits the action of PKCζ by interfering with the substrate binding site in the catalytic domain (Shen and Buck, 1990). Figure 9(a) shows the effects of these inhibitors and RO on NMDA-mediated cell death in transfected HEK cells. As shown previously, cells exposed to NMDA alone (Fig. 9a; NMDA), or PMA and NMDA (Fig. 9a; PMA + NMDA) had significantly enhanced cell death compared with control cultures. Pre-exposure to pseudo Z prior to treatment with PMA and NMDA had no effect on cell death (Fig. 9a; PseudoZ + PMA + NMDA). Preincubation with pseudo A prior to treatment with PMA and NMDA significantly reduced cell death compared to PMA and NMDA-treatment alone (Fig. 9a; PseudoA + PMA + NMDA; $p < 0.001$). The reduction in cell death seen with exposure to the cPKC inhibitor, Pseudo A, was similar to that achieved with the pan-specific PKC inhibitor RO (Fig. 9a; RO + PMA + NMDA) and was also similar to the cell death found with NMDA

Figure 9: Cell death of NR1A/2A-transfected cells that were pre-exposed to different PKC inhibitors and prolonged PMA treatment.
(a) Cell death of NR1A/NR2A-transfected cells pre-exposed to different PKC inhibitors. NMDA-treated, or PMA and NMDA-treated cells had a significantly higher cell death compared with control cells. In all treatment conditions where PMA+NMDA were present, PMA was applied as a pre-treatment and NMDA was added subsequently. Pretreatment with RO, or pre-treatment with pseudo A (Ca^{2+}-dependent PKC inhibitor) prior to exposure to PMA and NMDA significantly reduced cell death compared with treatment with PMA and NMDA alone ($p < 0.001$ for RO and for pseudo A). Pre-treatment with pseudo Z (PKCζ inhibitor) prior to PMA and NMDA treatment did not affect cell death. *** Significantly different from control ($p < 0.001$); * significantly different from control ($p < 0.05$); ## significantly different from NMDA alone ($p < 0.05$); pre-exposure to RO ($p < 0.001$); and pre-exposure to Pseudo A ($p < 0.001$). Data derived from duplicate batches of transfected cells for each condition performed in at least 3 separate experiments.
(b) Cell death in NR1A/NR2A-transfected cells pre-exposed to prolonged PMA treatment. Pretreatment with PMA for different time periods prior to exposure to NMDA reflects a 'downregulation' of PKC. A 10-min preincubation in PMA prior to NMDA (PMA + NMDA 10 min) led to increased cell death compared with NMDA alone (NMDA). A longer preincubation time than 10 min resulted in a reduction in cell death to, at most, a similar level as NMDA exposed cells without PMA pre-exposure. Data derived from duplicate batches of transfected cells for each condition performed in at least 3 separate experiments. **** Significantly different from control ($p < 0.001$); *** significantly different from control ($p < 0.005$); ** Significantly different from control ($p < 0.01$); * significantly different from control ($p < 0.05$); # significantly different from NMDA alone, or 20, 30 and 180 min pre-exposure to PMA ($p < 0.005$).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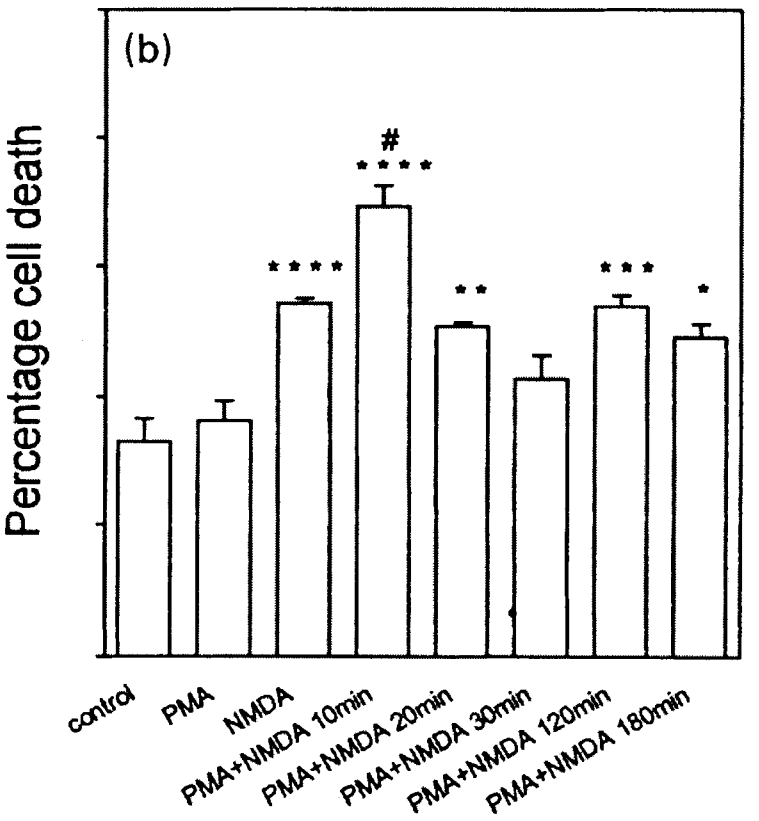
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PMA+NMDA 180min

stimulation in the absence of PMA (Fig. 9a; NMDA). As the only cPKC isoform expressed by transfected HEK cells was β1, this indicates that β1PKC mediates the PMA potentiation of NMDA toxicity in HEK cells.

3.3.4 PKC down-regulation reduces cell death in NR1A/NR2A-transfecte cells

PKC activity in many cell types is reduced by longer term exposure to PMA ('down regulation'). To determine whether down-regulation of PKC could alter cell death in transfected HEK cells, cells were preincubated with PMA using different exposure times (from 10 min to 3 h) prior to NMDA treatment. As shown previously, exposure to PMA 10 min prior to NMDA (Fig. 9b; PMA + NMDA 10 min), increased cell death compared with NMDA alone (Fig. 9b; NMDA). Increasing the duration of PMA pre-exposure led to a reduction in the percent of dying cells. At 20 and 30 min following PMA treatment prior to NMDA exposure (Fig. 9b; PMA + NMDA 20 min; PMA + NMDA 30 min), cell death was significantly lower than when NMDA was applied 10 min following PMA treatment (Fig. 9b; PMA + NMDA 10 min; $p < 0.005$). A 3 h pre-treatment with PMA prior to NMDA stimulation produced a similar amount of cell death as NMDA alone and was significantly different from 10 min PMA pre-treatment (Fig. 9b; PMA + NMDA 180 min; $p < 0.005$). These results suggest that down regulation of PKC leads to similar percentages of cell death as occur with exposure to NMDA alone.

3.3.5 The C1 region of NR1 does not contribute to cell death

Previous work has indicated that the C1 region of NR1 is heavily phosphorylated by PKC and regulates the phorbol ester-sensitivity of the NR1 subunit (Logan et al., 1999). To evaluate the role for the C1 domain in the modulatory effect of PMA in

NMDA-toxicity we compared cells transfected with a splice variant of the NR1 which did not contain the C1 domain (NR1C) with cells transfected with NR1A, which contains the C1 region. As shown in Figure 10, there were no significant differences between the cytotoxic effect of cells transfected with NR1C/NR2A and NR1A/NR2A subunits of the NMDA receptor. The percentage cell death was very similar under control conditions, following NMDA-stimulation, PMA and NMDA-treatment, or with pretreatment with RO (Fig. 10). Thus, deletion of the C1 region did not alter the cytotoxic effect of NMDA, or change the modulatory effect of PMA in this system.

3.3.6 The C terminus of NR2A subunit contributes to PMA-mediated cell death

To explore the role of the NR2A subunit in the modulatory effect of PMA in NMDA-mediated cell death, we compared the cytotoxicity of cells transfected with NR1/NR2A and those transfected with NR1 and a truncated NR2 subunit having a deletion in amino acids between 1267 and 1458 ($NR2A_{\Delta 1267\text{-}1458}$; Grant et al., 1998). HEK cells transfected with this construct and NR1 have been previously shown not to exhibit PMA-induced enhancement of the NMDA-evoked Ca^{2+} transient (Grant et al., 1998). As shown in Fig. 10, HEK cells transfected with NR1/$NR2A_{\Delta 1267\text{-}1458}$ (NR1/NR2A') had a similar percentage of cell death following NMDA stimulation as NR1/NR2A. However, with PMA and NMDA treatment, no increase in cell death was seen in NR1/$NR2A_{\Delta 1267\text{-}1458}$-transfected cells, compared with NMDA treatment alone (Fig. 10; PMA + NMDA). Using the NR1/$NR2A_{\Delta 1267\text{-}1458}$ construct, pretreatment with RO gave a significant reduction in cell death compared with NMDA + PMA, or NMDA treatment (Fig. 10). This result suggests that NR1/$NR2A_{\Delta 1267\text{-}1458}$ NMDARs might be maximally potentiated by PKC, either directly or indirectly, prior to PMA exposure. The modulatory effect of

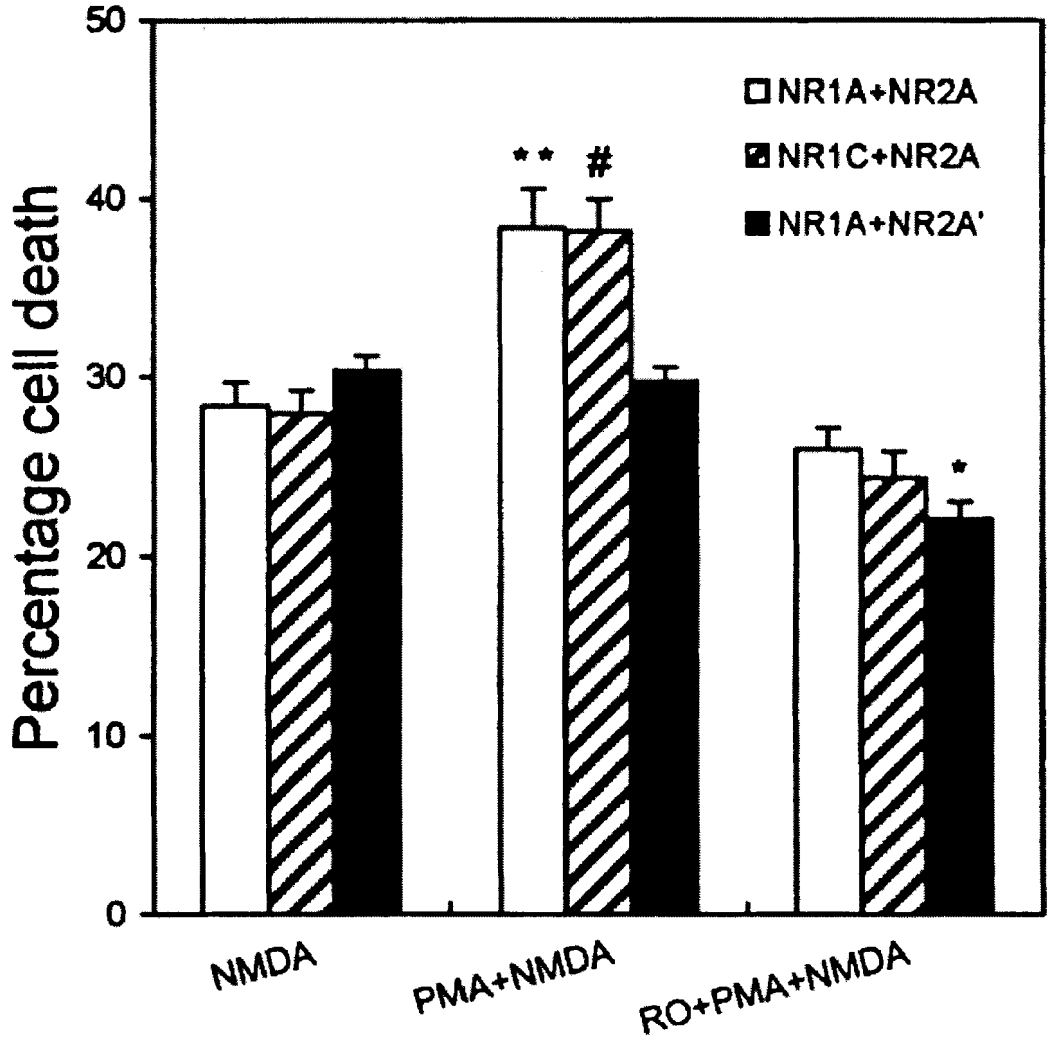

Figure 10: Cell death in NR1A/NR2A-, NR1C/NR2A- and NR1A/NR2AΔ1267-1458 (NR1A/NR2A')-transfected cells.

Cell death of NR1C/NR2A-transfected cells treated with NMDA, PMA and NMDA (PMA + NMDA) or RO 32-0432 prior to PMA and NMDA (RO + PMA + NMDA) were not significantly different from cell death of NR1A/NR2A-transfected cells given the same treatments. Percentage cell death of NR1A/2A' was not different from NR1A/NR2A or NR1C/NR2A following NMDA treatment (NMDA). NMDA-mediated cell death in NR1A/NR2A' was unaffected by preincubation in PMA (PMA + NMDA). Exposure to RO significantly reduced NMDA-mediated cell death in $NR1/NR2A_{\Delta 1267\text{-}1458}$ (RO + PMA + NMDA). ** Significantly different from cells transfected with the same constructs and treated with NMDA alone ($p < 0.005$), or RO + NMDA + PMA ($p < 0.001$); # Significantly different from cells transfected with the same constructs and treated with NMDA alone, or RO + NMDA + PMA ($p < 0.001$); * Significantly different from cells transfected with the same constructs and treated with NMDA alone, or NMDA + PMA ($p < 0.005$). Data derived from duplicate batches of transfected cells for each condition performed in at least three separate experiments.

PMA on NMDA-mediated cell death was abolished by deletion of the C terminus region of NR2A.

3.4 Discussion

HEK-293 cells transfected with NMDA receptor subunits have been used extensively as an *in vitro* model to study the mechanism of the modulation of NMDA receptor currents (Raymond et al., 1994; Grant et al., 1998) and NMDA-mediated cytotoxicity (Cik et al., 1994; Anegawa et al., 1995; Raymond et al., 1996). We have used these cells for the present studies as the homogeneous cell population facilitates biochemical measurements and assessment of cell death and also permits transfection with known NMDA receptor subunits. One limitation of these cells is that, as HEK cells are not neurons, it is possible that they might not contain the same population of protein kinases and other cellular constituents necessary for NMDA-mediated toxicity. However, as previous work has shown that transfected HEK cells demonstrate NMDA receptor-mediated currents (Raymond et al., 1994), changes in free cytoplasmic Ca^{2+} concentrations (Grant et al., 1998) and cell death in response to NMDA stimulation (Raymond et al., 1996), these cells appear to mimic the responses observed in neurons. Furthermore, as these cells express only NMDA receptors, they provide an opportunity to study the mechanism of cell death following NMDA receptor activation in the absence of confounding effects from other EAA receptors. Previous experiments by Raymond et al. (1994) have demonstrated that HEK cells transfected with NR1/NR2A subunits identical to those used in the present experiments show more than 300% potentiation of NMDA receptor-mediated currents for at least 20 min, following dialysis of purified PKC into the cytosol.

In this study we evaluated whether PKC activation potentiates NMDA-induced cell death using HEK cells transfected with NMDA receptor subunits. We find that PKC activities and PKC protein are present in unstimulated, transfected HEK cells and that NMDA augments PKC activity as determined by increased histone H1 phosphotransferase activity. These results are similar to those found in neurons and neuronal cell lines when activated by glutamate (Fukunaga et al., 1992; Hasham et al., 1997). We also obtained indirect evidence for PKC activation by phorbol esters in HEK cells, as we observe an increase in the bandshift of ERK1, which has been attributed to phosphorylation of ERK1 (Fiore et al., 1993), an event associated with the activation of ERK1 by PKC. Furthermore, we also observe an increase in ERK1 and ERK 2 phosphorylation by PMA and NMDA using phospho-ERK1/2 antibodies.

Death of HEK cells was determined using trypan blue exclusion. This method suffers from the limitation that it is likely not all of the HEK cells in the cultures were transfected by NR1/NR2A subunits. Transfection efficiencies were generally in the range of 60−80%, which is similar to those reported in other studies using these cells (Raymond et al., 1996). This level of transfection efficiency would have underestimated the effects observed had all HEK cells been transfected. As observed in other studies, HEK cells transfected with NR1/NR2A subunits die even in the presence of NMDA antagonists, an effect which has been attributed to incomplete NMDA receptor antagonism. With NMDA and glycine stimulation, cell death increases substantially, as has been observed previously (Raymond et al., 1996). Our data also confirms the observations that cell death is greater in NMDA-treated HEK cells that are co-transfected with cDNAs for NR1 and NR2A, compared with cells transfected with NR1A alone (Raymond et al., 1996). In this

study we did not attempt to determine if the cell death was due to necrosis or apoptosis.

A novel finding in our study is that pre-exposure to PMA significantly augments death of NMDA-treated transfected HEK cells. The increased cell death results from PKC activation as it was blocked by the specific PKC inhibitor, RO. Furthermore, the potentiation of NMDA-mediated cell death by PMA was inhibited by PseudoA, a specific inhibitor of cPKC isoforms. In view of the absence of α and γ PKC isoform expression in transfected HEK cells, this effect appears to be mediated primarily by PKCβ1. Additionally, the potentiation of NMDA-mediated cell death could be inhibited by PKC down-regulation.

These data confirm and extend the results of several previous studies. Favaron et al. (1990) found that pretreatment of cultured cerebellar granule cells with gangliosides, which inhibit PKC translocation, decreased glutamate- and kainate-mediated neurotoxicity. Furthermore, primary cultures of rat cerebellar neurons can be protected from glutamate neurotoxicity by PKC inhibitors, whereas an inhibitor of cyclic nucleotide-dependent protein kinases had no effect (Felipo et al., 1993). Our data are also consistent with the observations of Manev et al. (1990) who found that down-regulation of PKC in cerebellar granule cells protects neurons from glutamate-induced neurotoxicity. As these studies used primary cultures containing neurons having a number of EAA receptor channel types it was not possible to draw specific conclusions about the contributions of given receptor subunits.

The present data deviate from results obtained using cultured primary rat cortical neurons after 8 days *in vitro* (DIV). Durkin et al. (1997) reported that NMDA-mediated cell death increased in neurons that were pretreated with the PKC inhibitor,

staurosporine. As in the present experiments, they did not observe any significant effect of PMA or PKC inhibitors on cell death in the absence of NMDA stimulation. However, in contrast to the transfected HEK cells our study, NMDA application to cortical neurons in culture for 8 days, showed little toxicity in the absence of PKC inhibitors (Durkin et al., 1997). The findings of Durkin et al. (1997) are unique in that many studies have shown that NMDA is toxic for neurons which have been cultured for 12–14 DIV, even in the absence of PMA (see Sattler et al., 1999). These observations suggest that differences in neurotoxic pathways exist in some classes of embryonic neurons during *in vitro* development. As cultured embryonic cortical neurons express mainly the NR1/NR2B subtype of NMDA receptor before 8 DIV (Zhong et al., 1994), and may also express NR1 splice variants other than the NR1A used in this study (Sugihara et al., 1992), specific effects of PKC activation by staurosporine on these different NMDA receptor subtypes after NMDA stimulation may contribute to the apparently contradictory results. Consistent with the lack of NMDA-mediated toxicity found by Durkin et al. (1997) in 8-DIV cortical neurons, Zeron et al. (2001) have also shown lower levels of NMDA-mediated cytotoxicity in HEK cells expressing NR1A/NR2B, compared with those expressing NR1A/NR2A. Moreover, Zeron and colleagues (2001) found a substantial increase in NMDA-induced cytotoxicity and apoptosis for NR1A/NR2B, but not NR1A/NR2A-expressing cells in the presence of an apoptosis-inducing stimulus. As staurosporine is sometimes used to induce apoptosis, the effects of this drug on NMDA-induced neuron death expressing mainly NR1A/NR2B may be due to an interaction separate from that of PKC on the NMDA receptor current. The present results may correlate best with neurons expressing NR1/ NR2A subtype NMDA receptors.

Another explanation for the discrepancies between the results of Durkin et al. (1997) and our findings is that the stimuli employed by Durkin and colleagues may have also produced oxidative stress in their cultures. In addition to the effects of glutamate at stimulating ionotropic glutamate receptors, glutamate is also capable of producing oxidative stress by inhibition of cystine uptake at the glutamate/cystine transporter and subsequently reducing levels of intracellular cysteine and glutathione, promoting oxidative stress (see Maher, 2001). Furthermore, oxidative stress produced by glutamate is sometimes seen in neurons that have been in culture for limited periods, before ionotropic receptors are fully expressed. We suggest that PKC can exert multiple effects on excitotoxicity in cultured neurons. In the present study, we have attempted to isolate the specific effects of PKC on the NMDA receptor.

The mechanism by which PKC activation potentiates the toxicity of NMDA was not determined in these experiments. Consensus sequences for PKC phosphorylation are found in several distinct sites on the NMDA receptor, including several serine residues located at the alternatively spliced C1 region (Tingley et al., 1993). Specifically, phosphorylation of Ser890 in the C1 region produces a reduction of PKC potentiation, whereas mutations of other PKC phosphorylation sites (Ser889, Ser896 and Ser897) have no significant effect on phorbol ester stimulation (Zheng et al., 1999). To explore the relevance of the C1 exon in PKC potentiation of NMDA-mediated cell death in our system, we compared cell death in NR1A/NR2A-transfected cells with those in NR1C/NR2A-transfected cells (NR1C splice variants do not contain the C1 region). Our results indicate that PMA potentiates NMDA-mediated cell death in both splice variants and that the absence of the C1 region had no effect on cell death in transfected HEK cells,

consistent with the relatively minor effect of Cl on potentiation of NMDA currents by PKC.

Sites for PKC-induced phosphorylation have also been identified on the C-terminus of NR2A (Leonard and Hell, 1997). The physiological effects of expression of the C-terminal domain of NR2A are variable and dependent on the cell type studied. For example, Zheng et al. (1999) expressed an NR2A C-terminal deletion mutant in *Xenopus* oocytes having all serines and threonines removed downstream of the last transmembrane segment. They found that NR2A C-terminal mutants co-expressed with $NR1_{100}$ had reduced NMDA currents compared with $NR1_{100}$/NR2A when barium was used in the external solution. The deletion mutants still manifested PKC potentiation of NMDA receptor currents (Zheng et al., 1999). In contrast, Grant et al. (1998) observed that expression of a truncated NR2A subunit (Δ1267-1458) with NR1A in HEK cells was associated with a larger rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to glutamate, than in NR1A/NR2A-expressing cells. However, when PMA was coapplied with glutamate, the $[Ca^{2+}]_i$ response was much smaller in the NR1A/$NR2A_{\Delta 1267\text{-}1458}$-transfected cells than when exposed to glutamate without PMA. Thus, PMA inhibited the NMDA receptor-mediated $[Ca^{2+}]_i$ response of the NR1A/$NR2A_{\Delta 1267\text{-}1458}$-expressing cells (Grant et al., 1998).

Our data are consistent with the results of Grant et al. (1998), to the extent that we do not observe a potentiation effect of PMA on NMDA-mediated cell death in NR1A/$NR2A_{\Delta 1267\text{-}1458}$-transfected cells. This observation suggests that PMA is interacting directly or indirectly with a site on the C-terminus of NR2A to promote NMDA-mediated toxicity. This effect does not rule out the possibility of other

interactions between PKC and NMDA receptors but suggests that it is the interaction with the NR2A C-terminus which is most critical for enhanced toxicity. One concern about these experiments is that the truncated NR2A protein might have aberrant properties that would not necessarily occur in a normally functioning cell.

The mechanism underlying the PMA potentiation of NMDA excitotoxicity is unclear. One possibility could be that cell death is related to NMDA-evoked elevations in $[Ca^{2+}]_i$. However, previous work has shown that in the absence of PMA, NMDA-mediated changes in $[Ca^{2+}]_i$ are greater in NR1A/NR2A$_{\Delta 1267\text{-}1458}$-transfected cells than in cells transfected with NR1A/NR2A (Grant et al., 1998). Yet, in our results, NMDA-mediated cell death at 6 h was not significantly different in these two groups. In addition, we observed no change in NMDA-induced cytotoxicity for NR1/NR2A$_{\Delta 1267\text{-}1458}$ in the presence or absence of PMA, whereas PMA application markedly reduced NMDA-stimulated elevations of $[Ca^{2+}]_i$ for NR1/NR2A$_{\Delta 1267\text{-}1458}$ (Grant et al., 1998). Together, these data suggest that the modulatory effect of PMA on NMDA-mediated toxicity in NR1/NR2A-transfected HEK cells is not tightly correlated with its effect on $[Ca^{2+}]_i$. Other factors, such as localized elevation of Ca^{2+} in the vicinity of the critical domains of NMDA receptors, or the activation of 'downstream' protein kinases may be important for the regulation of cell death. Like other authors, we do not believe that PMA action on NMDA-mediated toxicity is exerted by indirect effects on intracellular Ca^{2+} stores (Grant et al., 1998), or by a change in the Mg^{2+} block of the NMDA receptor channel (Chen and Huang, 1992). We have not evaluated the possibility that PMA potentiation could occur through activation of the src family of tyrosine kinases which indirectly mediate phosphorylation of serine residues on the C-terminus region of NR2A (see MacDonald et

al., 1998).

The physiological consequences of PKC activation are highly variable and cell type-dependent. For instance, in neurons possessing EAA receptors, PKC activation is often associated with cell death (Favaron et al., 1990; Felipo et al., 1993). In other cell types that do not express EAA receptors, such as mammalian oligodendrocytes, PKC activation is associated with process outgrowth and does not lead to cell death (e.g., Yoo et al., 1999).

The present observations indicate that PKC activation potentiates NMDA-mediated toxicity in a cell line expressing NMDA receptors. These data provide implications for neurodegenerative disorders in humans.

CHAPTER 4
PROTEIN KINASE AND PROTEIN PHOSPHATASE EXPRESSION IN AMYOTROPHIC LATERAL SCLEROSIS SPINAL CORD*

4.1 Summary

The Kinetworks™ multi-immunoblotting technique was used to evaluate the expressions of 78 PKs, 24 protein phosphatases PPs and phosphorylation states of 31 phosphoproteins in thoracic spinal cord tissue from control subjects and patients having the sporadic form of ALS. In both the cytosolic (C) and particulate (P) fractions of spinal cord from ALS patients as compared with controls, there were increased levels of calcium/calmodulin-dependent protein kinase kinase (CaMKK; C=120%↑/P=580%↑; % change, compared with control), extracellular regulated kinase 2 (ERK2; C=120%↑/P=170%↑), G protein-coupled receptor kinase 2 (GRK2; C=140%↑/P=140%↑), phospho-Y279/216 glycogen synthase kinase 3 α/β (GSK3α/β; C=90%↑/P=220%↑), protein kinase B α (PKBα; C=360%↑/P=200%↑), phospho-T638 PKCα/β (C=630%↑/P=170%↑), cGMP-dependent protein kinase (PKG; C=100%↑/P=75%↑), phospho-T451 dsRNA-dependent protein kinase (PKR; C=2600%↑/P=3330%↑), ribosomal S6 kinase 1 (RSK1; C=750%↑/P=630%↑), phospho-T389 p70 S6 kinase (S6K; C=1000%↑/P=460%↑), and protein-tyrosine phosphatase 1 δ (PTP1δ; C=43%↑/P=70%↑). Cytosolic increases in phospho-α-S724/γ-S662 Adducin

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(C=15650%↑), PKCα (C=100%↑) and PKCζ (C=190%↑) were found in ALS patients as compared with controls, while particulate increases in cAMP-dependent protein kinase (PKA; 43%↑), protein kinase C β (PKCβ; 330%↑), and stress-activated protein kinase β (SAPKβ; 34%↑) were also observed. Cyclin-dependent kinase-associated phosphatase (KAP) was apparently translocated, as it was reduced (31%↓) in cytosolic fractions but elevated (100%↑) in particulate fractions of ALS spinal cord tissue. Our observations indicate that ALS is associated with the elevated expression and/or activation of many protein kinases, including PKCα, PKCβ, PKCζ and GSK3α/β, which may augment neural death in ALS, and CaMKK, PKBα, Rsk1, S6K, and SAPK, which may be a response to neuronal injury that potentially can mitigate cell death.

4.2 Introduction

There has been considerable interest in the expression of protein and lipid kinases in the CNS of humans and other animals. Many of these kinases regulate important cellular functions such as ion channel activity, neurotransmitter receptor properties, axon transport and cell survival (Smart, 1997; Battaini, 2001). Perturbations in the activities or expression of protein and lipid kinases have been reported in a variety of human neurodegenerative disorders such as ALS (Lanius et al., 1995; Wagey et al., 1998), Alzheimer's disease (Lanius et al., 1997) and Parkinson's disease (Ferrer et al., 2001).

In ALS, abnormalities in the activities or expression of PKC, PI3-K, CDK5 and SAPK have been reported in CNS tissue from patients who died with ALS, compared with control patients (Lanius et al., 1995; Migheli et al., 1997; Bajaj et al., 1998; Nagao et al., 1998; Wagey et al., 1998).

Protein kinases (PKs) are activated by various stimuli, for example by changes in the concentration of second messengers such as intracellular Ca^{2+} ($[Ca^{2+}]i$), calmodulin, or cAMP. PKs can also be activated by phosphorylation, usually by another kinase, although autophosphorylation may be possible. Consequently, kinases can stimulate other 'downstream' kinases in a cascade-like fashion.

In the present study we have evaluated the expression of a wide variety of PKs in spinal cords from patients who died with ALS and control subjects who died without evidence of neurological disease. PKs and protein phosphatase (PPs) expression was analyzed by applying a novel proteomics screening technique called Kinetworks™ which involves using multi-immunoblotting with prevalidated antibodies against 78 PKs, 24 PPs and 31 phosphoproteins.

4.3 Results

4.3.1 Multi-kinase/phosphatase analysis

To evaluate the expression of a large number of PKs, phosphoproteins and PPs in human spinal cord, Kinetworks™ protein profiling was performed, employing multi-immunoblotting with prevalidated antibodies against 78 PKs, 31 phosphoproteins and 24 PPs. Cytosolic and particulate fractions of thoracic spinal cord tissue were pooled from 5 patients who died without known neurological disorders (control) and tissue from 7 patients who died with ALS (ALS). Examples of immunoblots of PKs (Figs 11a-d), PPs (Fig. 11e) and phosphoproteins (Fig. 11f) are shown in Fig. 11 for cytosolic samples from ALS and control tissue. Each lane of these immunoblots is probed with cocktails of one to three different, in-house validated antibodies for PKs, PPs or phosphoproteins (phosphorylation site-specific). The multiple bands in each lane reflect the expression of

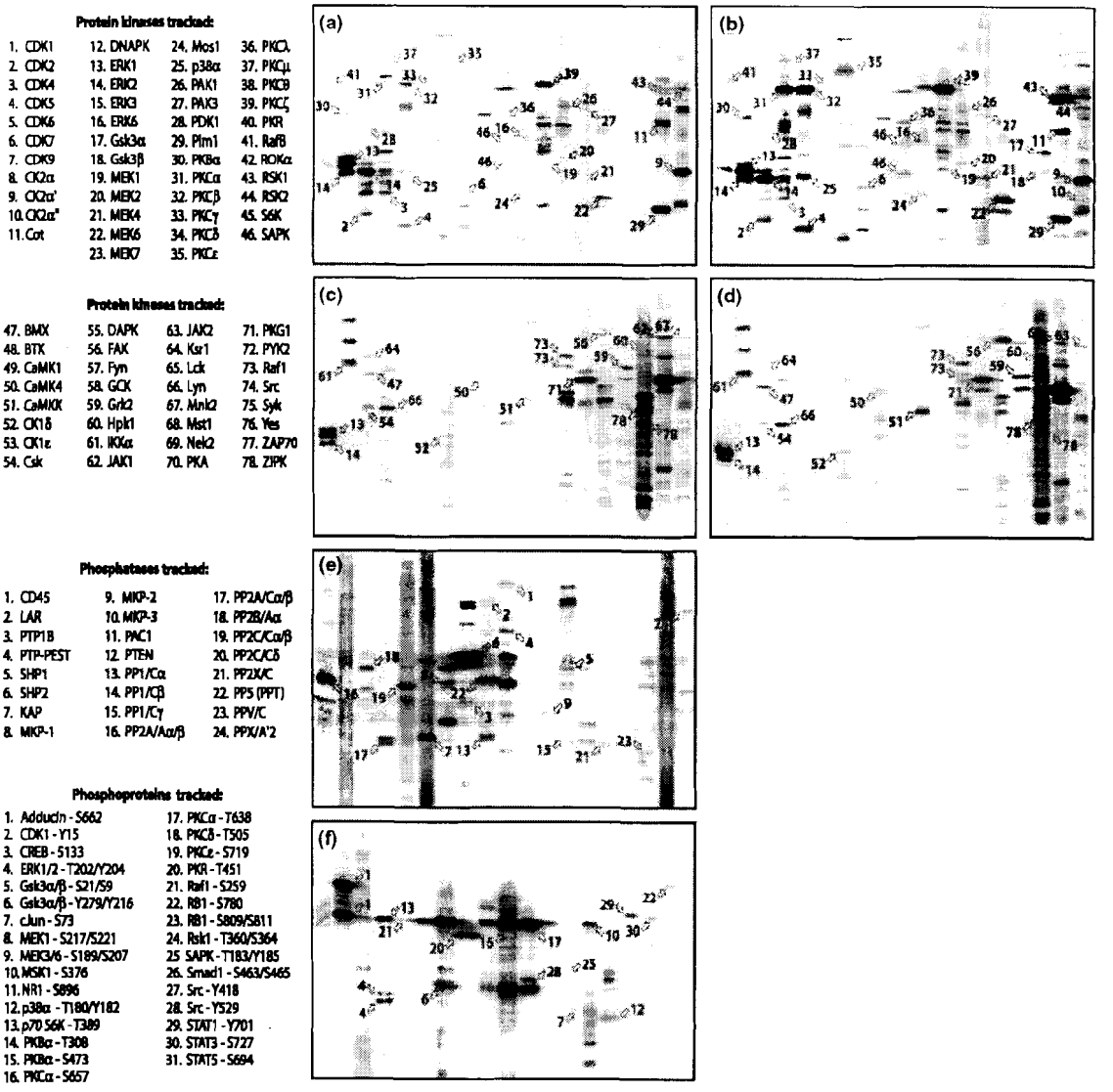


Figure 11: Examples of multi-immunoblots of Kinetworks™ screens of protein kinases, protein phosphatases and phosphoproteins.
Kinetworks™ KPKS 1.0 Protein Kinase (a-d), KPPS 1.1 Protein Phosphatase (e) and KPSS 1.1 Phosphoprotein (f) Screens of 78 protein kinases, 24 protein phosphatases and 31 phosphoproteins in individual (a-d) and pooled (e and f) samples of cytosolic fractions from human spinal cord tissue obtained from patients who died with ALS (b and d-f) and controls (a and c).

each of these target proteins and some unidentified cross-reactive proteins in the samples.

Figure 12 provides quantitative information regarding the levels of expression profiles of PKs (Figs 12a and b) and PPs (Figs 12c and d), as well as the phosphorylation states of specific phosphoproteins (Figs 12e and f) in pooled samples of cytosolic and particulate fractions from human spinal cord tissue obtained from patients who died with ALS and controls. For instance, in both cytosolic and particulate fractions, the following PKs were highly expressed: casein kinase 2 (CK2), cancer Osaka thyroid oncogene (COT), PKCβ, PKCζ, PKG and ZIP kinase (ZIPK). Expression of CaMKK, ERK2, GSK3α/β, KAP and PTP1δ were evident. Also seen were CDK5, phospho-S6K and other kinases (Fig. 12). The patterns of expression of PKs and PPs can differ profoundly between mammalian tissues, but the human spinal cord results were generally similar to what is observed in mouse brain and spinal cord (Chapter 6; Hu et al., 2003a).

4.3.2 Expression of specific PKs, phosphoproteins and PPs

The Kinetworks™ analysis was used to select those specific PKs, PPs and phosphoproteins that demonstrated large differences in either expression or phosphorylation between ALS patients and controls. While striking differences were observed, many of the detected proteins, e.g. BMX, CK2, COT, GSK3α, JAK1, Lyn, MEK1, MEK2, MOS, PAK1, ZIPK, MKP1, PP2A/Aα/β, PP2Cα/β, and phospho-S73 c-Jun were not appreciably altered between the ALS and control samples (Fig. 12). From the results of the Kinetworks™ study, we chose to re-examine the expression of 20 PKs, PPs and phosphoproteins in both cytosolic and particulate fractions of thoracic spinal cord tissue from seven individual ALS patients and five individual control subjects, by Western blots analysis. The specific PKs, phosphoproteins and PPs chosen for re-analysis

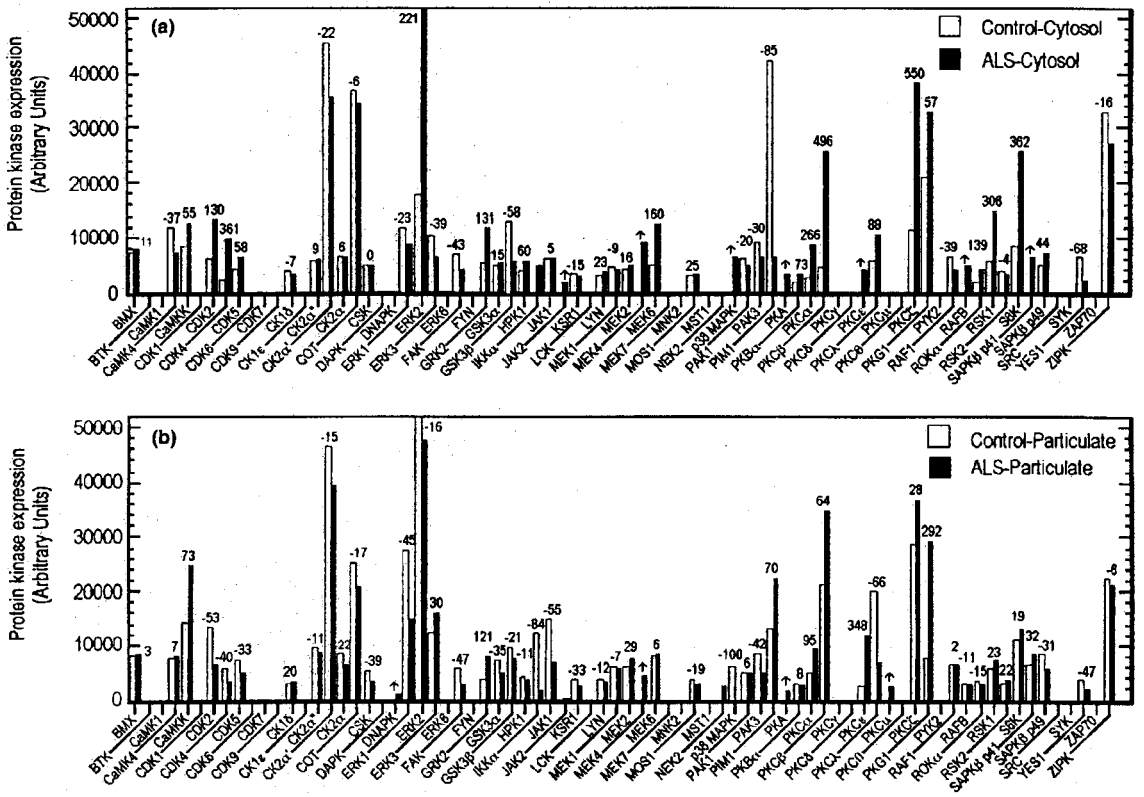


Figure 12: Kinetworks™ profiles of protein kinases, protein phosphatases and phosphoproteins in pooled human spinal cord tissue obtained from patients who died with ALS and controls.
(a,b) protein kinases, (c,d) protein phosphatases, (e,f) phosphoproteins, (a,c,e) cytosolic fraction, (b,d,f) detergent-solubilized particulate fraction, (black bars) ALS and (white bars) controls. The expression or phosphorylation level of each protein in the ALS tissue is shown as a percentage of that in control, which is taken as 100 percent. The arrow symbol indicates not detected in cytosol samples.

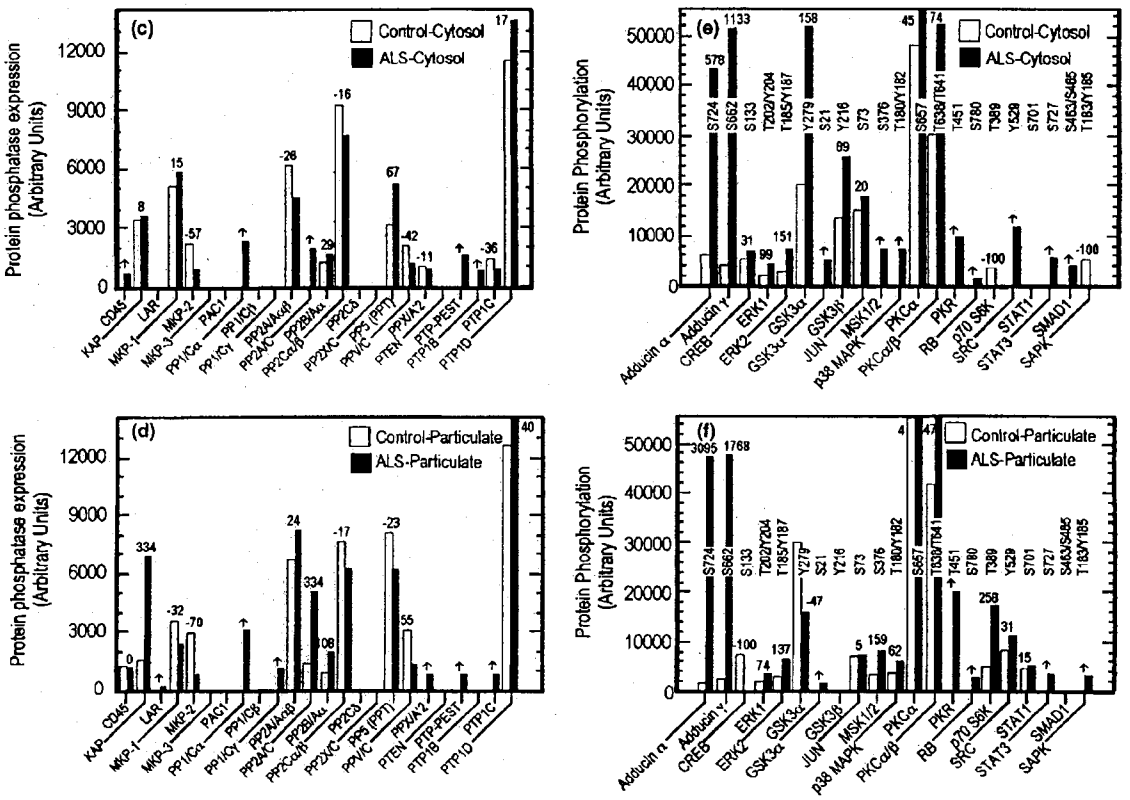


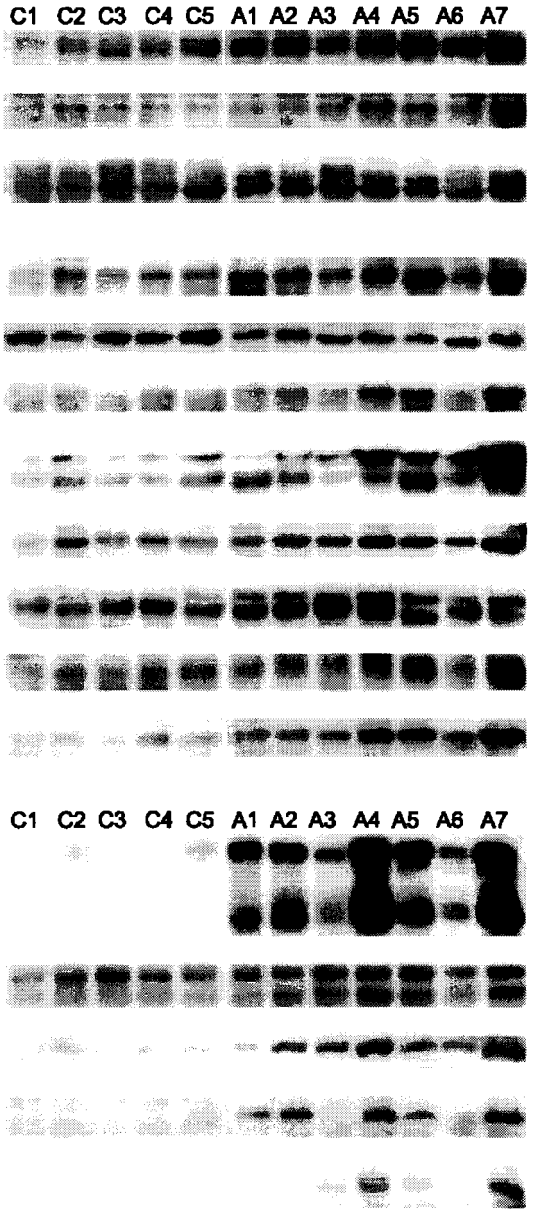
Figure 12 (Continued)

generally comprised those showing the greatest differences between ALS and control tissue in the Kinetworks analyses. In other cases, the expression of given proteins were studied because they were of particular interest based on previous work.

Among the 20 proteins we evaluated, we found significantly elevated expressions and/or phosphorylation of the following PKs, in cytosolic fractions from ALS tissue compared with controls: CaMKK p56/p52, ERK2 p39/p37, GRK2, phospho-GSK3α p44 (Y279), phospho-GSK3β p40 (Y216), PKBα, PKCα, phospho-PKCα/β (T638), PKCζ, PKG p75/p70, phospho-PKR (T451), RSK1 and phospho-S6K (T389) (Fig. 13). However, ERK1 p40 was observed to be significantly reduced in cytosolic fractions from ALS spinal cord tissue. Phospho-adducinα/γ p120/p80 (S724/S662) and PTP1δ were also increased in the ALS cytosolic samples. In particulate fractions of spinal cord tissue we found significantly elevated expression of the following PKs in ALS samples compared with controls: CaMKK p56/p52, ERK2 p39/p37, GRK2, phospho-GSK3α p44 (Y279), phospho-GSK3β p40 (Y216), PKA, PKBα, PKCβ, phospho-PKCα/β (T638), PKG p75, phospho-PKR (T451), RSK1, phospho-S6K (T389) and SAPKβ (Fig. 14). We also found significant increases in expression of the phosphatase PTP1δ in particulate fractions of ALS spinal cord tissue compared with controls (Fig. 14). There seemed to be a translocation of cyclin-dependent kinase associated phosphatase (KAP) since it was reduced in cytosolic fraction but elevated in particulate fractions of ALS spinal cord tissue compared with controls (Fig. 13 and Fig. 14).

4.4 Discussion

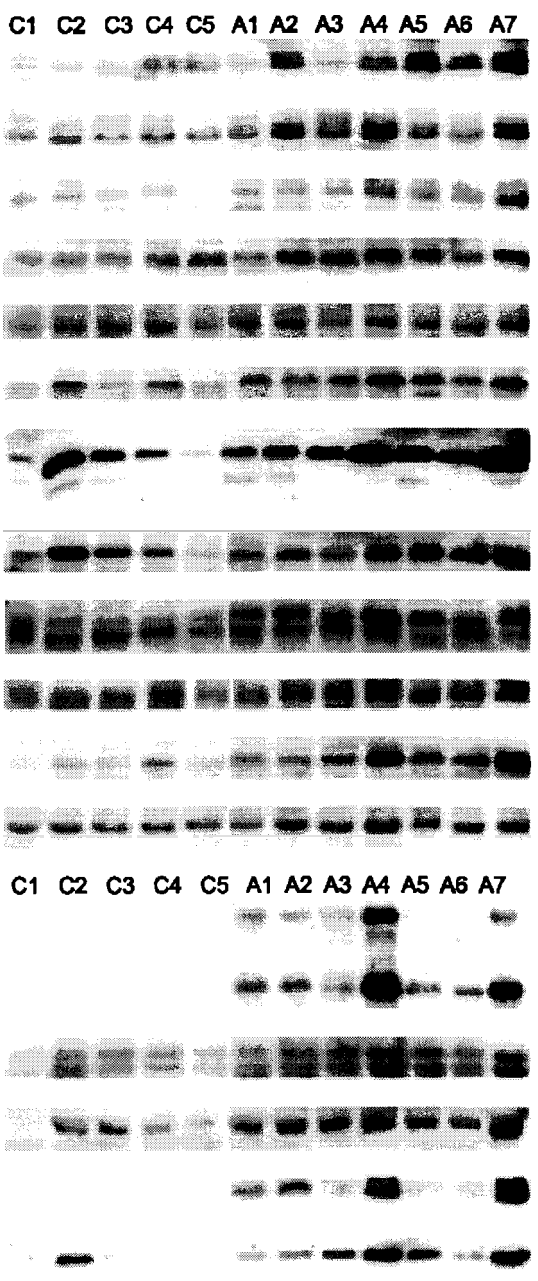
The data presented here demonstrate for the first time the expression profile of 78



Protein kinase / phosphatase	Control Mean ± S.Dev. (n=5)	ALS Mean ± S.Dev. (n=7)
CaMKK p56	1416 ± 330	5497 ± 2131***
CaMKK p52	5884 ± 2988	10291 ± 1940*
CDK5 p29	880 ± 317	1505 ± 889
ERK1 p40	1229 ± 662	437 ± 674*
ERK2 p39	1296 ± 349	2598 ± 930***
ERK2 p37	2482 ± 680	5686 ± 1966***
GRK2 p80	1096 ± 450	2603 ± 1022***
KAP p31	8833 ± 2882	6079 ± 1511*
PKB alpha p62	199 ± 140	911 ± 622*
PKC beta p89	324 ± 223	2710 ± 4108
PKC alpha p83	308 ± 105	614 ± 349*
PKC zeta p83	2298 ± 1251	6600 ± 4070*
PKG p75	451 ± 592	5188 ± 3426**
PKG p70	8876 ± 4163	13701 ± 4684*
PTP1 delta p68	1181 ± 303	1691 ± 391*
RSK1 p82	535 ± 294	4553 ± 3475*
Protein phosphorylation		
Adducin alpha p120 (S724)	77 ± 85	12637 ± 13983*
Adducin gamma p80 (S662)	38 ± 26	5481 ± 6598*
GSK3 alpha p44 (Y279)	762 ± 364	1247 ± 464*
GSK3 beta p40 (Y216)	177 ± 87	575 ± 288**
PKC alpha/beta p83 (T638)	264 ± 153	1927 ± 1455*
PKR p68 (T451)	14 ± 13	381 ± 299**
S6K p64 (T389)	7 ± 10	78 ± 91*

Figure 13: Expression levels of selective protein kinases and phosphatases and protein phosphorylation in the cytosolic fraction of the thoracic spinal cord tissues from ALS patients and control subjects.

The significant changes of expression between control and ALS are indicated: * $p < 0.05$; **$p < 0.01$; *** $p < 0.005$. C, control; A, ALS.



	Control Mean ± S.Dev. (n=5)	ALS Mean ± S.Dev. (n=7)
Protein kinase / phosphatase		
CaMKK p56	329 ± 404	4996 ± 4441*
CaMKK p52	1248 ± 548	5771 ± 4441*
ERK2 p39	10 ± 22	162 ± 158*
ERK2 p37	309 ± 78	712 ± 431*
GRK2 p80	202 ± 79	486 ± 365*
KAP p31	729 ± 228	1501 ± 631**
PKA p38	1127 ± 273	1609 ± 358*
PKB alpha p62	672 ± 467	2001 ± 1047**
PKC beta p89	2414 ± 2498	10330 ± 10211*
PKC alpha p83	447 ± 259	447 ± 360
PKC zeta p83	3521 ± 3755	3999 ± 1929
PKG p75	1649 ± 595	5631 ± 2290***
PKG p70	3512 ± 905	3379 ± 1505
PTP1 delta p68	1585 ± 290	2698 ± 679***
RSK1 p82	784 ± 848	5688 ± 4985*
SAPK beta p47	3594 ± 535	4828 ± 1116*
Protein phosphorylation		
Adducin alpha p120 (S724)	18 ± 13	321 ± 495
Adducin gamma p80 (S662)	14 ± 2	1218 ± 1972
GSK3 alpha p44 (Y279)	468 ± 182	1359 ± 727**
GSK3 beta p40 (Y216)	300 ± 163	1123 ± 754*
PKC alpha/beta p83 (T638)	2261 ± 1686	6173 ± 1634***
PKR p68 (T451)	42 ± 48	1441 ± 1412*
S6K p64 (T389)	140 ± 240	780 ± 844*

Figure 14: Expression levels of selective protein kinases and phosphatases and protein phosphorylation in the particulate fraction of the thoracic spinal cord tissues from ALS patients and control subjects.

The significant changes of expression between control and ALS are indicated: * $p < 0.05$; **$p < 0.01$; *** $p < 0.005$. C, control; A, ALS.

protein kinases, 24 protein phosphatases and 31 phosphoproteins in human thoracic spinal cord tissue using a quantitative and accurate proteomics screening technique. This normative data may help identify protein kinases and phosphatases present in the spinal cord that could be important for regulatory functions.

The protein profile found in human thoracic spinal cord revealed similarities and differences from mouse spinal cord (Hu et al., 2003a). For instance, the following PKs are present in human spinal cord but appear to be more highly expressed in murine spinal cord: CDK5, ERK1, ERK3, MEK4, MOS1, PKCβ, PKCε, RSK1 and SAPKβ. In contrast, other PKs are more highly expressed in human spinal cord, for instance: COT, ERK2, Pim1, PKG1, ZIPK (Fig. 12). Similarly, there were profound differences in the expression of protein phosphatase 1 catalytic α and γ subunits, and the MAP kinase phosphatase MKP1 between human and mouse. The relative differences in PK and PP expression between these species may be important variables in determining why human and murine tissues undergo different pathological responses to similar stimuli. Differences in PK expression may also be relevant for the use of cell culture models to study neurological disease. For instance, in contrast to spinal cord cells, we found that HEK cells express PKCμ and do not express PKCα (Wagey et al., 2001a). Thus, use of HEK cells as a culture model for spinal cord tissue might be inappropriate where certain PKC isoforms may exert a regulatory role.

In this study we have also examined protein expression in the neurodegenerative disease, ALS, which has prominent effects on spinal cord motoneurons, interneurons and descending tracts (Eisen and Krieger, 1998). Pooled samples of cytosolic and particulate tissues from ALS patients and controls were compared using the screening method. The

results being pooled are by necessity qualitative and were designed to identify specific proteins for more extensive quantitative study. We confirmed observations derived from the proteomics screening technique by employing Western blot analyses. To further elucidate our findings, we wished to determine if changes of PK and PP expression and protein phosphorylation fit into a coherent pattern which might implicate the involvement of specific PK pathways in ALS.

4.4.1 PKs in ALS

Analyses of the PK and phosphokinase profiles of spinal cord tissue revealed the general feature that ALS tissue is characterized by increased expression of a wide variety of PKs and phosphokinases.

PKC

We found that Ca^{2+}-dependent PKC (PKCα or PKCβ) and PKCζ are over-expressed in cytosolic and particulate fractions of ALS spinal cord tissue compared with controls. These findings are similar to our previous observations to the extent that we had found that the expression of Ca^{2+} and lipid-dependent PKC isoforms were significantly increased in spinal cord tissue from ALS patients compared with controls (Lanius et al., 1995). In a previous study we did not attempt to quantify individual PKC isoforms, but rather used an antibody that reacted equally well to PKCα, -β and -γ (Lanius et al., 1995). The present experiments extend the previous analysis by examining isoform-specific PKC expression. In addition to increased protein levels of PKCα in cytosolic and PKCβ in particulate fractions, we also found over-expression of PKCζ in ALS in the present study.

The significance of the elevated expression of different isoforms of PKC is

unclear. First, it is not established which cell type or types have increased expression of PKC. The elevated PKC expression could occur in the motoneurons that are affected in ALS, but also could occur in interneurons, or non-neuronal cells. The present study did not address this issue. Second, the elevated PKC expression could be a consequence of regenerative activity from non-neuronal cells, or a manifestation of inflammation within the CNS, which has been claimed to occur in ALS (McGeer and McGeer, 1998). An immunocytochemical study of PKC in spinal cord tissue from ALS and control patients at autopsy has demonstrated that in control spinal cord intense PKC immunoreactivity is seen in certain large motoneurons, but that this immunoreactivity is decreased in spinal motoneurons from ALS patients (Nagao et al., 1998). If that study is correct, this indicates that the elevated PKC expression arises in interneurons, or in non-neural cells.

It is possible the increased PKC expression found in spinal cord tissue from ALS patients is involved in the disease. Increased PKC activity is believed to be an important event leading to neurotoxicity and inhibitors of PKC are reported to reduce glutamate-induced toxicity (Felipo et al., 1993). There is also evidence that riluzole, a neuroprotectant compound that appears to modify the clinical course of ALS, has antagonistic actions on the activation of PKC (Noh et al., 2000). We have hypothesized that elevations of free intracellular Ca^{2+}, produced by excitatory amino acids or other stimuli may stimulate PKC, which in turn, will influence the viability of motoneurons in ALS (Krieger et al., 1996). This effect may be dependent on the specific receptor types present on motoneurons or other cells (Wagey et al., 2001a).

ERK1/2 signalling pathway

In the present study we found significant elevations of ERK2 p39 and ERK2 p37

expression together with a relatively small decrease of ERK1 p37 expression in spinal cord tissue from ALS patients compared with controls. This observation is at variance with a previous study where we did not find evidence for a difference in ERK2 expression between ALS and control tissue (Wagey et al., 1998). This inconsistency may be caused by the use of different anti-ERK2 antibodies in these two studies. Moreover, there were higher levels of the activated forms of both ERK1 and ERK2 in the cytosolic and particulate fractions from pooled ALS spinal cord samples compared with controls (Figs 12e and f). Interestingly, we also observed increased expression of RSK1 in both cytosolic and particulate fractions in ALS tissue. RSK1 has been reported to be a downstream target of the MEK-ERK signalling pathway, with a pro-survival role by directly phosphorylating the pro-apoptotic protein, Bad (Shimamura et al., 2000). Elevated expression of both ERK2 and RSK1 in ALS may indicate a role for this signalling pathway in producing neuronal survival. Some studies have indicated that protection from cell death is dependent on the relative expression of ERK1/2, compared with p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) (Xia et al., 1995; Maher 2001). In this study, increased cytosolic and decreased particulate expression of p38 MAPK was observed in ALS spinal cord (Figs 12a and b), but the levels of phosphorylated and activated p38 MAPK were enhanced in ALS, both in the cytosolic and particulate fractions (Figs 12e and f).

PI3-K signalling, CaMKK and GSK3

We found elevated levels of PKBα and phospho-S6K in both cytosolic and particulate fractions from ALS spinal cord, compared with controls. Taken together with our previous observations of increased activity of PI3-K in the particulate fraction and an

increase of S6K protein in cytosolic fractions of ALS spinal cord (Wagey et al., 1988), we believe that there is evidence for involvement of the PI3-K-PKB-p70S6K signalling pathway. Like the ERK signalling pathway, the PI3-K-PKB-p70 S6K signalling pathway also has a protective role in response to neuronal injury. Besides, CaMKK p56 and CaMKK p52 were also elevated in both cytosolic and particulate fractions of ALS spinal cord. As activation of PKB by CaMKK is believed to be important in the protection of neurons from apoptosis during development (Soderling, 1999), the increased expression of these two isoforms of CaMKK may indicate their neuroprotective role in the PI3-K-PKB-p70 S6K signalling pathway.

It has been found that GSK3 is a target downstream of PI3-K-PKB pathway in the regulation of apoptosis in primary human erythroid progenitors and that an increase of GSK3 activity may regulate commitment to cell death through a caspase-independent pathway, which results in a conformational change in Bax (Somervaille et al., 2001). Selective small-molecule inhibitors of GSK3 are also reported to protect both central and peripheral nervous system neurons in culture from death induced by reduced PI3-K pathway activity (Cross et al., 2001). Our findings of an increase in the cytosolic levels of phospho-Y279 GSK3α and phospho-Y216 GSK3β in ALS may indicate a similar proapoptotic role for GSK3 in the pathogenesis of ALS.

SAPK and cyclin-dependent kinase 5 (CDK5)

We observed increased expression of SAPK in particulate fractions of ALS spinal cord tissue, compared with controls, both in a kinase screen and by evaluation with Western blot analysis. Previous studies have reported evidence for activation of SAPK in ALS tissue. Migheli et al. (1997) have examined the immunohistochemical expression of

c-Jun, JNK/SAPK and nuclear factor-κB and found that immunoreactivity for JNK/SAPK was increased in ALS spinal cord compared with controls. SAPK immunoreactivity was increased, particularly in astrocytes and was generally low in motoneurons. The increased JNK/SAPK immunoreactivity was accompanied by increased expression of NF-kappa B. The finding of increased SAPK in our data is consistent with these observations. However, we are not able to determine the cell type responsible for the elevation in SAPK.

CDK5 was initially implicated in the pathogenesis of ALS by the observation that it was present in most of the Lewy body-like inclusions (LI) of a sporadic ALS patient. It was proposed that CDK5 might be involved in the cytoskeletal disorganization in LI-containing neurons by phosphorylating NF proteins (Nakamura, 1997). This idea was supported by the subsequent finding that CDK5 is present in affected motoneurons in ALS and it phosphorylates the NF-H to generate epitopes for antibodies that label NF accumulations in ALS, which finally leads to neuron death (Bajaj et al., 1999). Another study by Bajaj et al. (1998) showed that CDK5 is associated with lipofuscin in motoneurones in ALS where the biogenesis of lipofuscin is believed to be linked to oxidative stress which may be part of the pathogenic process in ALS. We found CDK5 expression in cytosolic and particulate fractions of spinal cord tissue in ALS, and there was a trend towards increased expression of CDK5 in the ALS cytosolic samples, but this was not statistically significant in our study.

4.4.2 Adducin

Adducins are a family of heteromeric membrane-associated cytoskeletal proteins composed of α subunits associated with β or γ subunits. Previous studies have

demonstrated that adducin is involved in the assembly of the spectrin-actin network in erythrocytes (Derick et al., 1992) where it colocalizes with spectrin at sites of cell-cell contact in epithelial tissues (Kaiser et al., 1989). Adducin, together with actin and other actin-binding proteins including spectrin and myosin, are major cytoskeletal components in the dendritic spines of neurons (Morales and Fifkova, 1989; Seidel et al., 1995). Phosphorylation of adducin by PKC or other PMA-activated kinases in the myristoylated alanine-rich C kinase substrate (MARCKS)-related domain of adducin inhibits its activity in promoting actin capping and recruiting spectrin to actin filaments in many cells, including the dendritic spines of neurons (Matsuoka et al., 1998). Our observations of elevated phospho-Adducin-α and -γ in cytosolic fractions of spinal cord tissue from ALS patients together with significantly increased expression and activation of PKC is interesting and may indicate that PKC is phosphorylating the MARCKS-related domain of Adducin in ALS tissue. In particulate fractions from ALS tissue, phospho-Adducin levels also appeared higher than in controls, but these levels did not reach statistical significance. We suggest that the phosphorylation of Adducin by PKC may play an important role in causing neuron death in ALS by affecting the basic structural scaffolding of neuronal dendritic spines. This profound change in the phosphorylation of Adducin may be a useful marker in ALS tissue.

In conclusion, this study has identified some of the PKs, PPs and phosphoproteins present in human spinal cord. Although the expression of these proteins is similar to rodent spinal cord, there are differences which may be of particular relevance for the use of rodent models of neurological diseases. Spinal cord tissue from patients who have died with ALS has up-regulation of a number of PKs and phosphokinases such as CaMKK,

phospho-GSK3α/β, ERK2, PKCα, PKCβ, PKCζ, RSK1 and SAPK. It is unclear as to why these proteins are overexpressed. However, our data has revealed that both pro- and anti-apoptotic PK pathways alter their expression. It is possible that the final outcome for individual cells is the relative balance of pro- and anti-apoptotic signalling evoked by a pathogenic stimulus. It may also be that the equilibrium between these death and survival factors is a determinant in the time course which individual cells follow in surviving, or succumbing to a pathological insult.

CHAPTER 5
LUMBAR MOTONEURON FATE IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS*

5.1 Summary

Onuf's nucleus, a collection of motoneurons within the spinal cord, is often spared in the neurodegenerative disorder ALS. To assess whether these cells survive in a rodent model of this disease, motoneurons were counted in the spinal nucleus of the bulbocavernosus (SNB; an homologous structure to Onuf's), as well as in two other cell groups at the same level of the spinal cord, the dorsolateral nucleus (DLN) and the retrodorsolateral nucleus (RDLN). In mice displaying signs of neurodegeneration, both DLN and RDLN displayed significant motoneuron loss compared with controls; this cell loss was particularly exaggerated in the RDLN of animals displaying a rapid disease progression. However, no significant decline in motoneuron number was observed in SNB, and the perineal muscle bulbocavernosus, which is innervated by this nucleus, appeared to be unaffected. This was in contrast to the thigh muscles, which displayed significant atrophy. Overall, these data indicate that the spinal nucleus of the bulbocavernosus is spared from degeneration in an animal model of ALS, paralleling observations in patients suffering from this disease. Further study of this resistance to motoneuron loss may provide useful insights into the pathophysiology of the degenerative process.

* Previously published as: Hamson D.K., Hu J.H., Krieger C. and Watson N.V. (2002) Lumbar motoneuron fate in a mouse model of amyotrophic lateral sclerosis. Neuroreport. 13:2291-2294, with minor changes, used by permission of Neuroreport, Watson N.V., Hamson D.K. and Krieger C.

5.2 Introduction

Postmortem examination of patients with ALS has shown that motoneuron survival is extended within specific pools of motoneurons. For instance, motoneurons of Onuf's Nucleus (ON) in the sacral spinal cord, along with their target musculature, are often spared in patients with ALS (Mannen et al., 1977; Kiernan and Hudson, 1993; Pullen and Martin, 1995). However, microscopic examination of ON motoneurons reveals some abnormalities, of unknown significance, that are not found in healthy and unaffected individuals (Pullen and Martin, 1995).

In rats and mice, the functional and anatomical homologue of Onuf's nucleus is the spinal nucleus of the bulbocavernosus (SNB), consisting primarily of motoneurons innervating the bulbocavernosus (BC) and levator ani (LA) muscles, which are involved in copulation. The SNB is sexually dimorphic; it is almost completely absent in females, but in males, SNB motoneurons are indirectly rescued from naturally occurring cell death by androgens (especially testosterone) acting upon the target muscles during a critical period to initiate a survival signal that probably involves cytokines (Xu et al., 2001). SNB motoneurons remain androgen sensitive and morphologically labile well into adulthood, making them an attractive system in which to study the effects of androgens on cellular plasticity and gene transcription (Breedlove and Arnold, 1980).

Neighboring the SNB in the rat and mouse lumbar spinal cord (segments L5-L6) are the sexually dimorphic dorsolateral nucleus (DLN), innervating the penile ischiocavernosus muscle and the external urethral sphincter, and the sexually monomorphic retrodorsolateral nucleus (RDLN), innervating the extensor digitorum longus muscle in the foot (Jordan et al., 1991). The DLN is dependent upon testosterone

for its survival during a perinatal critical period, like the SNB, but the RDLN is not (Tobin and Payne, 1991).

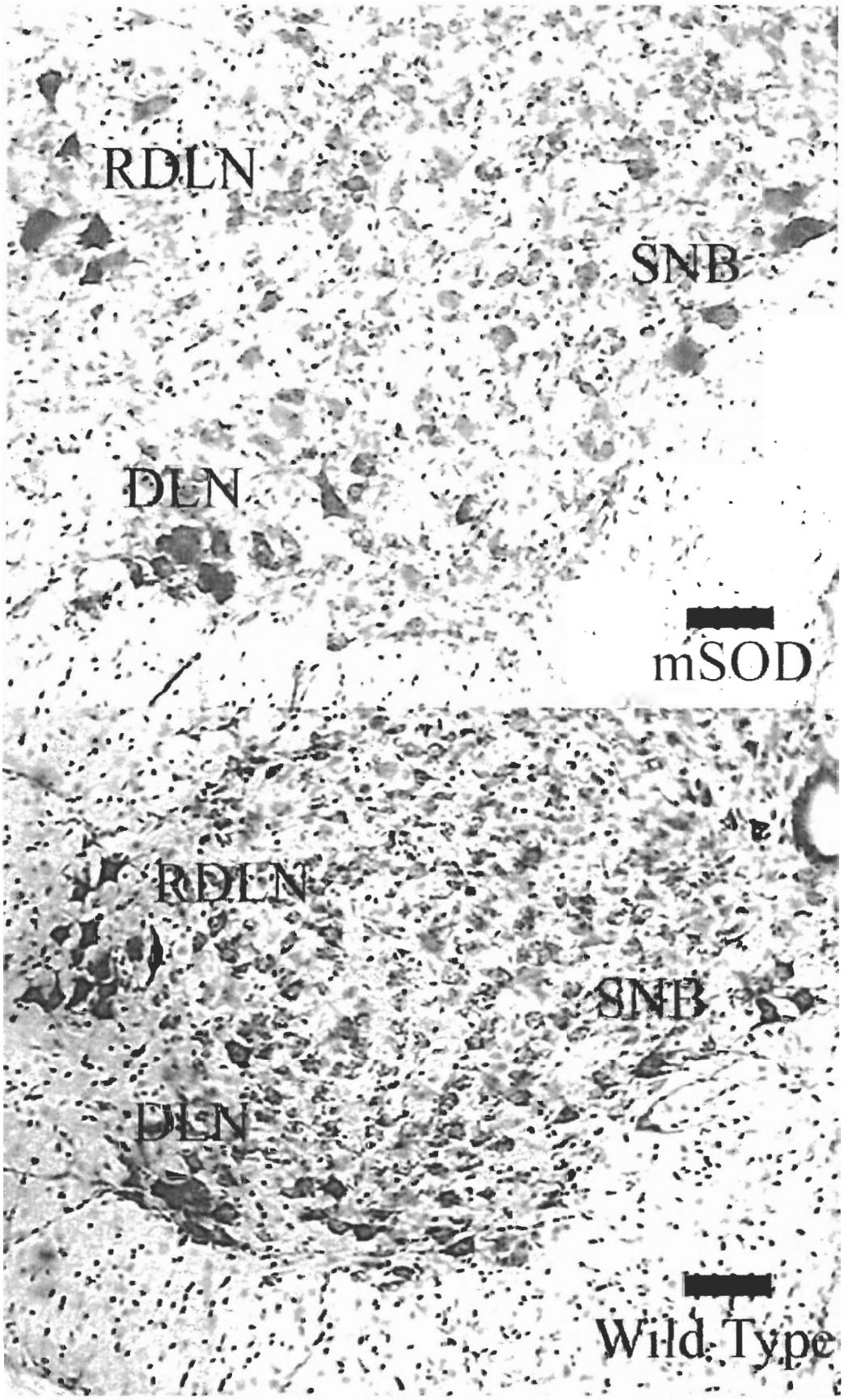
We evaluated the survival of motoneurons within the SNB, DLN and RDLN in mSOD mice to establish whether SNB survival was prolonged compared with DLN and RDLN. This was done to determine the extent to which SNB survival in the mSOD mouse is analogous to observations of ON in humans (Mannen et al., 1977; Kiernan and Hudson, 1993; Pullen and Martin, 1995). We also analyzed the extent of cell loss in the lumbar spinal cord as a function of the observed rate of disease progression following the onset of weakness.

5.3 Results

Transverse sections of lumbar spinal cord from mSOD and control mice containing the SNB, DLN and RDLN are shown in Figure 15 (as shown by arrows). As can be seen, there appeared to be a reduction in the total number of motoneurons in the mSOD mice compared to controls. Some neurons in the mSOD mice appeared vacuolated, especially those at the border between grey and white matter. Motoneuron numbers in SNB, DLN and RDLN for mSOD and control mice are presented in Figure 16. Analysis of the cell counts revealed a significant interaction between genotype and lumbar motoneuron cell type ($F_{(1,19)} = 13.33$, $p = 0.002$). Subsequent paired comparisons revealed a significant reduction in DLN and RDLN motoneuron number, relative to controls ($t = 3.886$, $p = 0.001$, and $t = 2.733$, $p = 0.013$, respectively; Fig. 16). SNB cell counts were not significantly different between control and mSOD spinal cords ($t = 1.686$, NS; Fig. 16).

Some of the mSOD mice declined very rapidly following symptom onset, while

Figure 15: Thionin stained representative transverse sections of mSOD (top) and wild type (bottom) animals showing the SNB, DLN, and RDLN.
Note the decreased number of DLN and RDLN motoneurons in the mSOD mouse compared with control, but with preserved SNB motoneuron number. As well, note the overall decrease in anterior horn cells in the ventral horns of the mSOD compared with the wildtype. Bar = 100 μm.


RDLN
SNB
DLN
mSOD
RDLN
SNB
DLN
Wild Type

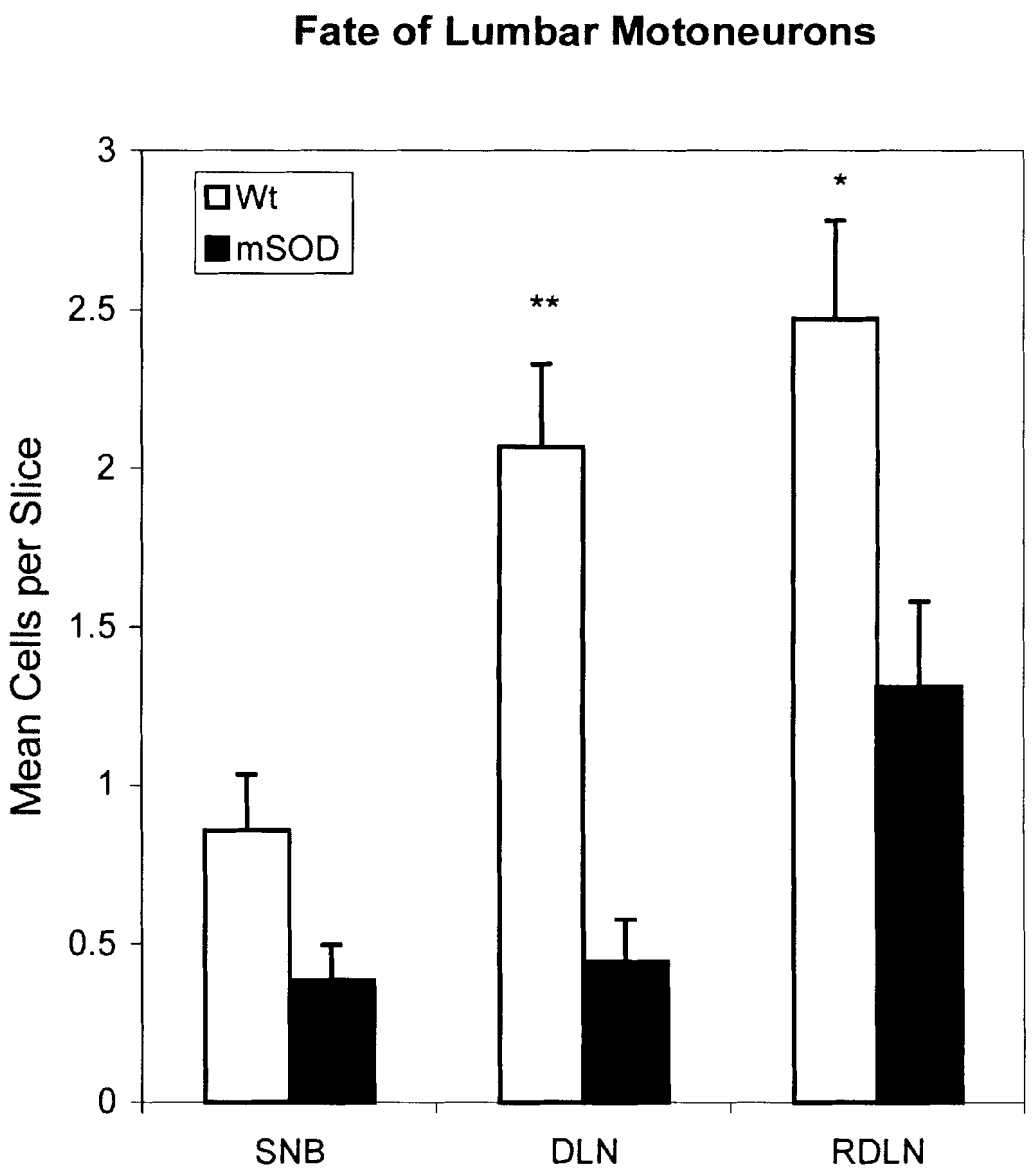


Figure 16: Motoneuron fate in the SNB, DLN, and RDLN of wild type (Wt) and mSOD mice expressed as mean number of cells per slice.
Cell counts differed between experimental and control animals in the DLN and RDLN, however, there was no statistically significant difference in SNB cell numbers. The significant differences in the numbers of DLN and RDLN between Wt and mSOD mice are indicated: *p = 0.013; **p = 0.001. Error bars indicate standard error of the mean (S.E.M.).

others exhibited a slightly less aggressive disease. We therefore divided the mSOD animals into two groups: those in which the development of mobility problems necessitated sacrifice at ~ 11 days after onset of weakness (fast disease progression group, FPG) and animals with slower disease progression that permitted a later sacrifice, at ~ 18 days (slower disease progression group, SPG). Significantly more RDLN motoneurons were lost in the FPG mSOD mice than in the SPG mice by the termination of the experiment ($t = 3.953$, $p < 0.01$; Fig. 17). In contrast, the rate of disease progression had no significant effect on the rate of cell loss in either the SNB or in the DLN ($t = 0.519$, $p = 0.626$; Fig. 17).

Dissection of some of the muscles innervated by these motoneuron pools revealed that the thigh muscles of affected animals were markedly atrophic, whereas the BC muscles of mSOD animals did not differ in gross weight from the BC muscles of control animals (means ± s.e.m. 0.081 ± 0.003 g, n = 4, and 0.089 ± 0.006 g, n = 4, respectively).

5.4 Discussion

Our results illustrate differential survival of lumbar motoneurons of the SNB, DLN and RDLN in mice expressing mutated SOD1. These data extend previous reports of enhanced motoneuron survival in ON in human ALS to the G93A mouse, confirming that these animals may suitably model the human disease. The further observation that in animals displaying slow disease progression, fewer RDLN motoneurons get lost, further indicates differential vulnerability of SNB, DLN and RDLN motoneurons in the mSOD animals. Interestingly, the BC muscles of the mSOD mice did not differ in weight from those of wt controls, suggesting that the entire SNB–BC neuromuscular system is spared in the affected animals.

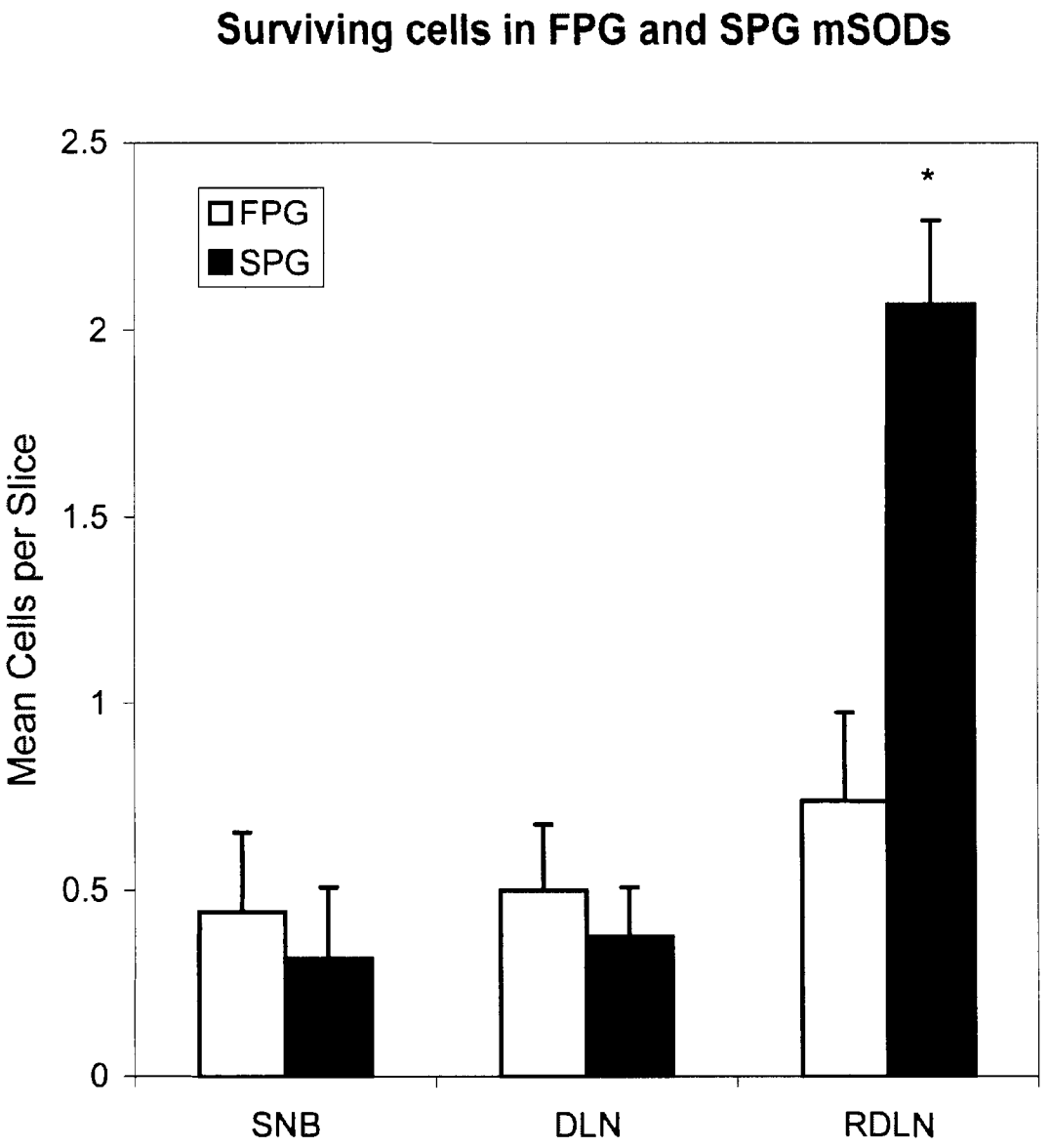


Figure 17: Number of surviving cells in the fast disease progression group (FPG) versus the slower disease progression group (SPG).
The FPG survived an average of 11 days after the first signs of disease onset, whereas the SPG developed disabling weakness approximately 18 days after onset. Asterisk indicates statistically significant difference from corresponding group; $p = 0.01$. Error bars represent standard error of the mean.

Although our data indicate that the SNB and its target muscles survive in the mSOD mouse, we can not rule out the possibility that if the animals were killed at a later time point, SNB motoneuron number would be reduced. There was a non-significant trend toward a decrease in the average number of SNB motoneurons in experimental animals as compared with wild type controls (Fig. 16). One interpretation is that our pre-determined endpoint for analysis of the experimental animals coincided with the onset of cell death in the SNB, but that death in the DLN and RDLN was already well under way. It is possible that with longer survival times we might have observed motoneuron death in SNB of mSOD mice, as well. In studies of respirator-maintained long-survival ALS patients, ON reportedly eventually succumbs to the disease process (Sasaki et al., 1992), suggesting that this nucleus is resistant, but not immune, to the pathophysiological processes of ALS.

The present data suggest that the SNB, DLN and RDLN differ in their vulnerability to neuron death in mSOD mice, with DLN motoneurons being the most vulnerable, regardless of disease progression rate. RDLN motoneurons are relatively preserved in slow-progressing mSOD mice, but would likely be reduced with time. SNB motoneurons are relatively resistant to motoneuron death in both slow- and fast-progressing mSOD mice. This differential vulnerability might reflect a gradient of expression for a motoneuron-selective protein or gene product that is involved in the neurodegenerative process. It could be argued that the mSOD and wild type mice differed in the amount of SNB cells present before or at the time hindlimb weakness developed. Mammals overproduce the number of needed cells within the nervous system and the excess is generally pruned out in an orderly process termed programmed cell death or

apoptosis. Within the SNB, ~ 25% of motoneurons die around the time of birth in normal male rats (Lance-Jones, 1982). If, during the critical period for SNB development, the mutant SOD1 product inhibited SNB cell death in mSOD mice, these animals would start out with more SNB cells overall than would wild type animals. If so, then with the onset of weakness a decline in SNB motoneuron number might be masked by the higher initial SNB motoneuron counts in the mSOD mice. However, there is no reason to suppose that the pruning process would be selectively affected in the SNB of mSOD mice and not the neighboring DLN and RDLN. Our finding of differential rates of cell loss among these nuclei therefore argues against this interpretation.

Why is the SNB resistant to degeneration when other motoneuron groups are severely affected? Either the SNB or its target muscles may regulate the expression or activity of a number of proteins involved in the pro- or anti-apoptotic pathways. As mentioned above, during development, androgens act on the bulbocavernosus muscle to attenuate apoptosis in the SNB, and it is possible that the sparing of SNB motoneurons in ALS occurs via a similar mechanism initiated in the BC muscles. For example, the BC muscle may play a role in modulating neuronal expression of pro-apoptotic enzymes in the SNB, and to a lesser extent in the DLN (which innervates the sexually dimorphic ischiocavernosus muscle that is anatomically contiguous with the bulbocavernosus), but not in the RDLN (which innervates a sexually-monomorphic foot muscle). It has recently been reported that activation of the pro-apoptotic cysteine protease caspase-1 takes place well before the onset of pathology in the spinal cords of mSOD mice, and also before the activation of a final executioner protease, caspase-3 (Pasinelli et al., 2000; Vukosavic et al., 2000). Activation of caspase-3 is concomitant with massive neuron loss in the ventral

horns of the mSOD animals (Pasinelli et al., 2000; Vukosavic et al., 2000). Under one model, gonadal steroids may be influencing the activation of these cysteine proteases, perhaps by regulating mitochondrial membrane permeability and cytochrome c release (Guegan et al., 2001). The increase of mitochondrial permeability by insertion of *bax* proteins into the outer membrane is an event that precipitates the release of cytochrome c into the cytosol, a process that is known to subsequently activate cysteine proteases and the cell death cascade in transgenic G93A mice (Guegan et al., 2001). A recent study reports that the target muscles regulate the expression of a family of survival signals (e.g., Bcl-2 family) in the SNB that dynamically inhibits bax activity (Zup and Forger, 2002). Gonadal steroid regulation of this process could thus explain the selective survival of the SNB/ON motoneuron pool in humans with ALS, but given the widespread androgen sensitivity of skeletal muscle, it is not clear why a similar mechanism is not observed in other motoneuron populations.

In summary, we find that motoneurons of the mouse homologue of ON, the SNB, survive in transgenic mutant SOD1 mice, while neighboring motoneuron pools degenerate, mirroring findings from studies of human ALS. This study helps to confirm that the G93A mouse model parallels disease progression in human ALS and permits further studies probing the unique properties of SNB neurons that confer the enhanced survival.

CHAPTER 6
PROTEIN KINASE AND PROTEIN PHOSPHATASE EXPRESSION IN THE CNS OF G93A mSOD OVEREXPRESSING MICE*

6.1 Summary

The expressions of 78 PKs, 24 PPs and 31 phosphoproteins were investigated by Kinetworks™ analysis in brain and spinal cord tissue of transgenic mice overexpressing G93A mSOD, a murine model of ALS. In the brains of affected mSOD mice we observed increased expression of PKA (111%↑compared with control), and protein phosphatase 2B (PP2B) Aα-catalytic subunit (calcineurin, 109%↑), and reductions in the levels of p21-activated kinase (PAK) 3 (76%↓) and protein phosphatase 2C (PP2C) Cβ-subunit (32%↓). Increased Ser259 phosphorylation of Raf1 (126%↑) in mSOD mice correlated with higher expression of p73 Raf1 (147%↑). There was also increased p73 Raf1 (69%↑) and Ser259 phosphorylation (45%↑) in the spinal cords of mSOD mice. While Adducin underwent enhanced phosphorylation (αS724, 90%↑; γS662, 290%↑) in mSOD brain, its phosphorylation was lower in the mSOD spinal cord (αS724, 53%↓; γS662, 46%↓). In spinal cords of affected mSOD mice, we also observed elevated expression of casein kinase 1δ (CK1δ, 157%↑), JAK2 (84%↑), PKA (183%↑), PKCδ (123%↑), p124 PKCμ (142%↑), and RhoA kinase (221%↑), and enhanced phosphorylation of ERK1, (T202/Y204, 90%↑), and ERK2 (T185/Y187, 73%↑), p38

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MAP kinase (T180/Y182, 1570%↑), and PKBα (T308, 154%↑; S473, 61%↑). There was also reduced phosphorylation of RB (S780, 45%↓; S807/S811, 65%↓), Src (Y418, 63%↓) and p40 SAPK/JNKβ (T183/Y185, 43%↓). Variability in the expression of kinases, phosphatases and phosphorylation of their substrates was observed even in mutant animals having a similar phenotype. The expression and phosphorylation differences between mSOD and control mice were dissimilar to those between ALS patients and controls. This finding indicates that the activation of protein kinases and phosphoproteins is different with neuron loss in the mSOD mouse compared with that seen in patients with the sporadic form of ALS.

6.2 Introduction

Mice overexpressing mSOD have been used extensively as a murine model of ALS to investigate the regulatory mechanisms underlying neuron loss in this neurodegenerative disorder of brain and spinal cord. There have been few studies evaluating changes in the expressions and/or activities of PKs in mSOD overexpressing mice. For example, G93A mSOD transgenic mice have decreased PI3-K and PKB immunoreactivity in the presymptomatic stage that precedes significant loss of spinal motor neurons in this animal (Warita et al., 2001). Another PK, JAK3, may be involved in the progression of motor neuron death in the G93A mSOD mouse as a specific inhibitor of JAK3, WHI-P131, delayed the onset of paralysis and increased survival in these mice (Trieu et al., 2000). The molecular mechanism for the neuroprotective role of JAK3 inhibition is probably through suppression of c-Jun, a transcription factor frequently induced in injured neurons and other cells. Expression of c-Jun is markedly

increased in the spinal cord of end-stage G93A mSOD mice (Jaarsma et al., 1996). Nguyen et al. (2001) reported a de-regulation of CDK5 activity associated with the hyperphosphorylation of tau and NF proteins in G37R mSOD mice. They found three G37R mouse lines exhibited perikaryal inclusions of NF proteins and suggested that these inclusions might act as a phosphorylation sink for CDK5 activity to alleviate ALS pathogenesis by reducing the detrimental hyperphosphorylation of tau and other neuronal substrates. All of these observations implicate a variety of PKs in the cause of neuron injury in the mSOD mouse and the relations between these and other PKs warrant further exploration.

We have used a multi-immunoblotting proteomics screening technique called Kinetworks™ to examine the expression profile of 78 PKs, 24 PPs and 31 phosphoproteins in the the brain and spinal cord of mutant mice over-expressing a human transgene coding for G93A mSOD found in patients with FALS. Brain and spinal cord tissue were collected when mSOD mice were at a stage with 'moderate-severe' clinical signs similar to that present in some ALS patients at the time of death. This would permit us to compare the profile of PKs, PPs and phosphoproteins present in mSOD mice to those observed in patients with ALS.

6.3 Results

6.3.1 Multi-immunoblotting of PKs, PPs and phosphoproteins in mouse CNS

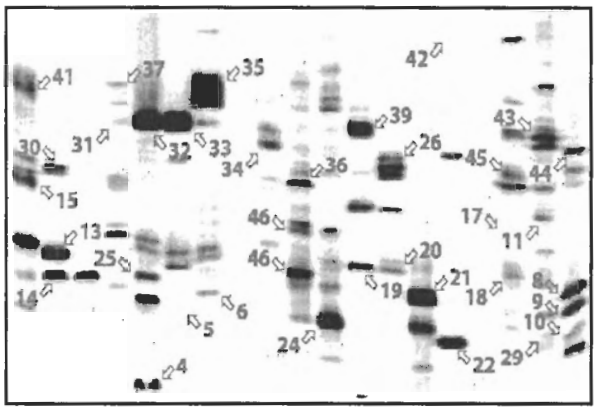
Using the Kinetworks™ multi-immunoblotting technique we evaluated 78 PKs, 24 PPs and 31 phosphoproteins in brain and spinal cords from mice overexpressing G93A mSOD and control littermates. Figure 18 shows examples of the multi-immunoblots of PKs (a and b), PPs (c) and phosphoproteins (d) in whole lysates of spinal

Figure 18: Examples of multi-immunoblots of Kinetworks™ screens of protein kinases, protein phosphatases and phosphoproteins in mouse wt spinal cord.
(a,b) Kinetworks™ KPKS 1.0 protein kinase, (c,d) KPPS 1.1 protein phosphatase, (e,f) KPSS 1.1 phosphoprotein. The identities of protein targets are indicated by arrows and numbers.

(a)

Protein kinases tracked:

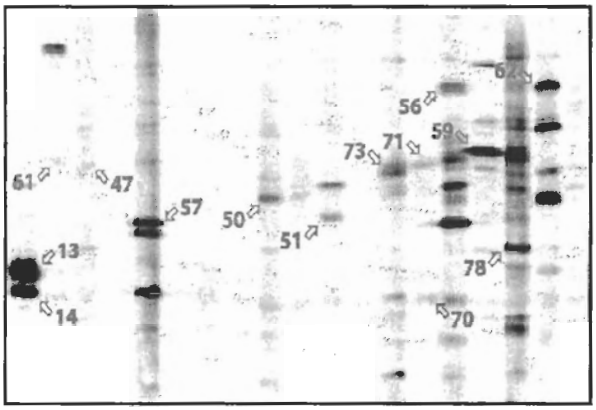
1. CDK1	12. DNAPK	24. Mos1	36. PKCλ
2. CDK2	13. ERK1	25. p38α	37. PKCμ
3. CDK4	14. ERK2	26. PAK1	38. PKCθ
4. CDK5	15. ERK3	27. PAK3	39. PKCζ
5. CDK6	16. ERK6	28. PDK1	40. PKR
6. CDK7	17. Gsk3α	29. Pim1	41. RafB
7. CDK9	18. Gsk3β	30. PKBα	42. ROKα
8. CK2α	19. MEK1	31. PKCα	43. RSK1
9. CK2α'	20. MEK2	32. PKCβ	44. RSK2
10. CK2α"	21. MEK4	33. PKCγ	45. S6K
11. Cot	22. MEK6	34. PKCδ	46. SAPK
	23. MEK7	35. PKCε	



(b)

Protein kinases tracked:

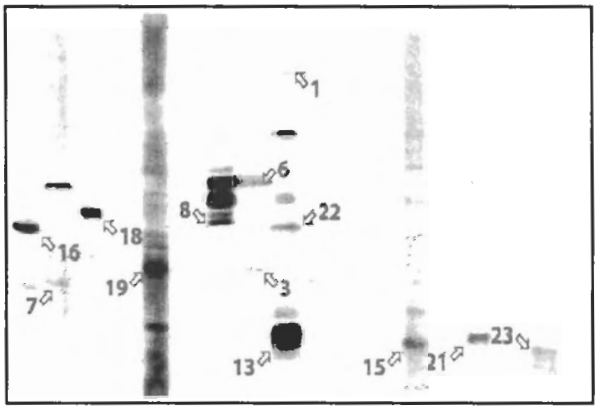
47. BMX	55. DAPK	63. JAK2	71. PKG1
48. BTK	56. FAK	64. Ksr1	72. PYK2
49. CaMK1	57. Fyn	65. Lck	73. Raf1
50. CaMK4	58. GCK	66. Lyn	74. Src
51. CaMKK	59. Grk2	67. Mnk2	75. Syk
52. CK1δ	60. Hpk1	68. Mst1	76. Yes
53. CK1ε	61. IKKα	69. Nek2	77. ZAP70
54. Csk	62. JAK1	70. PKA	78. ZIPK



(c)

Phosphatases tracked:

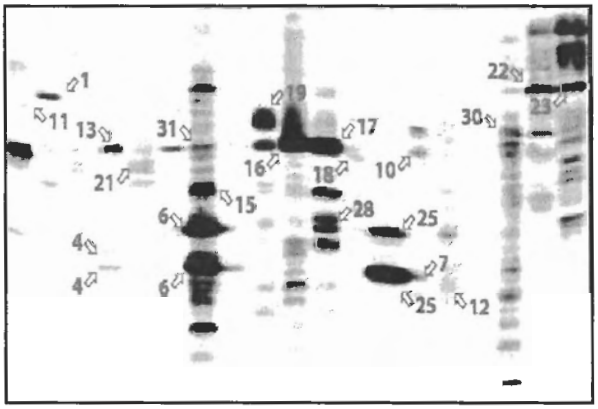
1. CD45	9. MKP-2	17. PP2A/Cα/β
2. LAR	10. MKP-3	18. PP2B/Aα
3. PTP1B	11. PAC1	19. PP2C/Cα/β
4. PTP-PEST	12. PTEN	20. PP2C/Cδ
5. SHP1	13. PP1/Cα	21. PP2X/C
6. SHP2	14. PP1/Cβ	22. PP5 (PPT)
7. KAP	15. PP1/Cγ	23. PPV/C
8. MKP-1	16. PP2A/Aα/β	24. PPX/A'2



(d)

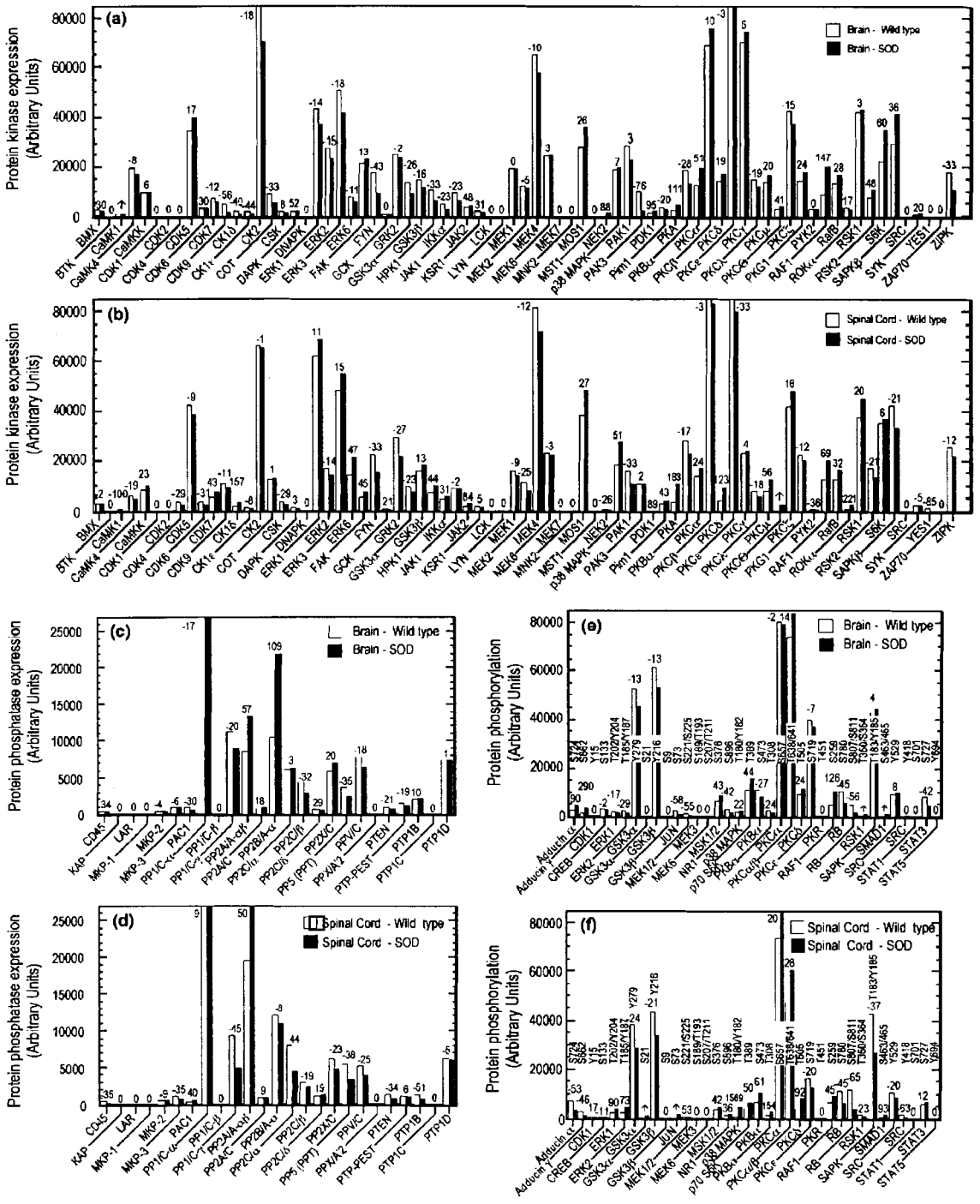
Phosphoproteins tracked:

1. Adducin - S662	17. PKCα - T638
2. CDK1 - Y15	18. PKCδ - T505
3. CREB - S133	19. PKCε - S719
4. ERK1/2 - T202/Y204	20. PKR - T451
5. Gsk3α/β - S21/S9	21. Raf1 - S259
6. Gsk3α/β - Y279/Y216	22. RB1 - S780
7. cJun - S73	23. RB1 - S809/S811
8. MEK1 - S217/S221	24. Rsk1 - T360/S364
9. MEK3/6 - S189/S207	25 SAPK - T183/Y185
10. MSK1 - S376	26. Smad1 - S463/S465
11. NR1 - S896	27. Src - Y418
12. p38α - T180/Y182	28. Src - Y529
13. p70 S6K - T389	29. STAT1 - Y701
14. PKBα - T308	30. STAT3 - S727
15. PKBα - S473	31. STAT5 - S694
16. PKCα - S657	



cord tissue from a littermate control. Many of the target proteins for these screens were clearly evident in the immunoblots of control spinal cord. Other target proteins that were not detected included the PKs: BTK, CaMK1, CDK1, CDK2, CDK4, CDK9, CK1δ, CK1ε, CSK, DAPK, DNAPK, ERK6, GCK, HPK1, JAK2, KSR1, LCK, LYN, MNK2, MST1, NEK2, MEK7, PAK3, PDK1, PKCθ, PKR, PYK2, SRC, Syk, YES and ZAP70, and PPs: LAR, PTP-PEST, SHP1, MKP-2, MKP-3, PAC1, PTEN, PP1Cβ, PP2ACα/β, PP2C/Cδ and PPX/A'2.

Neuropathological changes are prominent in the spinal cord of the mSOD mouse (Gurney et al., 1994) and we performed Kinetworks™ immunoblotting analyses using this tissue for both mSOD and control mice. Figure 19 shows the pooled results from the separate multi-immunoblotting analyses of the whole brain and spinal cord tissues of each of five mSOD transgenic mice and five wt littermate controls. Table 1 further shows the statistical significance of many of the changes in protein expression and phosphorylation that were evident between the mSOD and wt mice. The overall patterns of expression of PKs and PPs were very similar between the brains and spinal cords of wt mice. Notable exceptions were CaMK4 and PKCγ, which were expressed at threefold higher levels in brain as compared with spinal cord (Figs 19a and b), and the regulatory Aα/β subunit of protein phosphatase 2A, which was 130% more abundant in spinal cord (Figs 19c and d). Although the protein levels of PKCε were similar between wt brain and spinal cord, the specific phosphorylation of Ser719 of PKCε was 143% higher in brain (Figs 19e and f). These differences in expression likely occur because of variations in the specific cell types present in the brain and spinal cord. Within mouse brain, there are likely to be regional differences in PK expression. However, as shown in Figure 20, the



89Figure 19: Kinetworks™ profiles of protein kinases, protein phosphatases and phosphoproteins in brain and spinal cord of mSOD mice and control littermates.
(a,b) protein kinases, (c,d) protein phosphatases, (e,f) phosphoproteins, (a,c,e) brain, (b,d,f) spinal cord. Values are the averaged results from 5 mSOD mice (black bars) and 5 wt mice (white bars).

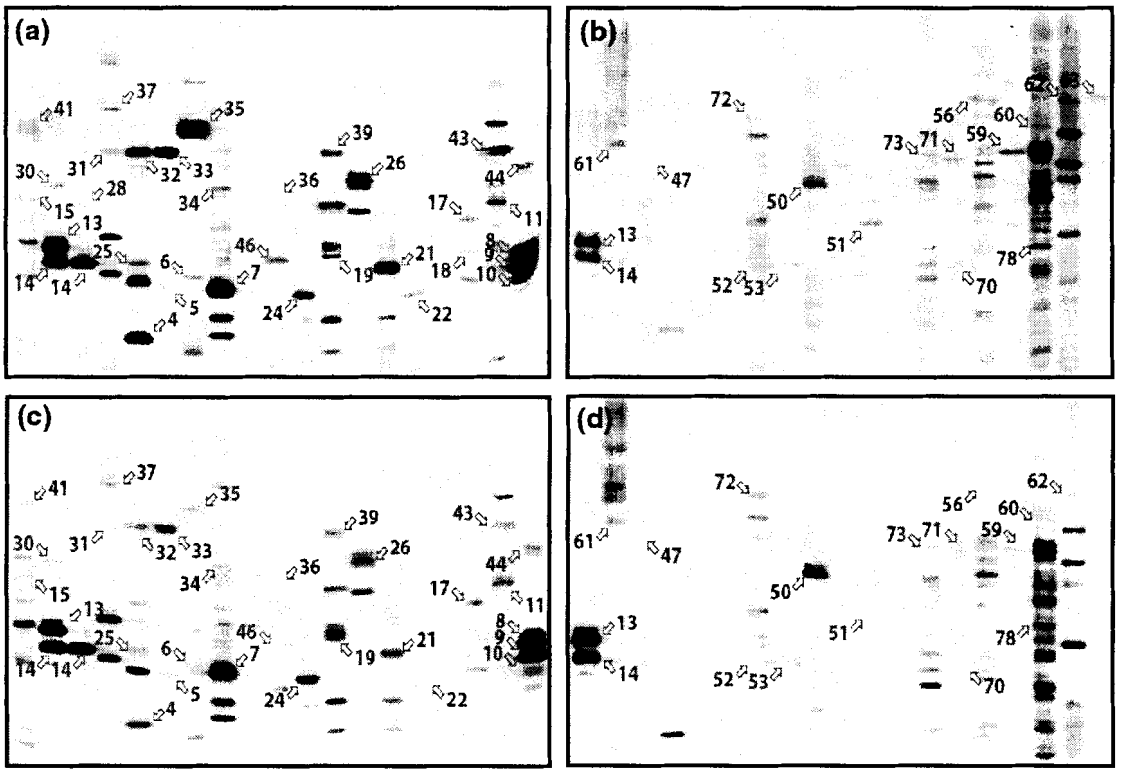


Figure 20: Kinetworks™ KPKS 1.0 protein kinase analyses of mouse wt frontal cortex (a and b) and striatum (c and d).
The identities of protein kinase targets indicatedby arrows and numbers are same as provided in Fig. 18.

Kinetworks™ KPKS 1.0 protein kinase expression patterns were very similar for mouse frontal cortex and striatum, although PKCβ, γ and ε, PAK1 and Mek4 were preferentially expressed in the frontal cortex.

PKCγ and PKCε were not significantly altered in expression or phosphorylation in the mSOD mice. There was a shift in the molecular mass of CaMK4 in the spinal cord of mSOD mice from 62 kDa to 64 kDa, which may arise from increased phosphorylation of this kinase or alternative splicing of this enzyme (Table 1). There may have also been a trend towards a 50% increase in the levels of PP 2A Aα/β in the brain and spinal cord of mSOD mice, but this change was not statistically significant (Table 1). While Adducin underwent enhanced phosphorylation (αS724, 90%↑; γS662, 290%↑) in mSOD brain, its phosphorylation was lower in the mSOD spinal cord (αS724, 53%↓; γS662, 46%↓) (Figs 19E and F).

In the brains of mSOD mice, there also appeared to be enhanced levels of PKA (111%↑), p73 Raf1 (147%↑), Ser259-phosphorylated Raf1 (126%↑), the band-shifted, phosphorylated p83 form of S6 kinase (130%↑), and PP 2B catalytic A subunit (109%↑). There were reductions in the amount of PAK3 (76%↓), PP 1 Cγ-subunit (20%↓), PP 2C Cβ-subunit (32%↓), Ser73 phosphorylated c-Jun (58%↓), and Ser780/807/811 phosphorylated RB (44–56%↓). However, only the increases in PKA and PP2B A subunit and reductions in PP1 and PP2C C subunit were statistically significant (Table 1). Like all the statistical analyses in this study, these differences were calculated as a *t*-test without correction for multiple tests.

In spinal cord tissues of mSOD as compared with wild-type mice, there were also statistically significant increases in CK1δ protein (157%↑), Thr202/Tyr204-

Table 1: Expression levels of protein kinases, and protein phosphatases and phosphorylation levels of protein kinases in the brain and spinal cord tissues of mSOD and control mice.

The protein kinases, phosphatases and phosphoproteins which had significant changes in the same tissue between experimental animals and controls are shown. Statistical analysis was performed of data in control and mSOD mice from same anatomic region, asterisks indicate the p-values. Expression levels shown constitute mean ± SD determined from multi-immunoblot determinations from each of five control and five mSOD mice. No correction for multiple statistical analysis was used. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Protein Kinase	Antibody Specificity	Control Brain Trace quantity Mean ± S.DEV	mSOD Brain Trace quantity Mean ± S.DEV	Control Sp. Cord Trace quantity Mean ± S.DEV	mSOD Sp. Cord Trace quantity Mean ± S.DEV
Calmodulin-dependent kinase 4 (CaMK4) p62	Pan	15434 ± 5002	14934 ± 2736	5982 ± 2917	1087 ± 1574**
Calmodulin-dependent kinase 4 (CaMK4) p64	Pan	3595 ± 4192	2649 ± 2447	0 ± 0	3713 ± 3816*
Casein kinase 1 (CK1) delta p39	Pan	2027 ± 2052	1210 ± 1165	1465 ± 1510	3768 ± 1233*
Extracellular regulated kinase 1 (Erk1) p42	Pan	43001 ± 14222	37144 ± 11461	62271 ± 14056	68924 ± 31755
	T202/Y204	1842 ± 1081	1520 ± 1604	2094 ± 1804	3969 ± 1119*
Extracellular regulated kinase 3 (Erk3) p56	Pan	22658 ± 17367	21706 ± 9508	7302 ± 4584	15794 ± 8709*
Extracellular regulated kinase 6 (Erk6) p47	Pan	7358 ± 5566	6518 ± 5110	14313 ± 4522	21105 ± 4833*
Fyn oncogene related to SRC p48	Pan	10963 ± 10866	2472 ± 2413	12872 ± 11945	10338 ± 10165
Glycogen synthase kinase 3 (GSK3) alpha p45	Pan	13599 ± 11865	10115 ± 7686	9531 ± 1554	7355 ± 3991
	Y279	52459 ± 5115	45608 ± 4315*	37841 ± 9285	28578 ± 4949*
Glycogen synthase kinase 3 (GSK3) beta p41	Pan	0 ± 0	0 ± 0	11319 ± 13812	13152 ± 12439
Glycogen synthase kinase 3 (GSK3) beta p42	Pan	14614 ± 11632	12317 ± 9386	4576 ± 2948	4803 ± 3999
Hematopoetic progenitor kinase 1 (HPK1) p90	Pan	10649 ± 10980	7092 ± 7502	6864 ± 2814	9873 ± 8459
p38 Hog MAP kinase p38	Pan	18921 ± 3466	20166 ± 4554	18116 ± 2107	27285 ± 6964*
	T180/Y182	1795 ± 2321	2191 ± 1975	293 ± 401	4884 ± 4799*
p21 activated kinase 3 (PAK3) p65	Pan	9672 ± 8874	2289 ± 5118	10535 ± 12299	10748 ± 11408
Protein kinase A (cAMP-depe. protein kinase) p38	Pan	2229 ± 1738	4705 ± 1737*	3797 ± 2407	10747 ± 11122
Protein kinase B (PKB) alpha p59	Pan	1027 ± 2296	3984 ± 5456	22355 ± 5484	18869 ± 6059
Protein kinase B (PKB) alpha p62	Pan	17590 ± 10006	9438 ± 6015	5456 ± 5904	4163 ± 5701
Protein kinase C (PKC) beta p83	Pan	0 ± 0	0 ± 0	28387 ± 27667	42889 ± 41765
Protein kinase C (PKC) beta p89	Pan	69277 ± 20709	76598 ± 17686	56648 ± 44092	40001 ± 49740
Protein kinase C (PKC) gamma p84	Pan	70418 ± 7505	74979 ± 17323	23033 ± 7082	23997 ± 4272
Protein kinase C (PKC) delta p80	Pan	14717 ± 5654	17508 ± 3224	4229 ± 4442	9412 ± 3659
Protein kinase C (PKC) mu p124	Pan	7499 ± 3831	10544 ± 2959	3452 ± 3997	8349 ± 3927*
Protein kinase G1 (PKG1) p75	Pan	14736 ± 16562	18337 ± 20107	22343 ± 24118	19627 ± 20776
Raf1 p73	Pan	5345 ± 2071	13208 ± 9812	7816 ± 1958	13231 ± 5565
S6 kinase p70 (S6K) p60	Pan	4303 ± 8245	5290 ± 9929	7121 ± 9171	11416 ± 11290
S6 kinase p70 (S6K) p64	Pan	10965 ± 9779	10870 ± 10051	11632 ± 10742	10897 ± 11067
S6 kinase p70 (S6K) p67	Pan	4431 ± 6248	9513 ± 10494	4622 ± 6655	5446 ± 5437
S6 kinase p70 (S6K) p81	Pan	0 ± 0	0 ± 0	2596 ± 5804	9242 ± 15974
S6 kinase p70 (S6K) p83	Pan	4147 ± 5718	9550 ± 9105	8828 ± 8122	0 ± 0*
Stress activated protein kinase (JNK; SAPK) beta p40	Pan	13911 ± 12075	15059 ± 14378	22844 ± 14890	18885 ± 12113
	T183/Y185	31411 ± 12430	31754 ± 16048	33644 ± 13020	19167 ± 10429*
Stress activated protein kinase (JNK; SAPK) beta p47	Pan	10712 ± 9395	17173 ± 14194	11709 ± 7367	4597 ± 6132
	T183/Y185	11469 ± 6177	12807 ± 9119	9184 ± 5495	7887 ± 5942
Protein phosphatases					
Phosphatidylinositol 3'-phosphatase (PTEN) p54	Pan	942 ± 111	741 ± 171	1237 ± 220	820 ± 231*
Protein phosphatase 1 - C subunit-gamma p30	Pan	11107 ± 838	8851 ± 287*	9267 ± 1391	5068 ± 630**
Protein phosphatase 2A - A subunit p49	Pan	8488 ± 5642	13324 ± 6261	19570 ± 4255	29389 ± 10120
Protein phosphatase 2B - C subunit - alpha p53	Pan	10427 ± 1851	21834 ± 4873*	11996 ± 1655	10978 ± 2047
Protein phosphatase 2C - C subunit - beta p47	Pan	4502 ± 367	3058 ± 342***	3056 ± 978	2476 ± 536
Protein phosphatase 5 - C subunit p48	Pan	3681 ± 1141	2398 ± 542	5420 ± 910	3338 ± 630*
Other phosphoproteins					
Jun p40	S73	2360 ± 2188	999 ± 2233	0 ± 0	1734 ± 1709
Retinoblastoma (RB) p126	S780	10576 ± 9093	5862 ± 5324	11159 ± 10059	6154 ± 5099
	S807/811	4584 ± 10250	2014 ± 2813	11971 ± 11540	4211 ± 4223

phosphorylated ERK1 (90%↑), p56 ERK3 protein (116%↑), ERK6 protein (47%↑), p38 MAP kinase protein (51%↑), Thr180/Tyr182-phosphorylated p38 MAPK (1570%↑), PKCδ protein (123%↑), PKCμ protein (56%↑), p73 Raf1 protein (69%↑), and Ser73 phosphorylated c-Jun (> 200%↑). Statistically significant reductions in expression in mSOD mice were noted for Tyr279 phosphorylated GSK3α (24%↓), the band-shifted, phosphorylated p83 form of S6 kinase (100%↓), Thr183/Tyr185-phosphorylated p40 JNK MAPK (43%↓), PP 1 Cγ subunit (45%↓), PP 5 C (39%↓), PTEN (34%↓), and Tyr418-phosphorylated Src (63%↓). There also appeared to be elevated expression of JAK2 (84%↑), RhoA kinase (221%↑), enhanced phosphorylation of ERK2 (T185/Y187, 73%↑) and PKBα (T308, 154%↑; S473, 61%↑), and reduced phosphorylation of RB (S780, 45%↓; S807/S811, 65%↓) and Src (Y418, 63%↓) in the spinal cords of mSOD mice (Table 1). Examination of these results reveals that most of the differences in expression between mSOD and control spinal cord were in the direction of increased expression of PKs and decreased expression of PPs.

6.4 Discussion

In this work we analyzed the expression of 78 distinct PKs, 24 PPs and 31 phosphoproteins in the CNS of mSOD mice and unaffected littermates that did not express human mSOD either clinically or by PCR detection. These results provide interesting normative data on the expression of these proteins in the rodent CNS.

6.4.1 Regional expression of PKs and PPs

We analyzed protein expression in both brain and spinal cord to determine if there

were differences between these two distinct CNS regions. We found that some differences were evident, for example, GSK3β p41 and PKCβ p83 were expressed in spinal cord but not apparent in whole brain (Table 1). However, mouse frontal cortex clearly contained GSK3β and PKCβ (Fig. 20). The PKBα p59 isoform was highly expressed in spinal cord, with much lower expression in brain while the PKBα p62 isoform and PKCγ p84 were more highly expressed in brain than spinal cord (Table 1). These changes have not been described previously and indicate possible PK and PP candidates that may determine differences between regulatory pathways in brain and spinal cord.

Only a few previous studies have examined the expression of PKs and PPs in rodent CNS. A study of PI3-K, PKB, p70 S6K and ERK expression in the spinal cords of mice having the progressive neuronopathy mutation (pmn) and control mice revealed that these four kinases were highly expressed in total lysates of spinal cord and that p70 S6K was reduced in pmn mice (Wagey et al., 2001b). Previous work has also reported changes in the expression and/or activities of PKB, PI 3-K and CDK5 in spinal cord tissue from mice over-expressing mSOD, compared with controls (Nguyen et al., 2001b; Warita et al., 2001).

6.4.2 PK and PP expression in mSOD mice

Comparison of over 133 proteins in brain tissue from mSOD and control mice revealed only statistically significant elevated expression of PKA and PP2B/A-α catalytic subunit and reduced expression of Y279-phosphorylated GSK3α, PP1/C-γ and PP2C/C-β in mSOD mice. As was evident in Table 1, standard deviations of PKs, PPs and phosphoproteins were large, not only for mSOD animals having a similar phenotype, but

also for controls. We were particularly surprised by the variability in expression of proteins such as Fyn, hematopoietic progenitor kinase 1 (HPK1) in brain, and PAK3, PKG1 and S6K in spinal cord. We do not know why this variably is present but it may reflect animal to animal variability, or limitations with the protein screening technique. Variability appeared to be similar in mSOD and control tissue.

We did not perform corrections for multiple sample analysis, which would have removed the statistical significance of these data. Corrections for multiple analyses were not done as to do so would require large number of animals and antibodies to be used for the analysis, which was not technically feasible. In spinal cords of affected mSOD mice, we observed elevated expression of CaMK4, CK1δ, T202/Y204-phosphorylated ERK1, ERK3 p56, ERK6, S73-phosphorylated Jun, Raf1, p38 MAPK, T180/Y182 phosphorylated p38 MAPK, PKCδ and PKCμ and reduced expression of Y279-phosphorylated GSK3α, p83 S6K, T183/Y185-phosphorylated SAPKβ, PTEN, PP1/C-γ and PP5/C. The only consistent, significant change in protein expression between mSOD and control mice in spinal cord and brain were the alterations in expression of Y279-phosphorylated GSK3α and PP1/C-γ. All of these specific phosphorylation events are associated with enzymatic activation of the target proteins.

6.4.3 Evaluation of specific PK and PP species in mSOD mice

Generally, where statistically significant differences in expression were found between mSOD and control animals, these differences were in the direction of elevated expression of PKs and phosphoproteins, and reduced expression of PPs in mSOD animals. For instance, in spinal cord mSOD mice had elevated expressions of CaMK4, CK1γ, T202/Y204-phosphorylated ERK1, ERK3, ERK6, S73-phosphorylated c-Jun,

Raf1, p38 MAPK, T180/Y182-phosphorylated p38 MAPK, PKCδ, and PKCμ. In contrast to the at least 11 PKs and phosphoproteins that were elevated (out of 109), only 4 PKs and phosphoproteins had reduced expression in spinal cord (e.g. CaMK4, p83 S6K). Furthermore, PP expressions for PTEN, PP1 Cγ-subunit, PP5 C-subunit were significantly reduced in spinal cords of mSOD animals. These observations indicate that in the event an equilibrium exists between the expression of PKs and PPs in mSOD animals, the balance of expression may be shifted towards increased protein phosphorylation. The presence of specific alterations in PKs and PPs in mSOD mice implicates a role for some of these aberrant proteins in the neurodegeneration in mSOD mice.

Our data reveal increased expression of Raf1 and activation of ERK1 in spinal cord tissue from G93A mSOD mice (Table 1). This observation could implicate the Raf1→MEK1/2→ERK1/2 signaling pathway as being abnormally regulated in this mutant mouse. Raf1 is a serine/threonine kinase that is activated by Ras, producing translocation to the plasma membrane where subsequent modifications lead to its full activation (Leevers et al., 1994). The Ras→Raf1→MEK1/2→ERK1/2 pathway can be triggered by a variety of extracellular signals through the activation of specific membrane receptors which may be involved in cellular differentiation, proliferation and survival. Since Xia et al. (1995) provided evidence for the neuroprotective role of ERK1/2 against apoptosis in P12 cells, numerous studies have supported a neuroprotective effect of ERK1/2 in neurons (Encinas et al., 1999; Hetman et al., 1999; Han and Holtzman, 2000; Tsuji et al., 2000; Kuroki et al., 2001; Jin et al., 2002). However, ERK1/2 does not always seem to protect neuronal cells and under some conditions can mediate neuronal

injury (Alessandrini et al., 1999) and contribute to neuron death induced by oxidative stress (Satoh et al., 2000; Stanciu et al., 2000). We infer that the significant increase of Raf1 and T202/Y204-phosphorylated ERK1 in the spinal cord tissue of mSOD mice may result from increased demand of neurons to activate the Raf1→MEK1/2→ERK1/2 signaling pathway so as to protect them from death.

6.4.4 Differences in profiles of PKs and PPs in mSOD mouse and ALS

The G93A transgenic mouse model of ALS employed here is among the most extensively used models of ALS. Neurological disease in this transgenic mouse has a number of similarities to sporadic ALS in humans (Gurney et al., 1994). Presumably, it has much greater similarity in pathogenesis to those cases of familial ALS due to mutations in the gene coding for SOD1. However, differences may exist, which could be a consequence of the less developed state of the murine corticospinal tract and other descending tracts, compared with humans. These descending motor tracts are prominently affected in patients with ALS (Eisen and Krieger, 1998).

All the mSOD mice were studied at a stage where 'moderate' clinical involvement was evident by virtue of difficulty with ambulation, but righting was preserved and animals did not require assistance with feeding. We chose this stage of disease because we believed it would be comparable to the stage at which we obtained tissue from patients with ALS at postmortem (Hu et al., 2003b). Such patients may be extremely debilitated but generally have not been maintained on long term ventilation.

Comparison of differences in the profiles of PKs, PPs and phosphoproteins between CNS tissue from mSOD mice and controls are very different from those in thoracic spinal cord tissue from patients who died with ALS and controls (Table 2). Some

of these differences may be attributed to non-uniformity of the regions analyzed in human spinal cord (cytosolic and particulate fractions of thoracic spinal cord), versus whole spinal cord in mSOD and control animals; as well as the presence of a post-mortem delay in freezing human tissue. Additionally, as patients with sporadic ALS do not have mutations in the SOD gene they likely have differences in disease mechanism compared with patients with familial ALS, or to mice with mSOD mutations. In both mSOD mice and ALS patients we observe elevated expression and/or activation of different isoforms of ERK. The ERK1/2 signaling pathway may be activated in response to neuron injury in both human ALS and the mSOD mouse. However, the p38 MAPK is also elevated in mSOD mice, which was not found in ALS patients compared with controls (Table 2, or Hu et al., 2003b). It has been suggested by Xia et al. (1995) that the dynamic balance between growth factor-activated ERK and stress-activated p38 MAPK pathways may be important in determining whether a cell survives or undergoes apoptosis. The finding of increases in expression of ERK3, ERK6 and p38 MAPK in mSOD mice possibly demonstrate such an antagonistic roles between activating the ERK1/2 survival pathway in response to neuron injury and up-regulating p38 apoptotic pathway leading to neuron death.

Elevated expression of different isoforms of PKCs was observed in mSOD mice compared with those seen in ALS. PKCδ and PKCμ were elevated in mSOD spinal cord tissue while PKCα, PKCβ and PKCζ were increased in thoracic spinal cord of ALS patients (Table 2, or Hu et al., 2003b). The changes of other PKs and PPs in mSOD mice and ALS are quite different. In ALS patients, we found increased expression and/or activation of some of these kinases, such as PKCβ, PKCζ and GSK3α/β and attributed

	ALS compared with control		mSOD compared with control
Protein kinase	**Cytosol fraction**	**Particulate fraction**	**Whole lysates**
CaMKK	120% ↑	580% ↑	–
CK1 α	–	–	157% ↑
ERK2	120% ↑	170% ↑	–
ERK3	–	–	116% ↑
ERK6	–	–	47% ↑
GRK2	140% ↑	140% ↑	–
p38 MAPK	–	–	51% ↑
PKA	–	43% ↑	–
PKB α	360% ↑	200% ↑	–
PKC α	100% ↑	–	–
PKC β	–	330% ↑	–
PKC δ	–	–	123% ↑
PKC μ p124	–	–	142% ↑
PKC ζ	190% ↑	–	–
PKG	100% ↑	75% ↑	–
Raf1p73	–	–	69% ↑
RSK1	750% ↑	630% ↑	–
SAPK/JNK β	–	34% ↑	–
p70 S6K p80	–	–	100% ↓
Protein phosphatase			
KAP	34% ↓	100% ↑	–
PP1/C γ	–	–	45% ↓
PP5/C	–	–	38% ↓
PTP1 δ	43% ↑	70% ↑	–
PTEN	–	–	34% ↓
Protein phosphorylation			
Adducin α p120 (S662/S724)	15650% ↑	–	–
ERK1 p42 (T202/Y204)	–	–	90% ↑
GSK3 α p44 (Y216/Y279)	90% ↑	220% ↑	24% ↓
p38 MAPK (T180/Y182)	–	–	1570% ↑
PKC α/β (T638)	630% ↑	170% ↑	–
PKR (T451)	2600% ↑	3330% ↑	–
S6K (T389)	1000% ↑	460% ↑	–
SAPK/JNK β p40 (T183/Y185)	–	–	43% ↓

Table 2: Comparison of different expression profiles of protein kinases, protein phosphatases and phosphoproteins in spinal cord tissues between mSOD mice (compared with control littermates) and ALS patients (compared with control subjects).

Data derived from analyses of whole spinal cord and thoracic spinal cord tissues were used for mouse and human results, respectively. Only the protein kinases and protein phosphatases that had statistically significant changes in expression are included. ↑, increase; ↓, decrease; compared with control.

them a possible role of augmenting neural death in ALS (Hu et al., 2003b). Also, we observed increased expression and/or activation of CaMKK, PKBα, Rsk1, S6K and SAPKβ and suggested they might be a response to neuronal injury that potentially can mitigate cell death (Hu et al., 2003b). In contrast, we found reduced level of Y279-phosphorylated GSK3α/β in mSOD brain and spinal cord tissues, as well as a reduction of S6K p83 and T183/Y185-phosphorylated SAPKβ. The observation of increased CK1δ and S73-phosphorylated c-Jun in mSOD mice were not seen in ALS patients. Furthermore, we could not find any similarity in the expression profiles of PPs between mSOD mice and ALS patients. An elevated expression of protein phosphatase PTP1δ and a possible translocation of protein phosphatase KAP were found in ALS (Hu et al., 2003b), however, we observed decreased levels of PTEN, PP1/C-γ, PP5, PP2C/C-β but an increase of PP2B/A-α in mSOD mouse brain and/or spinal cord tissues. The specific roles of each of these PPs have not been determined.

In conclusion, differences in the profile of PKs, phosphoproteins and PPs expressed in the central nervous system of the mSOD mouse compared with control mice demonstrate a pattern that is dissimilar to that seen between ALS patients and controls. This indicates that the activation of these proteins in neuron death in the mSOD mouse is different from that seen in patients with the sporadic form of ALS.

CHAPTER 7
GENERAL DISCUSSION

The projects described here are different approaches to understand the cause of neuron death in ALS. For instance, I have evaluated the role of PKC activation in NMDA-mediated cytotoxicity to determine how altered PKC activity might be associated with cell death *in vivo*. Also, I have investigated whether there is an impairment in cell survival signaling pathways in ALS tissue. This has been done by measuring the expression levels of various PKs, phosphoproteins and PPs in postmortem spinal cord tissues from ALS patients, as well as in CNS tissues from G93A mSOD mice, an animal model of ALS. Furthermore, I have explored the fate of motoneurons in the lumbosacral spinal cord from mSOD mice, in order to determine whether SNB survives in mSOD mice the same way as their homologue, ON, does in patients with ALS. These findings should permit the identification of cell-specific proteins that influence whether motoneurons will survive, or die in ALS.

7.1 The role of signaling molecules in NMDA-mediated cell death

Considerable literature suggests that excitotoxicity is involved in the pathogenesis of ALS. I wished to examine the interaction between PKC activation and excitotoxicity and used a cell line (HEK) that I transfected with NMDA receptor subunits. The use of a cell line allowed the application of various combinations of wt and mutant NMDA receptor subunits. The HEK cells are a good model to use for such studies as they do not possess any endogenous NMDA receptors (Cik et al., 1993; Raymond et al., 1994). One

might argue that a kidney cell would not be a good model for studying the properties of neurons. However, these HEK cells possess most of the proteins that present in the nervous tissues of human and mouse (data from Kinetworks^{TM} screens of HEK cells, not shown). Also, a number of studies have already demonstrated that in HEK cells transfected with NMDA receptor subunits, there were NMDA-mediated currents, changes in the concentration of Ca^{2+} in cytoplasm, and cell death (Raymond et al., 1994, 1996; Grant et al., 1998), which are similar to what have been found in neurons upon NMDA receptor activation. I evaluated the death of HEK cells using trypan blue exclusion. Like other cell death assays such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay and lactate dehydrogenase (LDH) release assay, trypan blue exclusion is a simple and reliable assay and is still widely used (Friedman et al., 2003; Kamikubo et al., 2003; Koh et al., 2003).

The trypan blue exclusion cell death assays in HEK cells were performed 6 h after the cells had undergone various stimuli. This time point was chosen since the cytotoxic effect of NMDA exposure on NR1A/NR2A-transfected HEK cells was shown to be most evident at that time, as compared with non-treated cells. In preliminary studies I had measured cell death at different time points of 6 h, 12 h, 24 h, 36 h and 48 h after transfected cells were treated with NMDA, alone or together with a pre-incubation of PMA, or RO. The significant differences in cell death between treated and control groups tended to diminish at 12 h time points and vanish at 24 h after treatment (data not shown). At longer periods than 12 h after exposure of cells to NMDA, differences in cell death were less apparent between treated and control groups, with a tendency of decrease in the percentage of cell death in treated cells but an increase in non-treated cells. I interpret that

this effect is due primarily to proliferation of non-transfected cells in the cultures of treated cells. As indicated in Chapter 3, the transfection efficiency determined by β-gal staining was at the range of 60−80% and I estimate that less than 60% of HEK cells were successfully transfected with both NR1 and NR2 subunits. In the event that cells were not transfected with both subunits they would survive the exposure to NMDA and continue to proliferate. Thus, with increasing time in culture, cells not expressing NR1 and NR2 subunits would increase disproportionately and populate the culture. Evidence in favour of this possibility is provided by the observation of a 3−4% of cell death in NR1-transfected in HEK cells and the proliferation of these cells with time. Another possible reason for this decrease in difference of cell death between treated and control groups is that transfection itself is toxic, this explains why I have noticed an increase of cell death in non-treated NR1A/NR2A-transfected cells after 12 h. Also, it is likely that with time, cells might have developed some method to rescue themselves in transfected cells that were treated with NMDA, which could parallel what I have found in human ALS and mSOD mouse model of ALS.

Interestingly, these observations suggest that the whole process of NMDA receptor activation leading to cell death happens in less than 6 hours. Such a time period is similar to what has been reported in another study that the treatment of leukaemia cells with prostaglandin E1 induces the activation of cAMP pathway and thereby the apoptosis of leukaemia cells within 4−6 h after treatment (Ruchaud et al., 1997). The up-regulation of mRNAs for proteins that are involved in apoptosis is usually seen at 2−3 h after cells are undergone different pathogenic stimuli (Leff et al., 1996; Saitoh et al., 2001). Also, immediate early genes respond to external stimuli fairly quickly and they were seen to

up-regulate the expression of other proteins such as transcription factors as early as within less than 15 min (Blattner et al., 2000). Based on these previous findings, I conclude that it is very reasonable to measure the cell death at 6h after treatment and the significant changes in cell death between different groups of cells are believable. My data suggest a possible series of sequential events happening after cells underwent treatment, which include the activation of PK, phosphorylation and stimulation of nuclear factors including transcription factors, expression of early genes, and the up-regulation of proteins that contribute to cell death by early gene products.

Besides studying the effects of PKC activation, I also studied whether PI3-K and MEK had protective roles in NMDA-mediated cell death in HEK cells. The inhibition of PI3-K and MEK by the inhibitors, LY 294002 and PD 98059, respectively, did not induce cell death in NR1A/NR2A-transfected HEK cells. Both of these two inhibitors were applied for 20 min, either alone without NMDA, or prior to NMDA exposure, at a concentration of 25 μM (LY 294002) or 30 μM (PD 98059). The incubation with LY 294002 or PD 98059 alone by itself, or together with the exposure of cells to NMDA, had no significant cytotoxic effect on the transfected cells. Under the experimental conditions of this study, inhibitors of PI 3-K and MEK did not alter NMDA-mediated cytotoxicity. A possible explanation could be related to the absence of growth factors in the culture medium which might have activated the PI 3-K and MAPK pathways. However this is less likely the case since FBS was added to the culture medium. Another possibility is that PI3-K and MEK were already highly up-regulated in order to save the cells from dying, so the concentration of the inhibitors used may not have been high enough to exert any inhibitory effect, or the time of incubation was not sufficient for this cell type. Other

investigators have used higher concentrations and longer incubation periods of these inhibitors in a chick spinal cord motoneuron culture (Dolcet et al., 1999). Also, as the HEK cell line used in this study is of neoplastic origin, it may be less sensitive to drugs or inhibitors compared with neurons *in vivo* (see Tiffany-Castiglioni et al., 1999).

The postsynaptic protein PSD-95 and signaling molecule nNOS were of interest in my study since they are both closely associated with the NMDA receptor. PSD-95 binds to a conserved region on the C-terminus of NR2 subunit and associates with nNOS via a PDZ-PDZ interaction. It is very likely that PSD-95 translocates nNOS close to the NMDA receptor channel and causes the activation of nNOS by Ca^{2+} which fluxes through the activated NMDA channel. NO, the catalytic product by nNOS from arginine, is known to be a cause of oxidative damage of neurons (see review: Dawson and Dawson, 1996; Chabrier et al., 1999). However, PSD-95 may act as a double agent since PSD-95 itself protects cells from NMDA cytotoxicity, likely through decreasing the glutamate sensitivity of NMDA receptor channels and by inhibiting the potentiation of channel activity by PKC (Yamada et al., 1999b; Rutter and Stephenson, 2000). I studied the role of PSD-95 and NOS in NMDA-mediated cytotoxicity in HEK cells through co-transfecting cDNAs for PSD-95 with NMDA receptor subunits into HEK cells, and by using an inhibitor of NOS in transfected HEK cells. In some experiments, I found the co-expression of PSD-95 with functional NMDA receptors in HEK cells did not enhance or reduce NMDA-mediated cell death significantly, although there was a slight increase in cell death. A simple explanation for this non-significant increase of cell death is that it is the result of an elevation of cell stress caused by the over-expression of a third protein. However, this result may tells us that PSD-95 is more involved in indirectly causing the

oxidative stress by helping to stimulate the production of NO by NOS. A pre-incubation of cells with L-NAME, a nonselective NOS inhibitor, at the concentration of 100 μM 30 min prior to NMDA treatment did not reduce cell death in transfected HEK cells compared with cells only exposed to NMDA. Thus NO-induced oxidative damage does not seem to contribute to NMDA receptor toxicity. However, this might not be what happens in neurons. Since nNOS is neuron-specific and not present in HEK cells (Charles et al., 1993; Bischof et al., 1997; Schmidt et al., 2001), the use of a nonselective inhibitor L-NAME in HEK cells did not actually inhibit nNOS but the basal NOS. However, although NOSs are believed to be ubiquitous in living organisms (see Luckhart and Rosenberg, 1999). Bischof et al. (1997) did not detect NOS in HEK cells. Future studies could be done to carry on this investigation further by co-expressing PSD-95 and nNOS together with NMDR receptor subunits in HEK cells. But there is a possibility that the expression of too many proteins may induce a background toxicity, which will mask the effect of PSD-95 and/or nNOS on NMDA-mediated cell death.

7.2 Phosphorylation of adducin by PKs and the distribution of phospho-Adducin

An interesting observation in my study is that concomitant with an increase in expression of PKC and phospho-PKC in spinal cord tissues from ALS patients, there are considerable elevations of phospho-Adducin-α and -γ expression in ALS spinal cord. Also, the activity of PKC was reported to be elevated in ALS spinal cord (Wagey et al., 1998). As Adducin is a substrate for PKC, I suggest that this increase in Adducin phosphorylation is a direct consequence of PKC activation. The phosphorylation of cytoskeletal proteins may have important consequences on their properties. For instance,

following glutamate exposure, neurons overexpressing the intermediate filament protein, NF, develop cytoskeletal changes consisting of neurofilamentous aggregates and a slowing of axonal transport which was believed to be caused by the phosphorylation of the side-arm domains of NF by MAPK (Ackerley et al., 2000). Therefore, the phosphorylation of a cytoskeletal protein, Adducin, by PKC may affect the physiological function of Adducin so as to lead to neuron death in ALS.

Adducins α, β and γ are a group of membrane-associated cytoskeletal proteins that are involved in the assembly of the spectrin-actin network by cross-linking actin with spectrin (Gardner and Bennett, 1987; Li et al., 1998), and bundling and capping actin filaments (Mische et al., 1987; Kuhlman et al., 1996). These proteins are encoded by α, β and γ Adducin genes, with ubiquitous expression of α- and γ-Adducin and a more restricted expression pattern of β-Adducin (Gilligan et al., 1999). Each of these Adducin subunits has an N-terminal globular head domain, a neck domain, and a C-terminal protease-sensitive tail containing a MARCKS-related domain (Joshi and Bennett, 1990; 1991; Dong et al., 1995; Suriyapperuma et al., 2000).

The interaction of Adducin with spectrin and actin is regulated by calcium/calmodulin (Gardner and Bennett, 1987), and by several protein kinases via phosphorylation. A number of studies have shown that PKC, PKA, Fyn, rho-associated kinase (rho-kinase) and myosin phosphatase can phosphorylate or dephosphorylate Adducin (Matsuoka et al, 1996; Kimura et al., 1998; Matsuoka et al, 1998; Fukata et al., 1999; Shima et al., 2001). It has been found that Adducin associates with PKC using *in vitro* studies by techniques such as overlay assays and co-immunoprecipitation (Chapline et al., 1993; Dong et al., 1995; Laustsen et al., 2001). The RTPS-serine (Ser726) is the

major PKC phosphorylation site in the MARCKS-related domain of Adducin. Phosphorylation of Adducin by PKC induces its translocation from membrane to cytosol (Kaiser et al., 1989; Gilligan et al., 2002), as well as inhibiting the activity of Adducin in promoting spectrin-actin complexes as shown by both *in vitro* and *in vivo* studies (Matsuoka et al., 1998). Also, adducin phosphorylated by PKC has been shown to be cleaved by calpain during platelet activation, or by caspase-3 in renal epithelial cells undergoing apoptosis (van de Water et al., 2000; Gilligan et al., 2002).

Adducin has been implicated in a number of physiological roles such as promoting platelet aggregation (Gilligan et al., 2002), maintaining membrane stability in red blood cells (Gilligan et al., 1999) and membrane ruffling and cell motility in MDCK cells (Fukata et al., 1999). Moreover, mutations of β-Adducin are reported to trigger hypertension (Marro et al., 2000). A recent study done by Gruenbaum et al. (2003) has identified and characterized an *Aplysia* cytoskeletal protein that is homologous to mammalian Adducin, *Aplysia* adducin (ApADD). This novel protein distributes mainly in the submembraneous region of *Aplysia* neurons and is phosphorylated by PKC both *in vivo* and *in vitro*. It is spectulated that ApADD may contributes to structural changes during long-term facilitation (LTF) that are a common feature of long-term synaptic plasticity and memory (Gruenbaum et al., 2003).

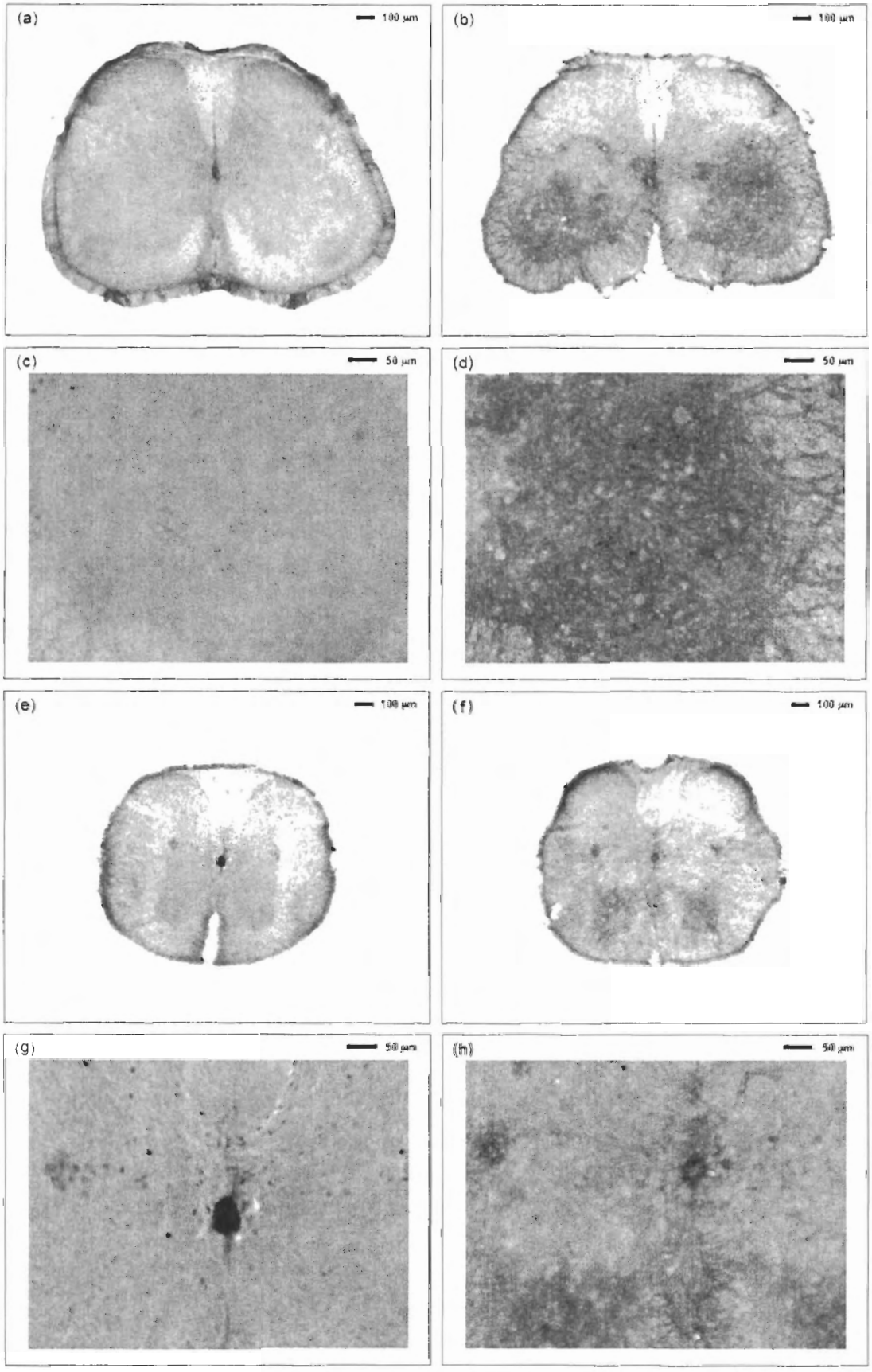
Given the close relationship between PKC and Adducin reported by previous studies and together with my own finding, I speculate that PKC phosphorylates Adducin in ALS spinal cord. In addition, I hypothesize that the phosphorylation of Adducin by PKC plays an important role in causing neuron death in ALS, likely by affecting the basic structural scaffolding of neuronal dendritic spines. To address this hypothesis, several

questions would need to be answered.

One question is the localization of Adducin in the CNS and what types of cells are responsible for the increase of phospho-Adducin expression in the spinal cord tissues from ALS patients. So far only a limited number of studies have examined the distribution of Adducin immunoreactivity. Seidel and colleagues (1995) studied the spatial and sub-cellular localization of α-Adducin in rat brain and found that α-Adducin is highly localized to regions with high synapse densities, such as the hippocampus, cerebral cortex and cerebellum. The sub-cellular localization of α-Adducin is distinct in hippocampus and cerebellum, with observations of α-Adducin immunoreactivity in dendrites and dendritic spines in the CA1 and CA3 regions of the hippocampus, while immunolabelling of α-Adducin is found in pre-synaptic terminals of parallel fiber terminals in the molecular layer of the cerebellum (Seidel et al., 1995). Seidel et al. (1995) also found significant immuno-labelling of α-Adducin in the processes of glial cells, both in hippocampus and cerebellum. Moreover, high levels of phospho-Adducin were detected in the dendritic spinces of cultured hippocampal neurons (Matsuoka et al., 1998). The α-isoform of adducin is also present in the PSD regions of rat and a decrease of α–Adducin was observed following excitotoxic stimulus (Wyneken et al., 2001). The β1-Adducin subunit is highly expressed in cerebellum and DRG tissues of rat (Ghassemi et al., 2001). Also, the expression of β-Adducin can be developmentally up-regulated by trophic factors like NGF and glial-derived neurotrophic factor (GDNF) (Ghassemi et al., 2001). There does not appear to be any previous immunolocalization studies of Adducin in spinal cord although the gene encoding γ-Adducin was found to be enriched in the dorsal horn of rat undergone persistent nociceptive input (Yang et al., 2001).

I have collected preliminary data on the localization of Adducin in spinal cord by immunohistochemical labeling of phospho-Adducin in human and mouse spinal cord tissues. In these limited experiments I did not detect any immunoreactivity for phospho-Adducin in the spinal cord from either ALS patients or control subjects. One explanation for these negative findings may be that the sections were fixed in PFA solution for long time periods (e.g., > 2 years) and this long period of fixation may have altered the epitope sites of the antigen. The distribution of phospho-Adducin immunoreactivity in murine spinal cord is shown in Figure 21. Immunoreactivity for phospho-Adducin is distributed in a generalized manner within the grey matter but markedly present in the superficial regions of the dorsal horn (lamina I; substantia gelatinosa) (Fig. 21a and e). Immunoreactivity was also observed in the cell bodies of the intermediolateral cell column (IML), as well as cells in the central canal (lamina X) (Fig. 21e and g). These findings were seen at various segments including cervical, thoracic, lumbar, and sacral levels, however only examples of lumbar (Fig. 21a) and thoracic (Fig. 21e) cords are shown. The differences in the pattern or intensity of phospho-Adducin labelling between mSOD and control mice were also studied. As shown in Figure 21a-g, there was an increased density of immunoreactivity of phospho-Adducin in mSOD spinal cord and this immunolabelling in mSOD mouse appeared in a more diffuse pattern compared with control mouse. Specifically, there was stronger immunoreactivity in mSOD mouse in the regions of lamina I, lamina VII, the central canal and ventral horns (Fig. 21b, d, f and h). Also, in some cases the immunolabelled cells appeared to have a neuronal morphology although I did not perfrom immunolabelling using neuron-specific markers. I also tried examining the immunolabelling of phospho-Adducin in the DRG of control and mSOD

Figure 21: The distribution of phospho-Adducin immunore activity in murine spinal cord.
The lumbar and thoracic sections of spinal cords from control (left) and mSOD mice (right) were immunolabelled with rabbit anti-phospho-Adducin antibody. (a and b) Lumbar sections of control (a) and mSOD (b) spinal cord; (e and f) Thoracic sections of control (e) and mSOD (f) spinal cord; c, d, g and h are regional magnifications of a, b, e and f, respectively.


(a)
100 µm
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100 µm
(c)
50 µm
(d)
50 µm
(e)
100 µm
(f)
100 µm
(g)
50 µm
(h)
50 µm

mice. As shown in Figure 22, phospho-Adducin was observed to be plasma membrane-associated as well as present in the cytosol of cells in the DRG. There seemed to be a trend of both an increase of protein levels and an elevated distribution of phospho-Adducin in the cytosol or even the peri-nuclear region in mSOD mouse compared with controls (Fig. 22b compared with a). This observation of a distribution of phospho-Adducin in DRG is consistent with a previous report that the β1 isofrom of Adducin is present in rat DRG (Ghassemi et al., 2001). To determine the cellular localization of Adducin in mouse spinal cord, further work needs to be done by doing double immunolabelling of phospho-Adducin with cell-specific markers such as the neuronal marker, NeuN, the microglial marker, Mrf-1 and the astrocyte marker, GFAP.

Another question I wish to address is whether PKC phosphorylation of Adducin really results in cell death. To pursue this issue, I could have performed experiments to study whether cell death induced by PKC activation are related to the phosphorylation and translocation of Adducin. This can be done by by using primary cultures of neurons, or cell lines. In preliminary results I found that PKC activation in HEK cells induces an increase of protein levels and also the translocation of phospho-Adducin from plasma membrane to cytosol (Fig. 23a). These data are similar to the findings of Matsuoka and colleagues (1998). Furthermore, the transfection of NMDA receptor subunits into HEK cells also causes similar changes in adducin to that of PKC activation (Fig. 23b). These observations are interesting and indicate that Adducin may be involved in NMDA-mediated cytotoxicity. However, this assumption is just based on limited preliminary data and needs to be validated by future studies.

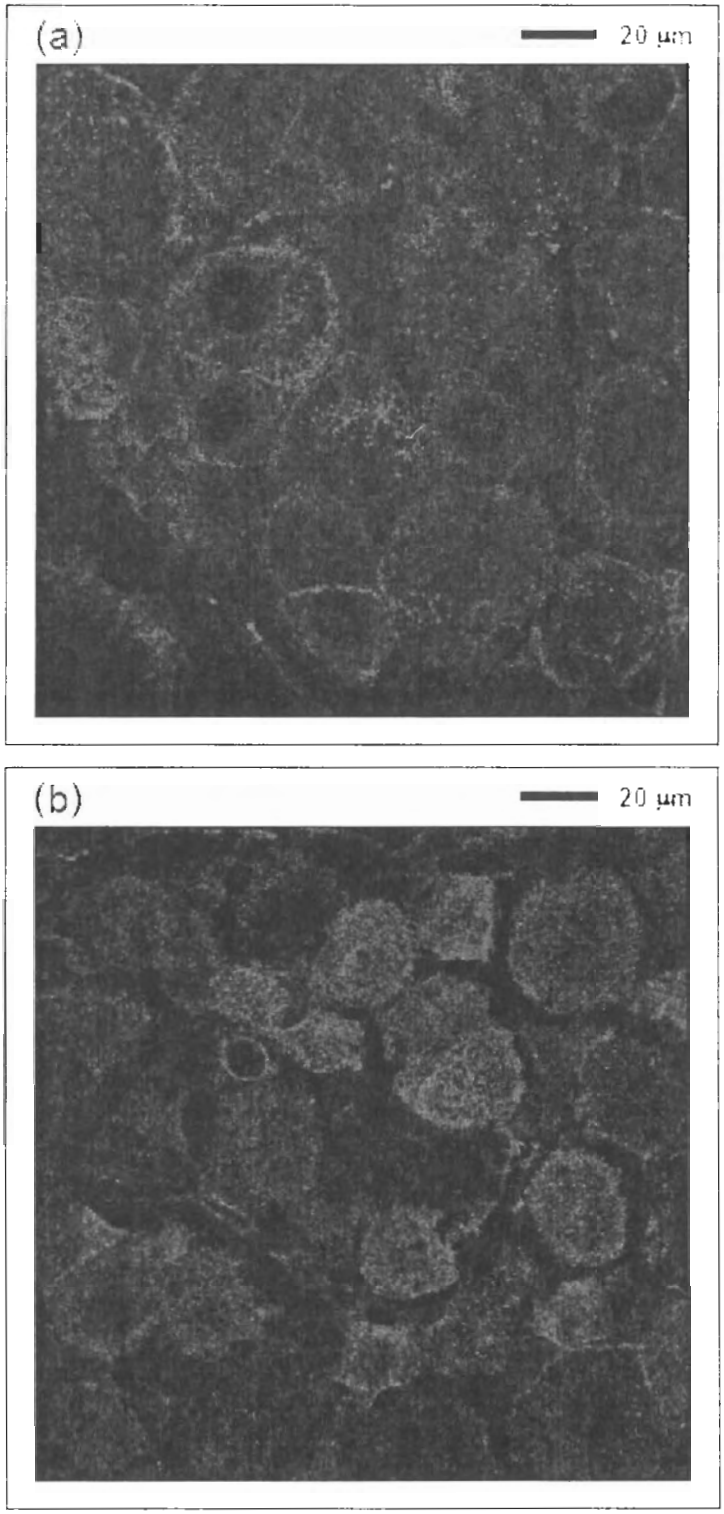

Figure 22: Immunolabelling of phospho-Adducin in dorsal root ganglion (DRG) of control and mSOD mice.
(a) control; and (b) mSOD.

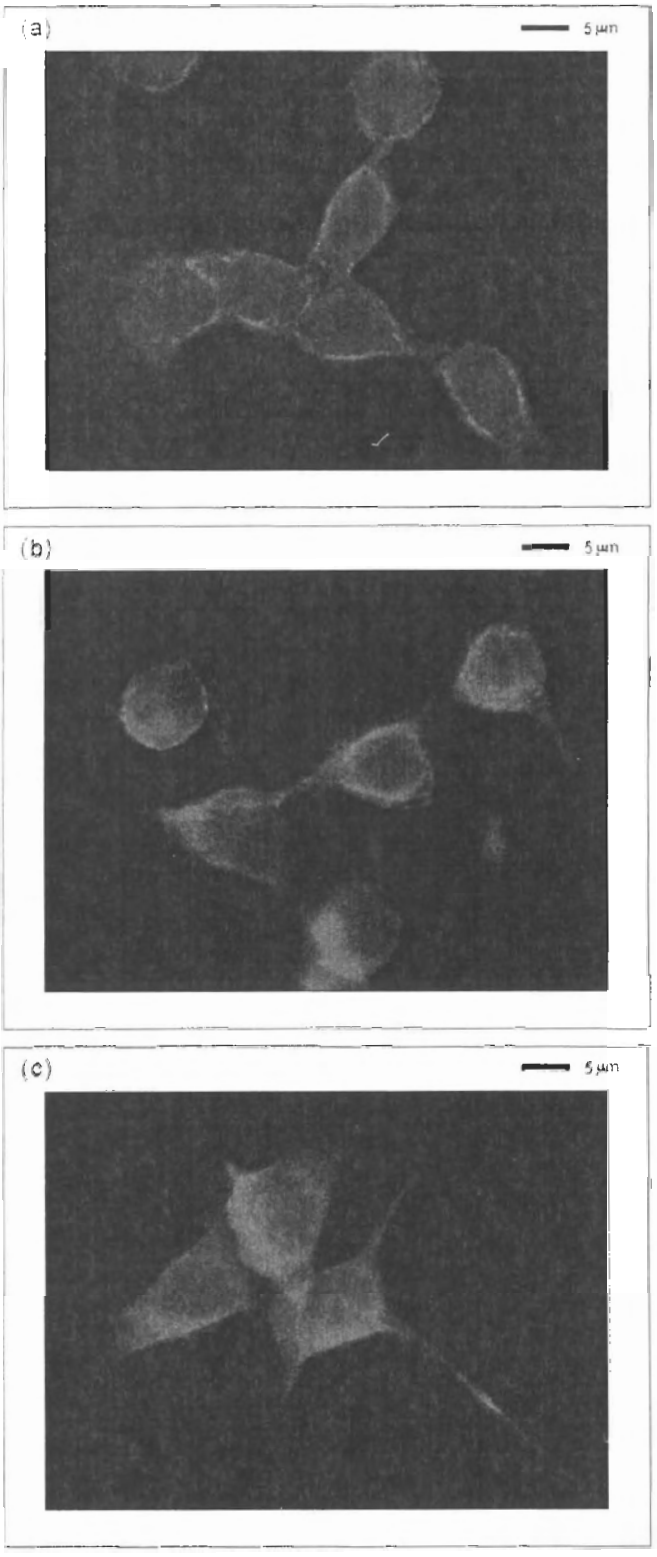

Figure 23: Changes in distribution and protein levels of phospho-Adducin in HEK cells.
(a) non-transfected and non-treated; (b) non-transfected but treated with PMA; (c) NR1A/NR2A-transfected but non-treated.

7.3 Advantages and limitations of human postmortem tissues and models of ALS

To examine the role of PKs and PPs in ALS, I have used human postmortem tissues, cell lines, and the mSOD mouse model of ALS. The value of doing studies using a variety of systems is that it can minimize the limitations from any particular model system.

An important factor relevant to the analysis of studies on post-mortem tissue is the effect of changes in protein expression with different DF intervals. A previous study in our lab has already examined this issue by evaluating protein expression and activity in the rat CNS at different times following death. This study was not intended to provide quantitative data on the relation between the DF interval and the expression levels and activities of a few PKs such as PKC in the rodent CNS, but rather to determine the extent to which changes in kinase expression and activity would be expected in the human CNS at postmortem, by using the rodent CNS as a model. It was observed that there were differences in the rates of decline in the expressions and activities of PKs after death (Hu et al., 2003b; Wagey et al., unpublished data). The decline in expression and activity of the same PK varies in different tissues as well. For instance, the protein level of S6K had a 10% reduction in brain and a 50% decrease in spinal cord at 3h DF interval, respectively. The activities of PK seemed to be more stable compared with the protein level of PKs, for example there was only 15% and 25% reduction in the activity of PI3-K in rat brain and spinal cord at 6 h DF interval, respectively. As a general observation, most of the PKs evaluated in rodent CNS do not have more than a 50% reduction in expression and activity within 6 h after death. We acknowledge that specific PKs or their activators can demonstrate changes in expression after death (Taniguchi et al., 2001) and

that we have not evaluated the effects of varying postmortem intervals in the expression of a number of PKs and PPs that demonstrate differences between ALS and control spinal cord tissue. However, the results from the study in the rodent CNS indicate that PK expression in postmortem human tissue does reflect the levels present during life, especially following short (< 6 h) DF intervals. Undoubtedly, measurement of PK expression in humans could be further complicated by medication effects, effects of chronic illness or pre-terminal hypoxia (a major problem in ALS). Furthermore, aging may also change expression levels of a number of PKs, PPs and phosphoproteins. Combining these DF results with our data from ALS spinal cord tissue, we demonstrate that a large variety of PKs can be detected in postmortem spinal cord and that spinal cord tissue having short DF intervals is reasonably representative of the PKs, PPs and their substrates present during life. Given these limitations, studies such as these that examine PKs in postmortem tissues from ALS patients only give indications of the pathology of ALS at the late-stage of the disease.

The pros and cons of using HEk293 cells have been already addressed in the discussion part of Chapter 3. Although HEK cells are not neurons and they may do not have exactly the same cellular components as what neurons have, my research findings from HEK cells together with previous studies by other researchers have demonstrated that HEK cell is an appropriate model for studying the function of NMDA receptor and the involvement of PKC in NMDA-mediated cytotoxicity. One benefit of the HEK cell model is that it is useful for studying the roles of different NMDA receptor isoforms, by transfecting different combinations of cDNA constructs of NMDA receptor subunits into HEK cells. Also, because of the homogeneity of HEK cells, this model is easier to use

for biochemical measurements and cell death assays than when using primary cultures having multiple cell types present in the culture.

Promising advances in the understanding of the pathogenesis of ALS have come with the creation of murine models of ALS. In particular, transgenic mice overexpressing mSOD to various degrees have been used extensively for studies relevant to ALS. The use of these models has been considerable. Especially popular have been the G93A overexpressing mice created by Gurney and colleagues (1994). The intense interest and use of these mice to understand ALS has made criticism of these models almost heretical. However, several major concerns can be made regarding the relevance of these models for ALS. First, because mice express various proteins differently from humans, one might reasonably expect differences in the profiles of protein kinases, or indeed other proteins, between humans and mice. Our data have shown that there are many differences in the profiles of PK, PP and phosphoprotein expressions in spinal cord tissue between control or mSOD overexpressing mice and human tissue (Hu et al., 2003a). This might have profound consequences for the nature and extent of activation of PK pathways triggered by stimuli in these two species. Second, it has been reported that elevated expressions of wt SOD1 in mice can be associated with pathological features and possibly motor impairments in aged animals (Jaarsma et al., 2000). Thus, pathological effects might be a consequence of the overexpression of a protein, and not a function of the underlying protein in 'physiological' amounts. Furthermore, recent work has shown that mSOD overexpressing mice have changes in the activities of the PP calcineurin (PP2B) that are probably a direct consequence of the overexpression of mSOD protecting calcineurin from inactivation (Wang et al., 1996; but see Ferri et al., 2001) (S. Li *et al.*, unpublished).

A third limitation of murine studies of ALS lies in the differences of the corticospinal pathways in humans and mice. Because ALS is associated with degeneration of neurons in corticospinal tract pathways, it might be reasonable to expect differences in the loss of these pathways between humans, who have a relatively well developed corticospinal tract, and mice, who have more limited pathways (Eisen et al., 1992; Zang and Cheema, 2002). Yet another concern is that several drugs that prolong the survival of mSOD transgenic mice failed to show any benefits for patients in clinical trials (Appel et al., 1988; Klivenyi et al., 1999; Andreassen et al., 2001; keep et al., 2001; Groeneveld et al., 2003). Taken together we suggest that the findings from transgenic mouse model of ALS should be cautiously interpreted.

7.4 PI3-K/PKB and MEK/ERK

My original hypothesis was that both PI3-K/PKB and ERK cell survival signaling pathways are impaired or down-regulated in the nervous system of patients with ALS and mSOD mice. PI3-K/PKB and ERK signaling pathways have been well known for their role in neurotrophin-regulated cell survival. The supporting evidences are numerous. To name a few, PI3-K/PKB signaling has been reported to contribute to neural cell adhesion molecule-mediated neuronal survival (Ditlevsen et al., 2003), the neuroprotection against glutamate cytotoxicity mediated by dopamine D2 receptor activation (Kihara et al., 2002), the insulin-like growth factor (IGF) 1-induced survival of cultured hippocampal neurons (Zheng et al., 2002), and its dual role in preventing injury-caused motoneuron death and promoting axonal regenerationg (Namikawa et al., 2000). Also, ERK pathway was found to play a role in protecting cultured spinal neurons against NO toxicity by prostaglandin (Kikuchi et al., 2002), in promoting neuronal survival under hypoxia in

primary cultures from murine cerebral cortex (Jin et al., 2002), and in regulating the neurotrophic effect of epidermal growth factor (EGF) in cultured rat hippocampal neurons (Abe and Saito, 2000). Comparing these two pathways, PI3K signaling is more important since it is believed to be responsible for 80% of neurotrophin-related cell survival (see Kaplan and Miller, 2000). Nonetheless, not all studies support the conclusion that PI3-K/PKB and ERK pathways are neuroprotective. PKB activation was found to be associated with neurotoxicity in a *Drosophila* model of spinocerebellar ataxia (Chen et al., 2003; Emamian et al., 2003). In this animal model, PKB was demonstrated to phosphorylate ataxin-1 and promote its binding to the 14-3-3 protein, which stabilizes ataxin-1 and slows its normal degradation and thereby induces neurodegeneration. Furthermore, ERK can also be associated with neuronal cell death with the evidence that glutamate-induced oxidative toxicity is attributed to activation of ERK in primary cortical neuron cultures and a hippocampal cell line (Satoh et al., 2000; Stanciu et al., 2000). As most of the existing evidence supports a close relation of these two pathways to neuronal survival, I expected that an impairment or down-regulation of these pathways would be found in human ALS and mSOD mouse. However, our data showed that the protein levels of PKBα, phospho-S6K, ERK2 and RSK1 were elevated in ALS spinal cord compared with controls, and these increases are quite remarkable. The changes of these two pathways in mSOD mouse are not as significant as that seen in human ALS. We observed increased expression of Raf1, ERK3, ERK6, and decreased protein level of S6K in mSOD spinal cord. Together with previous findings of increased activity and protein level of PI3-K in ALS spinal cord tissue (Wagey et al., 1998), we believe that both PI3-K/PKB and ERK pathways are activated and up-regulated in ALS. We assume these

changes are a response of cells to pathogogical stimuli which attempt to rescue neurons from death. This may indicate why trophic factors such as ciliary neurotrophic factor (CNTF), growth hormone and BDNF are not very promising in the treatment of ALS (Smith et al., 1993; Miller et al., 1996; the BDNF Study Group, 1999). As the cell survival pathways have already been highly activated, the administration of trophic factors will not further protect neurons by activating cell survival signaling.

7.5 Potential therapeutic agents

Our findings of abnormal expression levels of protein kinases in the CNS tissues from ALS patients and G93A mSOD transgenic mice may shed light on the development in new drugs for the treatment of ALS. Over the last decades, studies on therapeutic interventions for ALS have been expanding providing accumulating evidence for multiple causes of ALS. The therapeutic approaches that have been used in mSOD transgenic mouse models include pharmacological treatments, transgene overexpression, gene knockout, gene therapy and stem cell use (for examples, see Gurney et al., 1996; Hottinger et al., 1997; Kostic et al., 1997; Azzouz et al., 2000; Ende et al., 2000; Kong and Xu, 2000; Kuntz et al., 2000; Li et al., 2000; Keep et al., 2001; Kriz et al., 2002; Subramaniam et al., 2002; Sun et al., 2002; Kriz et al., 2003). As for the pharmacological treatment, a variety of molecules have been tested which can be grouped into anti-inflammatory agents, anti-apoptosis molecules, anti-glutamatergic compounds, antioxidants, regulators for metal ions and energy metabolism, trophic factors, and others. Among them, some of have been shown to prolong the survival of mSOD mice but have no effect on the onset of the disease, with riluzole as an example which acts possibly through inhibiting glutamate release or enhancing the high-affinity glutamate uptake

(Azbill et al., 2000; Gurney et al., 1996; Martin et al., 1993). However, some other compounds, for instance, the antioxidant vitamin E, can delay the onset of motoneuron disease but do not increase survival. Moreover, a few other molecules such as a copper chelator, d-penicillamine, not only delay the onset of disease but also extend survival of mSOD transgenic mouse (Hottinger et al., 1997).

Similar studies on pharmaceutical therapy have also been done in ALS patients by doing double-blinded, placebo-controlled trials but most of these have failed to demonstrate efficacy. One reason is that most of the drugs that have been found to be efficacious in mouse models of ALS were administrated before symptom onset, which is not feasible in SALS patients. Nonetheless, the glutamate antagonist, riluzole, did appear to improve the survival of ALS patients, although the degree of improvement is small with a marginal amelioration in patients with limb-onset disease but a better effect on bulbar-onset cases (Bensimon et al., 1994; Lacomblez et al., 1996). In 1995, riluzole was approved by the Food and Drug Administration (FDA) and it is the only drug that has been approved for the treatment of ALS.

We speculate that the inhibitors of PKs can retard or block the progression of motoneuron disease. An encouraging finding in favor of our hypothesis is that WHI-P131, a specific inhibitor of JAK3, increases the survival of G93A mice by more than two months (Trieu et al., 2000). Many of the protein kinase abnormalities evident in the ALS patients and mSOD mice might have an effect on the course of neurodegeneration. Inhibitors of PKCs are attractive new candidate drugs for treatment of ALS since my study has shown increased levels of PKC-α, β and ζ in ALS and PKC-δ and μ in mSOD spinal cord, compared with controls. The cPKC isoforms of this family (PKC-α, βI βII

and γ) generally have anti-apoptotic actions in many cell types against oxidative stress-induced cell death (Maher, 2001). Cleavage of the PKCδ isoform has also been linked to apoptotic death of neurons following oxidative stress and KCl-deprivation (Villalba, 1998; Anantharam et al., 2002). The proteolysis of PKCδ can be triggered by caspase-3 (Anantharam et al., 2002). We failed to detect PKC-δ in both the ALS and control spinal cords, but observed an increase of PKCδ in the mSOD mouse. It is of interest that recent investigations into the cause of diabetic neuropathy, a disorder of motor and sensory peripheral nerves associated with diabetes mellitus, have also identified increased activation of PKC in these tissues (Brownlee, 2001). In diabetic neuropathy, PKC activation might be secondary to increased superoxide production (Brownlee, 2001). Ruboxistaurin mesylate, a selective PKC inhibitor developed by Lilly Research Labs for treatment of diabetes, has undergone positive phase I clinical testing for diabetic neuropathy and might be a promising candidate for use in ALS patients.

Besides PKC, GSK3α/β, PKR and p38 MAPK are other good candidates for inhibition by pharmaceuticals since there are increased protein levels of phospho-GSK3α/β, GRK2, phospho-PKR in human ALS and elevated expression of both p38 MAPK and phospho-p38 in mSOD mouse spinal cord. Inhibitors of GSK3 are likely to have beneficial effect on the treatment of ALS since GSK3 was shown to be involved in cell death caused by growth factor withdrawl (Somervaille et al., 2001). Also, selective inhibitors of GSK3 have protective effects on neurons in both CNS and PNS (Cross et al., 1995). PKR is believed to induce stress-induced apoptosis in mammalian cells, which involves the down-regulation of protein synthesis by phosphorylating translational factor eIF-2α (Levin and London, 1978) and activation of the transcriptional factor NF-κB

(Kumar et al., 1994). Protein synthesis plays an important role in the viability and function of the cell. Also, the activation of PKR was found to induce apoptosis through FADD-mediated death signaling, by upregulating caspase 8 in a manner independent of Fas and TNF-alpha receptors (Balachandran et al., 1998; Gil and Esteban, 2000). Based upon these studies of PKR, we propose that an increase in expression of phospho-PKR in both cytosolic and microsomal fractions of ALS spinal cord may be relevant to the neuronal death in ALS with an apoptosis-inducing role of PKR. In the mSOD spinal cords, we noticed an increase in the level of p38 MAPK protein and elevations in the amount of active, phosphorylated p38, although such changes were not evident in our studies of the protein kinases in human ALS spinal cord. This activation of p38 MAPK in mSOD mouse was also observed by another group very recently. Tortarolo et al. (2003) reported that increased levels of phospho-p38 MAPK were present in spinal motoneurons and then in reactive glial cells along disease development. This may indicate a role of p38 MAPK in leading to neuron death in ALS since it was shown to be apoptotic in rat PC-12 pheochromocytoma cells undergone NGF withdrawal (Xia et al., 1995). However, this elevation of p38 MAPK might be part of a repair or distress signal from affected glial cells, such as microglia that could contribute to inflammation (Kriz et al., 2002). Microglia of mSOD overexpressing mice demonstrate greater immunoreactivity for p38 MAPK than do controls, which can be attenuated with the antibiotic minocycline (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002).

7.6 Conclusion and future studies

The cause of ALS has not yet been determined. However, it is believed that ALS is a heterogeneous disorder in which a variety of relatively distinct initiating factors such

as mutations in *sod1* gene, or other factors, lead to clinical manifestations by triggering a common cascade of 'downsteam' processes, ultimately resulting in neuron death. Figure 24 presents a schema of possible events leading to motoneuron death in ALS. My study has demonstrated that glutamate action on NMDA receptors causes cell death in a HEK cell line model of ALS, and that the activation of Ca^{2+}-dependent isofroms of PKC potentiate this NMDA-mediated toxicity, possibly throught its action at the C-terminus of NR2A subunit of NMDA receptor. Thus my work supports a connection between glutamate stimulation and the activation of phosphorylation of (Fig. 24). Stimulation of NMDA receptors induces an influx of Ca^{2+} through the NMDA receptor channel, which in turn produces an increase of cytosolic free calcium and thereby activates Ca^{2+}-dependent enzymes including PKC. Therefore, the stimulation of PKC by NMDA receptor activation exerts positively feedback on NMDA receptor cytotoxicity, which is speculated to be one of the causes of motoneuron death in ALS. Also, I have shown that abnormal protein phosphorylation is present in nervous tissues from patients with ALS and G93A mSOD mice. The aberrant regulation of PKs and PPs may be caused by oxidative toxicity and the mutation in *sod1* is one of the contributors. Consistent with my observation of involvement of PKC in NMDA-mediated cytotoxicity in HEK cells, Ca^{2+}-dependent isoforms of PKC were found to be up-regulated in both expression and activity in ALS tissue. These activated PKs and PPs could have injurious consequences on cell survival or even cause cell death by phosphorylating or dephosphorylating cellular substrates. It is suggested that cytoskeletal proteins such as NF and adducin, and NMDA receptors are several possible targets of these aberrantly activated PKs such as PKC (Fig. 24).

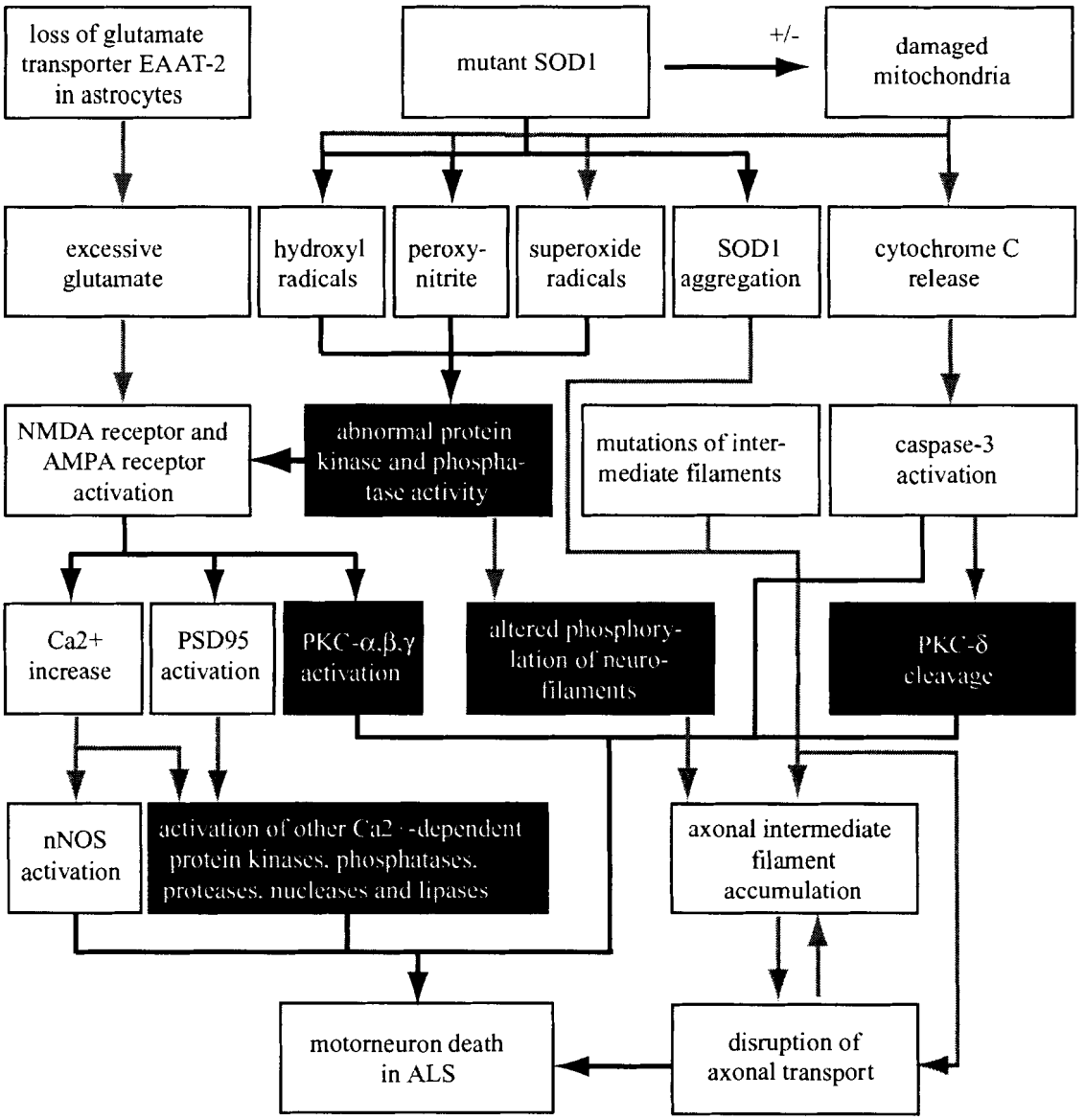

Figure 24: Hypothesized events leading to motoneuron death in ALS.
A variety of relatively distinct initiating factors such as loss of glutamate transporter function, mutations in the superoxide dismutase 1 (*sod1*) gene, or mitochondrial damage trigger a cascade of downstream processes producing motoneuron death through protein kinase activation. Events represented in filled rectangles associated with protein phosphorylation. Arrows indicate activating pathways. Abbreviations: EAAT-2, excitatory amino acid transporter-2; NMDA, N-methyl D-aspartate; PSD95, post-synaptic density 95 protein; PKC, Ca^{2+}-dependent protein kinase C; nNOS, neuronal nitric oxide synthase; +/-, mutations in SOD may act directly to damage mitochondria.

Some potential future studies have already been discussed in earlier sections. For example, the role of PKC-induced phosphorylation of Adducin in ALS needs further evaluation. I would also extend my preliminary data on the distribution of phospho-Adducin as well as different isoforms of Adducin. A further extension of my work would include the identification of the cell types manifesting various PKs and PPs that are overexpressed in the CNS tissues from mSOD mouse and spinal cord tissues from ALS patients, compared with controls, respectively. I would perform double-labelling to study the cell-specific changes in expression of a number of PKs such as PKC, GSK3α/β, PKR, which are believed to be related to cell death. The PKs that are involved in PI3-K/PKB and MEK/ERK cell survival pathways also need to be examined, so as to determine whether the up-regulation of these two pathways really happens in degenerating neurons. As phospho-p38 is a marker of activated microglia, the observation of an increase of protein levels of p38 MAPK and the active, phosphorylated p38 in the spinal cords of mSOD mice has raised the question of how microglia are involved in the pathogenesis of ALS. To address this issue, future studies could be done to investigate the relationship between microglial infiltration and neuronal damage, by transplanting green fluorescent protein (GFP)-expressing bone marrow cells that differentiate into microglia into G93A mSOD mice. Whether microglia contribute to neuronal loss could be explored by investigating if microglia infiltration precedes motoneuron loss in mSOD mice and by observing the anatomical distribution of the microglial infiltration in relation to dying neurons.

APPENDIX A

A list of the expression levels of each protein kinase that were screened using Kinetworks™ KPKS 1.0 in the spinal cord tissues of five control and five mSOD mice.
This gives an example of the variations in protein expression levels between each individual animal even in the same experimental group, which explains the presented large standard deviation when the differences in protein expression were compared between control and mSOD mice. SC: spinal cord; Ctrl: control; mSOD: mutant superoxide dismutase.

Kinase	SC Ctrl #1	SC Ctrl #2	SC Ctrl #3	SC Ctrl #4	SC Ctrl #5	SC-Ctrl Mean	SC mSOD #1	SC mSOD #2	SC mSOD #3	SC SOD #4	SC SOD #5	SC-mSOD Mean
BMX (75)	3505	4073	3605	1790	1202	2835	3889	3542	2360	1167	2906	2773
BTK (65)	0	0	0	0	0	0	0	0	0	0	0	0
CaMK1 (37)	0	2229	0	0	0	446	0	0	0	0	0	0
CaMK4 (62)	7791	8255	7863	1538	4466	5982	0	0	0	3439	1998	1087
CaMK4 (64)	0	0	0	0	0	0	3989	8874	5701	0	0	3713
CaMKK (52)	0	0	0	8348	7666	3203	0	6549	0	6884	7042	4095
CaMKK (56)	11072	7712	5664	0	0	4890	5801	5481	17818	0	0	5820
CDK1 (30)	0	0	0	0	0	0	0	0	0	0	0	0
CDK2 (31)	0	0	0	0	0	0	0	0	0	0	0	0
CDK4 (31)	0	0	0	0	0	0	0	0	0	0	0	0
CDK4 (38)	6202	3463	8292	0	0	3591	0	6501	6360	0	0	2572
CDK5 (29)	37483	31108	15626	65898	59543	41932	37838	36370	19873	45509	51970	38312
CDK6 (35)	5701	5157	6290	0	0	3430	4235	4430	3173	0	0	2368
CDK7 (37)	7572	6133	7708	4023	1510	5389	14435	8435	4325	1393	10009	7720
CDK9 (36)	15527	11109	12348	8697	4798	10496	13303	11549	5844	1772	14429	9379
CK1d (39)	3368	0	0	2562	1394	1465	2054	4437	3994	5253	3103	3768
CK1e (35)	2359	1648	0	1121	1210	1268	1160	2506	0	1074	1085	1165
CK2 (35)	17736	18237	26908	4650	3353	14177	8329	13503	5451	2532	14675	8898
CK2 (38)	9001	8237	11209	20547	21799	14159	20886	19619	6021	15326	29663	18303
CK2 (40)	27578	33546	38892	43619	44907	37708	43002	29064	39751	36330	41458	37921
COT (52)	21137	12808	10736	10849	6751	12456	22916	5558	14093	15549	5019	12627
CSK (44)	3877	4196	4012	3359	1533	3396	2300	3298	0	3523	2924	2409
DAPK (161)	0	0	0	2743	1669	883	1395	0	0	1213	1936	909
DNAPK (460)	0	0	0	0	0	0	0	0	0	0	0	0
ERK1 (42)	52089	56174	50195	69318	83578	62271	41242	50522	83365	118057	51434	68924
ERK2 (38)	23455	29079	28732	0	0	16253	11108	26084	33042	0	0	14047
ERK3 (56)	7718	6366	14809	4946	2671	7302	9686	25651	24972	10083	8578	15794
ERK3 (61)	9663	12552	19495	26928	21577	18043	3804	9285	9996	44533	31172	19758
ERK3 (64)	10967	15418	20264	38772	27827	22650	0	0	0	58176	39677	19571
ERK6 (47)	20532	15558	15320	8547	11610	14313	19773	18182	15675	27782	24111	21105
FAK (125)	3452	6778	5261	1910	8957	5271	10014	10952	11641	1636	3932	7635
FYN (46)	21400	10877	14084	0	0	9272	8004	9143	5808	0	0	4591
FYN (48)	23615	22763	17982	0	0	12872	11095	19748	20846	0	0	10338
GCK (92)	0	0	2870	0	0	574	0	3477	0	0	0	695
GRK2 (74)	16179	23460	24809	36676	45946	29414	11055	17225	20814	32705	25142	21388
GSK3a (45)	11112	9560	9854	6955	10176	9531	4274	10501	5501	3817	12682	7355
GSK3b (41)	5243	0	0	31044	20308	11319	5711	7129	0	26234	26687	13152
GSK3b (42)	7178	4747	7765	1708	1484	4576	10759	5782	0	2690	4783	4803

	SC	SC	SC	SC	SC	SC-Ctrl	SC	SC	SC	SC	SC	SC-mSOD
Kinase	Ctrl #1	Ctrl #2	Ctrl #3	Ctrl #4	Ctrl #5	Mean	mSOD #1	mSOD #2	mSOD #3	SOD #4	SOD #5	Mean
HPK1 (90)	6491	7859	10711	6326	2934	6864	24047	6642	11035	3480	4161	9873
IKKa (83)	0	0	0	13735	8464	4440	0	0	0	10505	18524	5806
JAK1 (122)	8388	8184	14974	6391	5846	8757	11350	5763	3061	7295	15555	8605
JAK2 (112)	0	0	3058	1792	3221	1614	2967	3303	3401	992	4213	2975
Ksr1 (97)	2820	0	2431	19230	16902	8277	5127	0	4047	13771	27650	10119
KSR1 (97)	2349	0	2380	2121	860	1542	3048	0	2443	958	1611	1612
LCK (46)	0	0	0	0	0	0	0	0	0	0	0	0
LYN (45)	0	0	0	0	0	0	0	0	0	0	0	0
MEK1 (42)	11670	11531	12814	23245	19570	15766	11497	9512	4652	18184	27557	14280
MEK2 (43)	7585	7077	7371	0	0	4407	6911	5653	6040	0	0	3721
MEK2 (42)	7774	6786	7763	5132	5586	6608	6567	5714	0	3459	6758	4500
MEK4 (36)	24994	0	0	0	0	4999	0	0	0	0	0	0
MEK4 (38)	39777	73264	59844	111413	97562	76372	77773	56255	82244	65224	77504	71800
MEK6 (34)	24073	29398	29937	17932	13711	23010	31946	29643	11430	8923	29777	22344
MEK7 (39)	0	0	0	0	0	0	0	0	0	0	0	0
MNK2 (55)	0	0	0	0	0	0	0	0	0	0	0	0
MOS1 (35)	45304	53241	50342	28733	13526	38229	44484	46687	87510	20112	44026	48564
MST1 (57)	0	0	0	0	0	0	0	0	0	0	0	0
NEK2 (57)	0	0	0	1502	2331	767	0	0	0	1871	958	566
p38 MAPK (39)	20493	18274	19539	17189	15085	18116	23549	27417	34631	17700	33130	27285
PAK1 (68)	13002	21091	19339	14438	10061	15586	16308	13327	6057	5833	10910	10487
PAK1 (73)	0	0	0	0	0	0	0	0	0	0	0	0
PAK3 (65)	17893	6553	28230	0	0	10535	22605	22727	8406	0	0	10748
PDK1 (58)	0	0	0	0	638	128	0	0	0	1208	0	242
Pim1 (37)	7764	0	6814	0	0	2916	6028	10256	4499	0	0	4157
PKA (38)	4568	4447	0	6553	3416	3797	0	4605	3735	24537	20857	10747
PKBa (59)	26007	30040	20351	18095	17284	22355	9121	24388	23043	20579	17212	18869
PKBa (62)	0	0	14031	5278	7971	5456	0	0	0	10476	10340	4163
PKCa (83)	7872	4690	7766	20248	27440	13603	0	6574	10201	37098	30488	16872
PKCb (83)	33652	47223	61062	0	0	28387	57743	61582	95121	0	0	42889
PKCb (89)	35745	44901	0	99679	102916	56648	0	12701	0	103495	83809	40001
PKCd (80)	0	10524	0	6081	4540	4229	12291	6309	6179	8042	14238	9412
PKCe (103)	46050	57071	0	69116	89994	52446	0	0	0	57696	45146	20568
PKCe (98)	41023	49352	85569	68612	94432	67798	72444	42471	70029	67970	45956	59774
PKCg (84)	22975	30866	29326	15414	16584	23033	28879	21970	28346	20694	20093	23997
PKCl (66)	6459	8217	8108	5498	8437	7344	5931	7578	5651	4134	6800	6019
PKCm (104)	5760	5582	8471	0	10413	6045	0	0	0	9963	9344	3861
PKCm (124)	3853	0	0	3640	9767	3452	7753	4204	7014	14844	7930	8349
PKCt (74)	0	0	0	0	0	0	0	4989	8112	0	0	2620

	SC	SC	SC	SC	SC	SC-Ctrl	SC	SC	SC	SC	SC	SC-mSOD
Kinase	Ctrl #1	Ctrl #2	Ctrl #3	Ctrl #4	Ctrl #5	Mean	mSOD #1	mSOD #2	mSOD #3	SOD #4	SOD #5	Mean
PKCz (83)	26443	35395	32975	0	0	18963	28220	22663	56872	0	0	21551
PKCz (92)	4406	3288	6464	52128	46079	22473	4104	8781	4028	59698	55794	26481
PKG1 (75)	3463	5014	5745	49049	48444	22343	4222	5034	4574	46329	37975	19627
PYK2 (111)	0	0	0	802	1740	508	0	0	0	599	1018	323
RAF1 (62)	0	1815	4853	4674	8461	3961	3090	0	0	3210	3365	1933
RAF1 (73)	9167	7812	10017	7133	4953	7816	15967	14020	20467	9503	6196	13231
RAF1 (80)	3697	0	0	0	0	739	4125	7710	5912	0	0	3549
RafB (98)	9284	9944	18476	10161	14049	12383	30472	19965	19436	8007	4023	16381
ROKa (154)	0	0	0	2148	1607	751	4650	2589	0	1497	3313	2410
RSK1 (77)	21791	0	0	0	0	4358	26656	29240	18866	0	0	14952
RSK1 (82)	25938	29751	23838	33988	28032	28309	30670	43657	24130	7543	8955	22991
RSK1 (85)	23771	0	0	0	0	4754	22140	0	12905	0	0	7009
RSK1 (93)	0	0	0	0	0	0	0	0	0	0	0	0
RSK2 (77)	18132	19742	22150	8689	11168	15976	19950	20241	5851	11483	9886	13482
RSK2 (91)	5961	0	0	0	0	1192	0	0	0	0	0	0
S6K p70 (60)	18301	0	15908	0	1393	7121	24609	20026	12444	0	0	11416
S6K p70 (64)	18080	22039	18040	0	0	11632	24522	19072	10891	0	0	10897
S6K p70 (67)	0	14465	8646	0	0	4622	12668	7130	7430	0	0	5446
S6K p70 (81)	12979	0	0	0	0	2596	0	36889	9319	0	0	9242
S6K p70 (83)	14933	13178	16027	0	0	8828	0	0	0	0	0	0
SAPKb (40)	31441	31253	37823	8642	5062	22844	35030	27735	13797	5480	12382	18885
SAPKb (44)	10439	10048	12314	0	0	6560	11383	10618	20028	0	0	8406
SAPKb (47)	18106	14397	18389	4065	3591	11709	0	14669	0	2395	5921	4597
SAPKb (52)	0	5454	0	0	0	1091	0	0	7406	0	0	1481
SRC (48)	0	0	0	0	0	0	0	0	0	0	0	0
SYK (75)	6631	0	5094	0	0	2345	4847	6315	0	0	0	2232
YES1 (55)	1380	1848	1865	0	0	1019	782	0	0	0	0	156
ZAP70 (80)	0	0	0	0	0	0	0	0	0	0	0	0
ZIP (45)	10868	16863	6611	8090	5332	9553	11421	16127	7868	4318	6131	9173
ZIP (49)	21102	28889	16652	6506	4677	15565	25441	9970	20418	4229	4089	12829

APPENDIX B
ETHICS APPROVAL

SIMON FRASER UNIVERSITY

OFFICE OF RESEARCH ETHICS

BURNABY, BRITISH COLUMBIA
CANADA V5A 1S6
Telephone: 604-291-3447
FAX: 604-268-6785

June 4, 2003

Mr. Jie Hong Hu
Graduate Student
School of Kinesiology
Simon Fraser University

Dear Mr. Hu:

Re: Protein kinase and phosphates protein expression in spinal cord tissues from patients with ALS
CIHR, NRPP
(Amyotrophic lateral sclerosis society of Canada; Muscular dystrophy)

I am pleased to inform you that the above referenced Request for Ethical Approval of Research has been approved on behalf of the Research Ethics Board. The approval for this project is for the term of the period of the grant, as defined by the funding agency. If this project does not receive grant support, the term of the approval is twenty-four months from the above date.

However, this approval is contingent on your receipt of Autopsy Consents and approvals from those institutions from which you receive the tissue. Any changes in the procedures affecting interaction with human subjects should be reported to the Research Ethics Board. Significant changes will require the submission of a revised Request for Ethical Approval of Research. This approval is in effect only while you are a registered SFU student.

Your application has been categorized as 'minimal risk" and approved by the Director, Office of Research Ethics, on behalf of the Research Ethics Board in accordance with University policy R20.0, http://www.sfu.ca/policies/research/r20-01.htm. The Board reviews and may amend decisions or subsequent amendments made independently by the Director, Chair or Deputy Chair at its regular monthly meetings

"Minimal risk" occurs when potential subjects can reasonably be expected to regard the probability and magnitude of possible harms incurred by participating in the research to be no greater than those encountered by the subject in those aspects of his or her everyday life that relate to the research.

.../2

Please note that it is the responsibility of the researcher, or the responsibility of the Student Supervisor if the researcher is a graduate student or undergraduate student, to maintain written or other forms of documented consent for a period of 1 year after the research has been completed.

Best wishes for success in this research.

Sincerely,

Dr. Hal Weinberg, Director
Office of Research Ethics

c: Dr. Charles Krieger, Supervisor

/jmy

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