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JIONG CAO

THE REGULATION AND ROLE OF STRESS-ACTIVATED PROTEIN KINASES (p38 AND JNK) IN NEURONAL CELL DEATH

Doctoral dissertation

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ABSTRACT

Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system. It has been reported that glutamate mediates cell death via NMDA receptors in both acute (e.g. stroke) and chronic (e.g. Alzheimer's disease) neurodegenerative insults. So the dissection of glutamate evoked signal transduction may have clinical significance for neuroprotection. The stress-activated protein kinases (SAPKs) JNK and p38 are implicated in neuronal apoptosis. However, it remains unclear whether p38 and JNK have differing roles dependent on cell type, on apoptotic stimulus, mechanism of cell death or whether they are redundant and each sufficient to induce identical forms of cell death. We found that apoptosis induced by withdrawal of trophic support and glutamate are mechanistically different in terms of caspase-activation, DNA fragmentation profile, chromatin morphology and dependence on *de novo* gene expression. Caspase-independent apoptosis induced by glutamate is accompanied by strong activation of p38, and dominant negative constructs and inhibitors of the p38 pathway prevent this apoptosis. In contrast, withdrawal of trophic support induces caspase-dependent death accompanied by JNK-dependent phosphorylation of c-Jun, and inhibition of JNK is sufficient to prevent the death induced by withdrawal of trophic support. Inhibition of p38 does not block withdrawal of trophic support-induced death nor does inhibition of JNK block glutamate-induced death. Furthermore, 10 μM SB203580 strongly inhibits neuronal JNK2/3, stress-induced c-Jun phosphorylation, and neuronal death in response to trophic withdrawal stress, affecting neither constitutive JNK1 activity nor total neuronal JNK activity, whereas 1 μM SB203580 inhibits p38 activity completely without effect on c-Jun phosphorylation. It reveals that neuronal stress (eg. withdrawal of trophic support) selectively activates JNK2/3 in the presence of mechanisms maintaining constitutive JNK1 activity, and this JNK2/3 activity selectively targets c-Jun, which is isolated from constitutive JNK1 activity. Nitric oxide (NO) is proposed as a downstream effector of excitotoxic cell death. PSD95 can recruit the calcium-dependent nNOS to the mouth of the calcium-permeable NMDA receptor, and depletion of PSD95 inhibits excitotoxicity. In our study, NOS inhibitors reduce both glutamate-induced p38 activation and the resulting neuronal death, while NO donor has effects consistent with NO as an upstream regulator of p38 in glutamate-induced cell death. Using a panel of decoy constructs targeting the PSD95-nNOS interaction, we found that this interaction and subsequent NO production are critical for glutamate-induced p38 activation and the ensuing cell death, which demonstrates that the PSD95-nNOS interface may provide a possible target for design of neuroprotective drugs. Rho GTPases (Rho/Rac1/Cdc42) activate JNK and p38 in certain type of cells. In our cerebellar granule neuron model, Rho is activated by increased intracellular calcium. It is required for the rapid glutamate-induced activation of p38α and the following neuronal death. Other Rho activators also activate p38 but are not sufficient to induce cell death, suggesting that requirements in addition to p38 activation exist for excitotoxic cell death. These observations reveal Rho as a novel and essential component of the excitotoxic cell death pathway.
Altogether, these studies demonstrate that p38α and JNK stress-activated protein kinases are required in different forms of neuronal death in response to different stimuli. The discovery of an interaction between nNOS and PSD95, and the essential role of Rho in glutamate induced activation of p38α and subsequent neuronal death may provide novel potential targets for the design of neuroprotective drugs.

National Library of Medicine Classification: WL 102.5, WL 104, WL 320, WL 359, QU 141
Medical Subject Headings: cell death; apoptosis; neurons; cerebellum; mitogen-activated protein kinases; caspases; glutamic acid; nitric oxide; nerve tissue proteins; nitric-oxide synthase; rho GTP-binding proteins

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Kuopio, April 2005

Jiong Cao

ABBREVIATIONS

AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride
AIF	apoptosis-inducing factor
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
AP-1	active protein1
ASK1	apoptosis signal-regulating kinase-1
ATF2	activating transcription factor 2
BDNF	brain-derived neurotrophic factor
BIR	baculovirus IAP repeat
CaMKII	calcium-calmodulin-dependent protein kinase II
CAPON	carboxy-terminal PDZ ligand of nNOS
CARD	caspase-activating recruitment domain
cGK	cGMP-dependent kinase
CNS	central nervous system
COX	cytochrome oxidase
CREB	transcription factor cAMP-response-element-binding-protein
crmA	cowpox virus cytokine response modifier A
CHOP	cyclic AMP element binding protein (CREB) homologous protein
DISC	death-inducing signaling complex
eNOS	endothelial NOS
Endo G	endonuclease G
ERK	extracellular-signal regulated kinase
FAD	flavin adenine dinucleotide
FCS	fetal calf serum
fmk	fluoromethylketone
GABA	gamma-aminobutyric acid
GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphophate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G-protein-coupled receptor
GST	glutathione S-transferase
GTP	guanosine triphosphate
HI	hypoxia ischemia
HSP27	heat shock protein 27
IAP	inhibitor of apoptosis protein
iGluR	ionotropic glutamate receptor
IMM	inner mitochondrial membrane
IL-1	interleukin-1
iNOS	inducible NOS
JIP	JNK interacting protein
JLP	c-Jun NH2-terminal kinase-associated leucine zipper protein
JNK	c-Jun N-terminal kinase
KD	kinase dead
LPA	lysophosphatidic acid

LTP	long-term potentiation
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MEF	murine embryonic fibroblast
MEF2	myocyte enhancer factor 2
MEM	minimal essential medium
mGluR	metabotropic glutamate receptor
MKK	MAP kinase kinase
MLK	mixed-lineage kinase
MLS	mitochondrial localization sequence
NGF	nerve growth factor
NMDA	*N*-methyl D-aspartate
NO	nitric oxide
nNOS	neuronal nitric oxide synthase
OMM	outer mitochondrial membrane
PAF	platelet-activating factor
PAK	p21-activated kinase
PARP-1	poly (ADP-ribose) polymerase-1
PBS	phosphate-buffered saline
PCD	programmed cell death
PDE	phosphodiesterase
PHAS-1	phosphorylated heat-and acid stable protein-1
PIN	protein inhibitor of nNOS
PSD	postsynaptic density
ROS	reactive oxygen specie
SAPK	stress-activated protein kinase
sGC	soluble guanylate cyclase
SMAC	second mitochondria-derived activator of caspase
SOD	superoxide dismutase
SRE	serum response element
SRF	serum response factor
TAB1	transforming growth factor-beta-activated protein kinase 1 (TAK1)-binding protein 1
TAK1	transforming growth factor-beta-activated protein kinase 1
TCF	ternary complex factor
TNF	tumour necrosis factor
VSCC	voltage-sensitive Ca^{2+} channel
zVAD	benzyloxycarbonyl-Val-Ala-Asp

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to by their corresponding Roman numerals:

I Coffey ET, Smiciene G, Hongisto V, Cao J, Brecht S, Herdegen T, Courtney MJ. (2002) c-Jun N-terminal protein kinase (JNK) 2/3 is specifically activated by stress, mediating c-Jun activation, in the presence of constitutive JNK1 activity in cerebellar neurons. J Neurosci. 22:4335-45.

II Cao J, Semenova MM, Solovyan VT, Han J, Coffey ET, Courtney MJ. (2004) Distinct requirements for p38alpha and c-Jun N-terminal kinase stress-activated protein kinases in different forms of apoptotic neuronal death. J Biol Chem 279(34): 35903-13.

III Cao J, Viholainen JI, Dart C, Warwick HK, Leyland ML, Courtney MJ. (2005) The PSD95–nNOS interface: a target for inhibition of excitotoxic p38 stress-activated protein kinase activation and cell death. J Cell Biol. 168: 117-126.

IV Cao J, Semenova MM, Courtney MJ. (2005) Rho mediates calcium-dependent activation of p38α and subsequent excitotoxic cell death. Submitted

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1. INTRODUCTION

Glutamate induced excitotoxicity is a form of neuronal death that can occur in a variety of brain regions subsequent to ischaemia insults or other neurodegenerative conditions, such as epilepsy, Parkinson's disease, Huntington's disease and Alzheimer's disease (Palmer and Widzowski, 2000). Although a primary role of the excessive or sustained intracellular Ca^{2+} accumulation and increased stimulation of ionotropic glutamate receptors have been implicated as a trigger for neuronal degeneration, they have not been considered as a useful basis for therapeutic intervention (Sattler and Tymianski, 2000). However, identification of the mechanisms by which calcium activates the downstream effectors may be important and necessary.

Increasing number of studies has showed that stress-activated protein kinases (SAPKs) may play a key role in neuronal cell death; however, it has been unclear about the relative roles of the JNK and p38 kinases in it. Xia and colleagues first suggested a role for both JNK and p38 for death induced by withdrawal of nerve growth factor in the PC12 cell line (Xia et al., 1995). Subsequent studies demonstrated that p38 contributes to axotomy-induced apoptosis of retinal ganglion cells, excitotoxicity induced apoptosis of cerebellar granule neurons, and ceramide-induced death of cortical neurons in primary cultured neurons (Kawasaki et al., 1997; Kikuchi et al., 2000; Willaime et al., 2001), while a role for JNK in developmental, trophic withdrawal-induced, excitotoxic, and MPTP-induced death has been substantiated in a variety of neuronal systems (Yang et al., 1997; Eilers et al., 1998; Kuan et al., 1999; Coffey et al., 2000; Harding et al., 2001). There are at least 10 isoforms of JNK expressed from Jnk1, 2 and 3 genes, which bind different substrates and proteins (Kallunki et al., 1994; Gupta et al., 1996). Although knock-outs have revealed distinct functions of the different gene products, studies for selective activation of endogenous JNKs are needed (Yang et al., 1997; Kuan et al., 1999). The JNK family regulates activity of c-Jun by phosphorylation of its N-terminus. However, the mechanisms of regulating c-Jun are still unclear, because total JNK activity does not increase in correlation with c-Jun regulation (Watson et al., 1998; Coffey et al., 2000).

It has also been found that JNK and p38 are implicated in physiological functions (Coffey et al., 2000; Chang et al., 2003), suggesting that they may not be the most suitable pharmalogical targets. This brought up the importance of identifying the pathway mediating calcium-evoked SAPK activation, potentially a rich source of targets for therapeutic interventions. Evidence from knock-out mice and other studies have demonstrated the contribution of nitric oxide and nNOS to glutamate-induced neuronal death (Huang et al., 1994; Dawson et al., 1996), in which the stress-activated protein kinase p38 can be activated within minutes by glutamate receptor activation (Kawasaki et al., 1997). The delayed p38 activation has been observed upon application of NO donors to neuronal cells, but the relationship between NO production and p38 in cell death still remains obscure (Lin et al., 2001; Bossy-Wetzel et al., 2004). The protein PSD95 permits specific coupling of glutamate receptor activity to production of NO by recruiting the calcium-dependent nNOS to the mouth of the calcium-permeable NMDA receptor-channel complex , which conducts neurotoxicity (Aarts et al., 2002; Aarts and Tymianski, 2003). Depletion of PSD95 with antisense oligodeoxynucleotides, or dissociating the

entire PSD95 molecule from the NMDA receptor with PDZ1-2 decoy constructs are found to be neuroprotective in cortical neurons and ischaemia models (Sattler et al., 1999; Aarts et al., 2002). However, PSD95 is known to link a large number of molecules to the NMDA receptor via its different domains, therefore PSD95 dissociation/ablation will disrupt additional functions of the molecule, thus causing side-effects.

The Rho family of GTPases includes Cdc42, Rac and Rho. Their role as molecular switches is critical for organisation of the actin cytoskeleton. By overexpression of dominant negative forms in withdrawal of trophic support, Cdc42 and Rac are suggested to be not only the activators of the JNK and p38 stress-activated protein kinases, but also contribute to neuronal death (Bazenet et al., 1998). In contrast, use of bacterial toxins indicates that Rac/Cdc42 GTPases but not Rho, are critical for neuronal survival (Linseman et al., 2001). Rho has been found to selectively activate p38γ but not p38α in cell lines (Zhang et al., 1995; Marinissen et al., 2001) and it is also known to be regulated by calcium in Xenopus brain (Li et al., 2002). However, the relationship between Rho activity or function and the neuronal response to toxic levels of calcium has not been studied.

In the present study, the distinct roles of p38α and JNK in different death mechanisms were investigated on cerebellar granule neuron culture model. Based on this, the upstream regulators of the p38α pathway in glutamate-induced neuronal death were further explored.

2. REVIEW OF THE LITERATURE

2.1 EXCITOTOXICITY

2.1.1 GLUTAMATE RECEPTORS

Excitotoxicity refers to neuronal cell death caused by activation of excitatory amino acid receptors. It has been considered to be a predominant mechanism of cell death in both acute and chronic neurological diseases, such as stroke, central nervous system (CNS) trauma, epilepsy and chronic neurodegenerative disorders. An excessive release and inadequate uptake of excitatory amino acids, mostly synaptic glutamate, can result in excitotoxicity.

Glutamate has been widely considered as the principal excitatory neurotransmitter in the mammalian central nervous system. Glutamate receptors are classified into metabotropic (mGluR) and ionotropic (iGluR) receptors. mGluRs are G-protein-coupled membrane receptors and have been shown to downregulate K^+ channels and upregulate non-selective cation channels, inhibit gamma-aminobutyric acid (GABA) receptor activity and potentiate iGluR function, resulting in enhanced neuronal excitability (Conn and Pin, 1997). iGluRs are ligand-gate channels permeable to monovalent cations and, in some cases, calcium ions. The ionotropic family of receptors can be further categorized into *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate receptors (Madden, 2002). Glutamate released from the presynaptic terminal binds and activates the ion channel receptors, which are in the postsynaptic membrane, resulting in postsynaptic excitation. In many cases, glutamate toxicity (especially in later phases of neuronal degeneration) can be attributed to excessive stimulation of the ionotropic receptors, especially the NMDA subtype of glutamate receptors (Choi et al., 1988).

The NMDA receptors are critical for learning, memory and development in the central nervous system (CNS). However, excessive activation of NMDA receptors contribute to pathological processes such as stroke, epilepsy and several neurodegenerative diseases (Lancelot and Beal, 1998). Abnormal bursts of excitatory synaptic transmission resulting in excess calcium flux may cause excitotoxicity (Choi, 1985). However, the level of intracellular Ca^{2+} is not the only determinant of NMDA-mediated toxicity. The increased intracellular Ca^{2+} influx coupling with the NMDA receptors is thought to be the key event to mediate cell death (Sattler and Tymianski, 2000).

The NMDA receptor is composed of four homologous subunits. There are three subfamilies of NMDA receptor subunits: one NR1 subunit, four NR2 subunits (2A- 2D) and two NR3 subunits. The NR1 subunit is essential for functional NMDA receptors, whereas four members of the NR2 subunits potentate channel activity and modulate functional properties (Madden, 2002). NMDA receptors require co-agonism by glycine or D-serine, which binds to the NR1 subunits, and glutamate, which binds to the NR2 subunits (Johnson and Ascher, 1987; Mothet et al., 2000). The integral channel of the NMDA receptor is highly permeable to both Na^+, which contributes to postsynaptic depolarization, and Ca^{2+}, which causes intracellular Ca^{2+} transients. Under normal resting

membrane potentials, NMDA receptor channels are blocked by physiological concentrations of Mg^{2+}. This block is voltage-dependent and is relieved by postsynaptic depolarization (Popescu and Auerbach, 2004). Together with high calcium permeability, this is the landmark property of NMDA receptors and forms the basis for its ability to trigger use-dependent changes in synaptic strength (synaptic plasticity).

Although there have been many reports of NMDA receptor activity contributing to neuronal death, there is also some evidence that NMDA receptors are neuroprotective. *In vivo* blockage of NMDA receptors cause increased apoptosis in the developing cerebellum (Monti and Contestabile, 2000) and it affects negatively the survival of granule cells *in vitro* (Ciani et al., 1997). Also, in the developing spinal cord cultures, NMDA receptor antagonists induce neuronal cell death by blocking electrical activity (Brenneman et al., 1990). Transcription factor cAMP-response-element-binding-protein (CREB) has been proposed as a mediator in the NMDA receptor-dependent survival (Mantamadiotis et al., 2002). After ischemia and glutamate exposure, NMDA receptor-mediated Ca^{2+} influx activates CREB by phosphorylation on its critical transcriptional regulatory residue, Ser-133, which is protective from the excitocixity challenges (Walton and Dragunow, 2000; Mabuchi et al., 2001).

Not all NMDA receptors are synaptic receptors. There are also extrasynaptic NMDA receptors, which lie outside of the synapse and have the death promoting effect on CREB (Hardingham et al., 2002). The activation of extrasynaptic NMDA receptors is unlikely to occur in normal physiological conditions, as glutamate release is synaptic and largely reversed by operation of neuronal glutamate transporters (Rossi et al., 2000). However, during pathological conditions, such as hypoxic/ischemic insults, glutamate homeostasis mediated by transporters fails dramatically. Instead of removing extracellular glutamate to protect neurons, transporters release glutamate to the small volume of extracellular space in brain which may increase extracellular glutamate concentration, stimulating extrasynaptic NMDA receptors and triggering neuronal death (Rossi et al., 2000; Hardingham et al., 2002). Ca^{2+} entry through synaptic NMDA receptors may induce CREB activity and expression of pro-survival genes encoded as brain-derived neurotrophic factor (BDNF) gene, which has been implicated in neuronal survival; whereas Ca^{2+} influx through extrasynaptic NMDA receptors, triggered by bath glutamate exposure or hypoxic/ischemic insults, may only transiently phosphorylates CREB, and does not induce BDNF expression, thereby promoting neuronal death (Rossi et al., 2000; Hardingham et al., 2002). It indicates that NMDA receptor localization affects the consequences of their activity. Interestingly, it has been found that although NMDA stimulation induces phosphorylation of CREB on Ser-133 (phospho-CREB) at all stages of development of hippocampal neuronal cultures, its kinetics changes with developmental maturity of the neurons. Stimulation of hippocampal neurons, which have been cultured for two weeks or longer, by NMDA causes only a transient phosphorylation of CREB on Ser-133 rather than the prolonged CREB phosphorylation found in immature neurons (Sala et al., 2000).

Table 1: Mammalian glutamate receptors

mGluRs	Downregulate K^+ channels; upregulate non-selective cation channels; inhibit GABA receptor activity and potentiate iGluR function, resulting in enhanced neuronal excitability.	
iGluRs	**NMDAR (NR1, NR2A-NR2D, NR3A, NR3B)**	Postsynaptic distribution; highly permeable to Ca^{2+} and Na^+; full activation of NMDA receptors requires the binding of glutamate and glycine or D-serine, and the release of the Mg^{2+} blockade of the channel; responsible for synaptic activity.
	AMPAR (GluR1-GluR4)	Postsynaptic distribution; permeable to Na^+ and K^+; also permeable to Ca^{2+} ions unless the receptor contains at least one GluR2 subunit; responsible for fast excitatory synaptic signalling.
	Kainate R (GluR5-GluR7, KA1, KA2)	Pre- and postsynaptic distribution: presynaptically, modulate the release of neurotransmitters; and postsynaptically, mediate excitatory synaptic signallings.

Abbreviations: mGluR, metabotropic glutamate receptor; iGluR, ionotropic glutamate receptor; NMDAR, *N*-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid; GABA, gamma-aminobutyric acid.

AMPA receptors, which are tetramers composed of four kinds of subunits (GluR1-4), are permeable to Na^+ and K^+. Most AMPARs contain GluR2 subunits which make the channel impermeable to calcium. This is due to an arginine residue in the channel pore, introduced by RNA editing to replace the genomically encoded glutamine residue (Hume et al., 1991). AMPARs lacking GluR2 subunits are relatively highly permeable to calcium (Jayakar and Dikshit, 2004). Although both NMDA and AMPA receptors are concentrated at postsynaptic sites of excitatory synapses, compared with the consistent feature of NMDA receptors, AMPA receptors are quite variable because of the dynamic changes in their distribution (Sheng, 2001). The delivery of AMPA receptors to synapses is induced by the activation of NMDA receptors and CaMKII.

Synaptic localisation of Ca^{2+}-impermeable GluR2 subunits is thought to be important in modulating the neurotoxic effects of AMPAR signalling. Studies have indicated that downregulation of GluR2 results in enhanced Ca^{2+} influx through newly synthesized AMPA receptors, which may increase neurotoxicity of endogenous glutamate and exhibit vulnerability to delayed death after ischemia (Bennett et al., 1996; Pellegrini-Giampietro et al., 1997). However, processes involved with some other proteins may also contribute to AMPA neurotoxicity, although untill now none of the AMPA receptor associated proteins have been found to play a role in excitotoxity. The PDZ domain-containing

proteins GRIP, ABP and PICK1, which interact with the C-terminal GluR2 of AMPA receptors directly, are found to be able to retain AMPA receptors at the synapses and this interaction appear to be very important for synaptic targeting and the stabilization of AMPA receptors (Daw et al., 2000). Recently, another AMPA receptor-binding protein, stargazin, is found to be required for synaptic targeting of AMPA receptors by interacting with the PDZ domains of synaptic PSD95 protein. The binding between stargazin and a synaptic MAGUK PDZ domain, for example PDZ domain of PSD95, is sufficient and necessary to retain complex of surface AMPA receptors with stargazin at the synapse (Schnell et al., 2002).

2.1.2 THE ROLE OF CALCIUM

Calcium ions (Ca^{2+}) are ubiquitous intracellular messengers governing a large number of cellular functions, such as the control of cell growth and differentiation, membrane excitability, exocytosis, synaptic activity and cell death. The major sources of intracellular Ca^{2+} ($[Ca^{2+}]i$) include flux through NMDA receptors or voltage-dependent Ca^{2+} channels (VDCCs) and release of Ca^{2+} from intercellular Ca^{2+} stores (Foster and Kumar, 2002). In normal physiology conditions, the resting free intracellular $[Ca^{2+}]i$ must remain at very low levels (around 100 nM, or 10 times lower than extracellular $[Ca^{2+}]$), so that physiological events can be triggered by relatively small or localized increases in $[Ca^{2+}]i$. However, in excitotoxicity, excessive release of glutamate leads to the disregulation of Ca^{2+} homeostasis which finally causes cell death. It has been generally agreed that glutamate receptor mediated neurotoxicity is mainly calcium-dependent (Choi, 1985; Garthwaite et al., 1986; Choi, 1987). Further repeated findings of the requirement of Ca^{2+} in neurodegeneration have lead to the calcium hypotosis that "neuronal Ca^{2+} overload leads to subsequent neurodegeneration" suggesting that neurodegeneration is simply caused by the quantity of Ca^{2+} entering the cell. However, it was also found that a general elevation in cytoplasmic calcium does not necessarily predict neurodegeneration (Dubinsky and Rothman, 1991). In addition, studies have been shown that Ca^{2+} loading through L-type voltage-sensitive Ca^{2+} channels (VSCCs) is not harmful, whereas similar Ca^{2+} increases via NMDA receptors were neurotoxic. These studies lead the "source specificity hypothesis" (Tymianski et al., 1993) suggesting that Ca^{2+} dependent toxicity is not simply a function of increased Ca^{2+} influx; rather it is regulated through distinct Ca^{2+}-signaling pathways linked to specific routes of Ca^{2+} influx.

2.1.3 THE POSTSYNAPTIC DENSITY (PSD)

In the 1950s, postsynaptic density (PSD) was discovered as a specialized electron-dense cytoskeleton structure in mature excitatory synapses by using an electron microscope (Palay, 1958). In the 80's and early 90's, some of the major protein constitutents of PSDs were identified, including PSD95. Subsequently, the cloning of cDNAs encoding PSD95 and related proteins led to the PSD95 family of membrane-associated guanylate kinases (MAGUKs). They are PSD95/SAP90 (Cho et al., 1992; Kistner et al., 1993), SAP97/hdlg (Muller et al., 1995), PSD93/chapsyn-110 (Brenman et al., 1996; Kim et al., 1996) and SAP102 (Lau et al., 1996). Each one has three tandem PDZ (PSD95/DLG/ZO-1)

domains in the amino-terminal portion followed by a src homology (SH) domain 3, and a carboxy-terminal yeast guanylate kinase (GuK) homology domain, each of which has been viewed as a site for protein-protein interaction (Sattler and Tymianski, 2000). The PDZ domains are named after three of the homologous proteins that contain them: PSD95, the *Drosophila* septate junction protein Discs-large (Dlg-A), and the epithelial tight junction protein zonula occludentes-1 (ZO-1) (Sattler and Tymianski, 2000). Through the PDZ mediated protein-protein interactions, MAGUK proteins may bind to ion channels or signaling proteins and cluster receptors at synapses to mediate downstream signaling (Kornau et al., 1997).

PSD95, which is a cytoskeleton-associated protein, was identified as an abundant and detergent-insoluble protein enriched in brain synaptosomal fractions (Cho et al., 1992; Kistner et al., 1993). By using the yeast two-hybrid method, researchers found the interaction between the second domain of PSD95 and the cytoplasmic COOH-terminal tail of the NR2 subunits (NR2A and NR2B). The PDZ domain binds to the conserved sequence of COOH-terminal domain containing the consensus terminal tSXV motif (where S is serine, X is any amino acid, and V is valine), which is common to NR2 subunits and is certain splice forms of NR1 (Kornau et al., 1995; Niethammer et al., 1996). Also in the forebrain, it has been found that there are interactions between the PDZ domains of PSD95 and NMDA receptors, both of which are very rich in the PSD fraction (Kornau et al., 1997). In addition, PSD95 mediates cell-surface clustering of Shaker-subfamily K^+ channels (Kim et al., 1995). Altogether these studies identified that the first two PDZ domains of PSD95 can participate in a domain interaction with ion channels that contain a C-terminal tSXV motif (Kim et al., 1995; Kornau et al., 1995).

PDZ domains of PSD95 can interact with several proteins including neuronal nitric oxide synthase (nNOS) (Brenman et al., 1996), synaptic Ras-GTPase-activating protein SynGAP (Chen et al., 1998) and neuroligin (Irie et al., 1997), thereby continuing the divergent intracellular signaling pathway. Neuroligin is a neuronal cell adhesion molecule which can bind to the third PDZ domain of PSD95 (Irie et al., 1997). SynGAP is specially expressed in neurons and is highly enriched in hippocampal neurons where it colocalizes with the scaffold protein PSD95 and NMDA receptors at synapses (Chen et al., 1998; Kim et al., 1998). The PDZ domain in the N-terminus of neuronal nitric oxide synthase (nNOS) binds to the second PDZ domain of PSD95 (Brenman et al., 1996), and the C-terminal domain of the NR2 subunit is able to independently bind to the first domain of PSD95, although *in vitro* NR2 may bind the first two domains of PSD95 (Niethammer et al., 1996). This binding may allow PSD95 to act as a scaffold protein which can form a ternary complex between the NMDA receptor, PSD95 and nNOS. In cultured cortical neurons, suppressing the expression of PSD95 selectively attenuated excitotoxicity triggered via NMDARs, but not by other glutamate or calcium ion (Ca^{2+}) channels (Sattler et al., 1999). In addition, the application of peptides which can block the interaction of subunit NR2B of NMDA receptors with PSD95 reduces glutamate-induced excitoxicity, thereby limiting the damage in focal ischemic brain and improving their neurological functions (Aarts et al., 2002). These data demonstrate the importance of this scaffold protein for conducting neurotoxicity *in vivo* and *in vitro*. However, contradictory results from studies in coexpression of PSD95 with NMDA receptors in human embryonic kidney (HEK) 293 cells indicate that PSD95 may play a protective role

against excitotoxicity by decreasing glutamate sensitivity of NMDA receptors (Rutter and Stephenson, 2000). Also Yamada and co-workers showed that expression of PSD95 decreased the sensitivity of the NMDA receptor channels to L-glutamate by injection of PSD95 cRNA into Xenopus oocytes expressing the NMDA receptors (Yamada et al., 1999).

PSD93 is highly enriched in brain and is postsynaptically expressed in cerebellar Purkinje neuron cell bodies and dendrites. Like PSD95, the PDZ motifs of PSD93 bind to nNOS as well as to the tSXV motif of NR2B (Brenman et al., 1996). Deletion of PSD93 does not change the expression of PSD95 and associated proteins under physiological conditions, but deletion of the PSD93 PDZ domain disrupts the interaction between PSD93 and NMDA receptors. Targeted disruption of the PSD93 gene reduces not only surface NR2A and NR2B expression, but also NMDA receptor-mediated excitatory postsynaptic functions. However, deletion of PSD93 does not alter the mortality or attenuate brain damage after hypoxia ischemia (HI) in neonatal mice (Jiang et al., 2003). Together with the finding that PSD93 deletion did not completely abolish platelet-activating factor (PAF)-induced neurotoxicity, it may suggest that PSD95 still interacts with NMDA receptor and nNOS, allowing normal NMDA receptor function in the PSD93 deletion model after neurotoxicity treatment (Jiang et al., 2003; Xu et al., 2004). Other protein targets for PDZ domain interactions with nNOS have also been identified. For example, CAPON (carboxy-terminal PDZ ligand of nNOS), which is highly enriched in brain and has numerous colocalizations with nNOS, competes with PSD95 for interaction with the nNOS PDZ domain through its C-terminus (Jaffrey et al., 1998). The N-terminus of nNOS also contains a domain for binding of a highly conserved small protein, termed PIN (protein inhibitor of nNOS), which destabilizes the nNOS dimers to inhibit its activity (Jaffrey and Snyder, 1996).

Alternative targets for triggering NMDAR-mediated Ca^{2+}-dependent neurotoxicity include a number of Ca^{2+}-sensitive second messengers, such as calcium/calmodulin-dependent protein kinase II (CaMKII). CaMKII is abundant in the PSD fraction (Kennedy, 2000). It has been established that neuronal CaMKII is regulated by Ca^{2+} influx via NMDA receptors (Fukunaga et al., 1992). Colocalization of nNOS, PSD95 and CaMKII in PSDs suggests the importance of regulating CaMKII phosphorylation of nNOS. PSD95 promotes phosphorylation of nNOS at residue Ser-847 which is mediated by endogenous CaMKII. This phosphorylation leads to a reduction of nNOS activity in neuronal cells (Komeima et al., 2000; Watanabe et al., 2003).

2.1.4 NEUROTOXICITY OF NITRIC OXIDE (NO)

Nitric oxide (NO) as a messenger molecule in the nervous system has been demonstrated to modulate the neuronal release of neurotransmitters *in vitro* and *in vivo*. It interacts with surrounding neurons not by synaptic transmission, but by diffusion between cells (Dawson et al., 1996). Nitric oxide (NO) has dual roles as neuronal messenger or neurotoxin. It has been implicated as a modulator of essential biological process through modification of cellular proteins. Most of the physiological actions of NO in the CNS are mediated by binding to Fe^{2+} in the heme of soluble guanylyl cyclase (sGC), the best

known physiological target for neuronal NO, which causes enzyme activation and cGMP accumulation (Dawson and Dawson, 1996; Bellamy et al., 2000). However, NO-sGC-cGMP is not involved in the genesis of neurotoxicity since inhibitors of guanylyl cyclase or cell permeable analogs of cGMP do not affect NMDA neurotoxicity (Dawson et al., 1993). Under pathophysiological conditions, such as excessive glutamate release, the excessive intracellular calcium accumulation leads to overactivation of Ca^{2+}-dependent enzymes and reactive oxygen species (ROS) are formed. The metabolic generation of superoxide ($O_2^{\cdot -}$) in the mitochondria is a major source of ROS in normal functioning cells (Nicholls and Budd, 2000). Superoxide dismutase (SOD) is an enzyme that scavenges the superoxide anion. However, the concentrations of SOD and $O_2^{\cdot -}$ are relatively constant in a given tissue. NO reacts with $O_2^{\cdot -}$ three-fold faster than SOD. So NO is capable of competing with SOD for available $O_2^{\cdot -}$ (Dawson et al., 1993). Therefore excess NO is most likely the primary molecule to react with the superoxide anion ($O_2^{\cdot -}$) forming peroxynitrite ($ONOO^-$), which leads to neuronal injury (Bonfoco et al., 1995; Sengpiel et al., 1998).

There is much evidence to suggest that glutamate neurotoxicity is, at least partially, mediated by NO upon activation of NMDA receptors through nNOS (Dawson et al., 1993). However, there are labs that are unable to reproduce the glutamate receptor activated NO-mediated neurotoxicity (Pauwels and Leysen, 1992; Garthwaite and Garthwaite, 1994). The discrepancies of exogenous NO in neurotoxicity may be related to the redox status of the cell. Lipton and colleagues pointed out that the redox status of the cell may determine whether NO formation is neurotoxic or neuroprotective after NMDA receptor activation (Lipton et al., 1993). An intracellular oxidizing environment favors formation of NO+ (nitrosonium ion). S-nitrosylation (transfer of NO+ equivalents to thiol groups) of the NMDA receptors results in a nitrosothiol derivative of the NMDA receptors, which downregulates receptor activity, and thus exhibits a neuroprotective effect. In contrast, a reducing intracellular environment favours reduction of NO+ to NO, which does not react with the thiol groups of the redox modulatory site of the NMDA receptor, but reacts with the superoxide anion ($O_2^{\cdot -}$) to form peroxynitrite ($ONOO^-$) leading to cell injury (Lipton et al., 1998). Normally, physiological intracellular conditions correspond more closely to the reducing situation. Furthermore, endogenous NO has been demonstrated to modulate NMDA receptors regulating the ion channel by S-nitrosylation of the NR2A subunit after NO+ transfer under physiological conditions. This suggested that endogenous NO-related species may have negative feedback to regulate the excess NMDA receptor related ion channel activity in physiological processes (Choi et al., 2000; Jaffrey et al., 2001).

In the mammalian organism, NO is synthesized by a family of three isoenzymes termed as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The endothelial form is responsible for cardiovascular action. The inducible form is found originally in macrophages and it is involved mainly in immunological processes (Dawson and Dawson, 1996). All these isoforms are present in the nervous system. However, the nNOS is the principal isoform in neurons (Dawson and Dawson, 1996). In the presence of oxygen and NADPH, all three NOS catalyse the oxidation of L-arginine to generate nitric oxide and L-citrulline. Under normal physiological conditions, nNOS and eNOS remain in inactive forms at resting intracellular Ca^{2+} level. However, they can be

activated by increasing levels of Ca^{2+} to maintain calmodulin binding. When Ca^{2+} concentration falls to the basal level, calmodulin is dissociated and renders the enzyme inactive. Therefore Ca^{2+} is supposed to be the major regulator of nNOS activity as it stimulates nNOS through interaction with calmodulin (Knowles et al., 1989). In the CNS, NO synthesis is mainly regulated by Ca^{2+} influx, particularly through postsynaptic stimulation of NMDA receptors by glutamate. Ca^{2+} transients arising from the activation of other receptors are presumably too diluted by the time they reach the vicinity of the enzyme. Therefore nNOS can only be ´´switched on`` by NMDA receptors (Garthwaite et al., 1989). nNOS has been found to be membrane-associated in axon terminals and over thick postsynaptic densities by electron micrography (Aoki et al., 1993). This membrane association of nNOS in neurons is mediated by the PDZ domain, as nNOS isoforms lacking this domain may only occur in soluble fractions of brain extracts (Brenman et al., 1996). The amino acid terminal of nNOSα, but not nNOSβ or γ, possesses a PDZ domain which can interact with the PDZ2 domain of PSD95 (Brenman et al., 1996). Thereby the scaffold protein PSD95 exposes nNOS directly to the influx of Ca^{2+} activated by NMDA receptors (Kornau et al., 1995). Deletion of nNOS gene *in vivo* and *in vitro* has shown less vulnerablility to neurotoxicity, which may support a role for neuronally produced NO in excitotoxity (Dawson et al., 1996; Ferriero et al., 1996).

2.2 TROPHIC SUPPORT WITHDRAWAL

Trophic support is critical for proper development and survival of the mammalian nervous system (Barde, 1989). Among the molecules shown to influence neuronal differentiation and survival are, for example, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) (Maisonpierre et al., 1990). However, in culture, neuronal survival can also be supported by a varity of agents in the absence of any neurotrophic factor. For instance, high K^+ has been suggested to influence neuronal development and phenotypic characteristics (Resink et al., 1992). Practically, trophic support withdrawal refers to K^+/serum deprivation. Cerebellar granule cells maintained in medium containing serum and 25 mM K^+ undergo an apoptotic death within 96 hr when switched to serum-free medium with 5 mM K^+ (Miller and Johnson, 1996). Removal of serum showed a fast-dying neuron population, while deprivation of K^+ alone resulted in a slow-dying neuron population (Miller and Johnson, 1996). The trophic withdrawal of neuronal cultures model has been used extensively in the study of the mechanism of neuronal programme cell death (PCD).

2.3 MAPKs

Mitogen-activated protein kinases (MAPKs) play an important role in transducing extracellullar signals into cellular responses (Davis, 1993). To date, three groups of MAPKs have been identified in mammalian cells, which are activated by dual phosphorylation on a threonine-Xaa-tyrosine motif (Raingeaud et al., 1995). The central

amino acid (Xaa) is a defining characteristic of each particular MAPK family, and it is glutamic acid in the case of the ERKs (Thr-Glu-Tyr), proline in the case of the JNK family (Thr-Pro-Tyr), and glycine in the case of the p38 family (Thr-Gly-Tyr) (Nishida and Gotoh, 1993; Derijard et al., 1994; Han et al., 1994; Zhou et al., 1995). The mammalian ERK cascade is generally involved in the control of cell proliferation and differentiation by mitogenic stimuli and growth factors (Boulton et al., 1991; Zhou et al., 1995), while JNK and p38 are regulated by environmental stress such as UV radiation, osmotic shock, and by proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) (Derijard et al., 1994; Han et al., 1994; Kyriakis et al., 1994; Lee et al., 1994; Minden et al., 1994; Sluss et al., 1994; Raingeaud et al., 1995). MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate cellular activities. Three families of protein phosphatases, named Ser/Thr phosphatases, Tyr phosphatases and dual specificity Ser/Thr/Tyr phosphatases, dephosphorylate MAPKs, thereby downregulating the activity of MAPKs (Tamura et al., 2002).

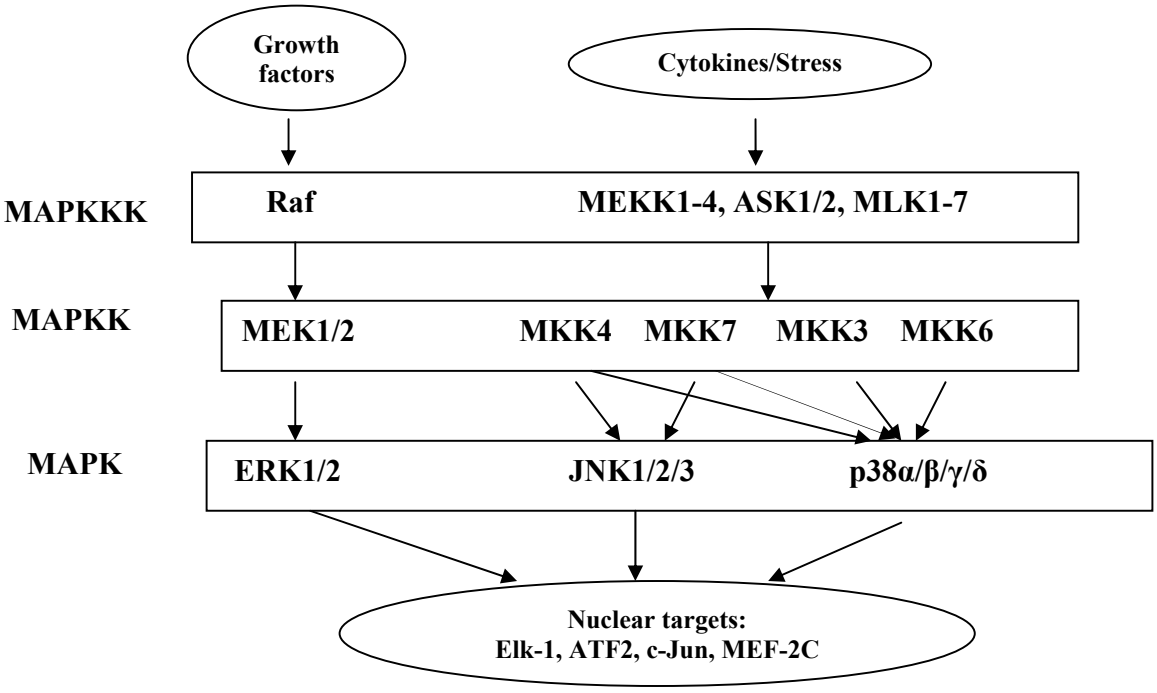

Figure 1. Pathways of MAPKs.
Abbreviations: MEKK, MAPK kinase kinase; MKK, MAP kinase kinase; MEK, MAPK/ERK kinase; ERK, extracellular-signal regulated kinase; JNK, c-Jun N-terminal kinase; ATF2, activating transcription factor 2 (modified from (Cowan and Storey, 2003))

2.3.1 C-JUN N-TERMINAL KINASES (JNKs)

JNKs (c-Jun N-terminal kinases) are activated in response to stresses such as osmotic stress, UV light and ribosomal inhibitors, and inflammatory cytokines such as tumour necrosis factor alpha (TNFα) and interleukin-1 (IL-1) (Derijard et al., 1994; Kyriakis et al., 1994; Sluss et al., 1994). Three genes encoding isoforms JNK1, 2 and 3 have been

identified, and alternative splicing of the genes yields 10 isoforms, four JNK1 isoforms, four JNK2 isoforms, and two JNK3 isoforms (Gupta et al., 1996). These ten isoforms are expressed in brain. JNK1 and JNK2 are expressed in most tissues, while JNK3 is expressed mainly in brain and heart with extremely low levels of expression in the kidney and testis (Mohit et al., 1995; Gupta et al., 1996). The difference between α and β isoforms in JNK1 and JNK2 has been shown particularly in substrate binding (Gupta et al., 1996). JNK3 has 39 amino acids at the N-terminus which are not present in JNK1 and 2. Knockout studies indicated important findings on the functions of JNK isoforms, particularly during development. Mice deficient in Jnk1, Jnk2, Jnk3, and Jnk1/Jnk3 or Jnk2/Jnk3 double mutants all survived normally. Mice mutants lacking Jnk1 and Jnk2 genes were embryonic lethal showing severe dysregulation of apoptosis in developing brains (Kuan et al., 1999). These mutants also had abnormal hindbrain due to a reduction of cell death, whereas increased apoptosis and caspase activation were found in the mutant forebrain (Kuan et al., 1999). However, Jnk3 knockout mice showed a reduction in seizure activity and hippocampal neuron apoptosis after the stimulation of the excitotoxic glutamate-receptor agonist kainic acid (Yang et al., 1997) and protection from brain injury after cerebral ischemia-hypoxia (Kuan et al., 2003). These results suggest that JNK1 and 2 regulate region-specific apoptosis during early brain development, while JNK3 may mediate the responses to stress stimuli in neurons. Furthermore, the JNK pathway also plays a role in mediating cellular apoptosis in response to stress (Xia et al., 1995).

Two different possible mechanisms of the proapoptotic actions of JNK have been brought up. One is that JNK may induce cell death by regulating the expression of death receptors (Faris et al., 1998). The other possible mechanism is that JNK signaling targets the mitochondria and regulates the release of cytochrome c (Kharbanda et al., 2000; Tournier et al., 2000). Bcl-2 family proteins have been identified as playing an important role in JNK–dependent apoptosis (Davis, 2000). JNK has been reported to translocate to mitochondria to phosphorylate and inactivate the antiapoptotic proteins Bcl-2 and Bcl-X_L in the apopototic response to stress (Kharbanda et al., 2000). Additionally, JNK can phosphorylate Bim (EL) at Ser-65 in order to potentiate its proapoptotic activity after NGF withdrawal in neurons (Whitfield et al., 2001; Putcha et al., 2003).

JNKs are activated by the upstream activators MKK4 (SEK1) and MKK7, which are dual specificity kinases that phosphorylate both the threonine (Thr) and the tyrosine (Tyr) residue of the Thr-X-Tyr motif of JNK (Sanchez et al., 1994; Holland et al., 1997). Interestingly, MKK4 and MKK7 preferentially phosphorylate JNK on Tyr and Thr, respectively (Lawler et al., 1998). Full activation of JNK1 (Lawler et al., 1998) and JNK3 (Lisnock et al., 2000) require phosphorylation by both MKK4 and MKK7. Once MKK7 phosphorylates Thr, MKK4 can phosphorylate Tyr (Lawler et al., 1998; Lisnock et al., 2000). It raises the possibility that other MAPKs may also require two or more MKKs to be activated fully (Lawler et al., 1998).

Under normal physiological conditions, JNKs are present in the cell nucleus and cytoplasm. Once activated, they translocate to the nucleus, where are the well characterized JNK substrates and transcription factors such as c-Jun (Pulverer et al., 1991), ATF2 (activating transcription factor-2) (Gupta et al., 1995), and Elk-1

(Whitmarsh et al., 1995). In cerebellar granule neurons, the activation of transcription factors might be mainly exerted by JNK2 and 3. JNK1 is not effective for phosphorylation of transcription factors, such as c-Jun, as it already shows elevated constitutive activity and is localized to the cytoplasm (Coffey et al., 2000; Coffey et al., 2002). Once activated, JNK phosphorylates serine-63 and -73 residues of c-Jun and increases the transcription activity of the active protein-1 (AP-1) complex (Derijard et al., 1994; Kyriakis et al., 1994), regulating gene expression in cell growth as well as in neuroprotection and regeneration (Herdegen et al., 1997).

Recent studies have supported a role for scaffold proteins in the activation of JNK. Four groups of potential scaffold proteins reported to be involved in complexes are CrkII, filamin, β-arrestin, and JIPs (JNK interacting proteins). CrkII has been demonstrated to assemble JNK signaling module in response to the activation of Rac1(Girardin and Yaniv, 2001). Filamin binds to MKK4 and TRAF2, as one of component of scaffold protein in JNK signaling pathway (Marti et al., 1997; Leonardi et al., 2000). β-arrestin binds to ASK1, MKK4 and JNK3, but not JNK1 or JNK2 (McDonald et al., 2000). JIPs are encoded by four genes. JIP1 and 2, which are highly expressed in the nervous system, have been identified as cytoplasm proteins binding to JNK to regulate its activity (Dickens et al., 1997; Yasuda et al., 1999). Both JIP1 and JIP2 contain a phosphotyrosine binding (PTB) domain and a JNK binding domain as well as a Src homology 3 domain (Meyer et al., 1999; Negri et al., 2000). JIP3 is structurally distinct from JIP1 and JIP2, and consists of an extended coiled-coiled domain (Ito et al., 1999; Kelkar et al., 2000; Lee et al., 2002). The JIP group of scaffold proteins selectively mediates signaling by the mixed-lineage kinase (MLK)-MAP kinase kinase 7 (MKK7)-JNK pathway (Dickens et al., 1997; Yasuda et al., 1999; Kelkar et al., 2000). JIP interacts with JNK and MKK7, but not with MKK4, to enhance the activation of JNK (Whitmarsh et al., 1998). An alternatively spliced form of JIP3, known as JSAP1 interacts with JNK, MKK4 and MEKK1 (Ito et al., 1999). However, JIP1 scaffold protein also modulates JNK signaling via association with protein phosphatases that target JNK (Willoughby et al., 2003).

CEP-1347 (KT7515), a K252a derivative, showed neuroprotective effects on some populations of neurons (Borasio et al., 1998; Maroney et al., 1999). It inhibits JNK activation by blocking members of the mixed lineage kinase (MLK) family which are the upstream activators of JNK (Maroney et al., 2001). SP600125 is another novel JNK inhibitor which completely blocks IL-1-induced accumulation of phospho-Jun and induction of c-Jun transcription, thereby reducing paw swelling in an inflammatory arthritis rat model, without interfering with ERK or p38 MAPK (Han et al., 2001).

2.3.2 P38 KINASES

There are five isoforms of p38 identified as p38α (Han et al., 1994), p38β (Jiang et al., 1996), p38β2 (Stein et al., 1997), p38γ (Li et al., 1996), p38δ (Jiang et al., 1997; Wang et al., 1997). p38α and p38β are the main isoforms expressed in brain (Jiang et al., 1996). P38γ is expressed in skeletal muscle (Li et al., 1996). p38δ is found in lung and kidney (Jiang et al., 1997). A group of substrates for p38 have been identified including MAPKAPK2/3 (MAPK-activated protein kinase-2/3), MNK1 (MAP kinase-interacting

kinase-1), ATF2 (activating transcription factor 2), CHOP (cyclic AMP element binding protein (CREB) homologous protein), MEF2 (myocyte enhancer factor 2), PHAS-1 (phosphorylated heat-and acid stable protein-1), and MBP (myelin basic protein). The isoforms differ in their substrate specificity, causing different functions: p38α-ATF2, Elk-1, PHAS-1, MAPKAPK2/3; p38β-ATF2 and MAPKAP2/3; p38γ-MBP; p38δ-ATF2 and PHAS-1 (Raingeaud et al., 1995; Jiang et al., 1996; Li et al., 1996; Wang and Ron, 1996; Cuenda et al., 1997; Han et al., 1997b; Wang et al., 1997; Waskiewicz et al., 1997). Once p38 gets phosphorylated, it phosphorylates transcription factors, such as ATF2 and CHOP-1, to regulate gene expression (Livingstone et al., 1995; Wang and Ron, 1996). Furthermore, once phosphorylated by p38, MAPKAPK2/3 can phosphorylate heat shock protein 27 (HSP27) (McLaughlin et al., 1996).

The upstream activators of p38 isoforms MKK3, MKK4, MKK6 dually phosphorylate Thr and Tyr in the activation loop of p38 *in vivo* and *in vitro* (Derijard et al., 1995; Raingeaud et al., 1995; Han et al., 1996; Raingeaud et al., 1996; Han et al., 1997a). In COS-7 cells, MKK3 or MKK4 strongly activates p38α, only modestly activate p38γ, but fail to activate p38β. MKK6 is suggested to activate all p38α, β and γ (Enslen et al., 1998). However, MKK3 is a more efficient activator of p38 than MKK4 (Enslen et al., 1998). MKK7 also has been reported to activate p38δ (Hu et al., 1999). The mechanism of specificity is the presence of a MAPK docking site in the N-terminus of MKKs and the sequence within the activation loop (T-loop) of individual p38 MAPK isoforms, which determines the selective formation of functional complexes between MKKs and different p38 MAPKs (Enslen et al., 2000).

Further upstream activators of the MKKs of p38 MAPK are MTK1 (MAP Three Kinase 1) (Takekawa et al., 1997), mixed lineage kinase-2 (MLK2) (Cuenda and Dorow, 1998), mixed lineage kinase-3 (MLK3) (Tibbles et al., 1996), dual leucine zipper-bearing kinase (DLK) (Fan et al., 1996), apoptosis signal-regulating kinase-1 (ASK1) (Ichijo et al., 1997), and transforming growth factor-beta-activated protein kinase 1 (TAK1) (Moriguchi et al., 1996). Overexpression of these MAP3Ks leads to activation of both p38 and JNK pathways, which may be the reason why p38 and JNK are often coactivated. Small G-proteins of the Rho family, Rac and Cdc42, have also been identified as potential regulators of the p38 MAPK pathway (Bagrodia et al., 1995; Zhang et al., 1995). Rac/Cdc42 may directly activate MLK1/2/3 which have been reported to have potential Rac/Cdc42 GTPase-binding (CRIB) motifs that can interact with the activated (GTP-bound) forms of Rac and Cdc42 (Tibbles et al., 1996; Nagata et al., 1998). The activation of PYK2, a focal adhesion kinase related to tyrosine kinase, leads to activation of p38 via MKK3 in PC12 cells (Pandey et al., 1999). Signaling modules can also be created by the interaction of the protein kinases with scaffold proteins. For example, Tiam1 and Ras-GRF1 are guanine nucleotide exchange factors (GEFs) that activate the Rac GTPase. JIP2 not only binds to the N-terminal region of both proteins, but also interacts with the Rac target MLK3 as well as MKK3, as a scaffold for the p38 MAPK cascade in COS cells (Buchsbaum et al., 2002). However, another activation mechanism for p38α has been suggested which MKK does not involve. The p38α is activated by interacting with non-MKK protein TAB1 (TAK1-binding protein 1), which leads to autophosphorylation of p38α (Ge et al., 2002).

Withdrawal of nerve growth factor (NGF) inducing apoptosis and activation of p38 MAPK pathways in PC12 cells was the first study to show the proapoptotic role of p38 (Xia et al., 1995; Kummer et al., 1997). p38 also contributes to neuronal apoptosis, such as axotomy-induced apoptosis of retinal ganglion cells (Kikuchi et al., 2000), excitotoxicity-induced apoptosis of cerebellar granule neurons (Kawasaki et al., 1997), and ceramide-induced death of cortical neurons (Willaime et al., 2001). However, it is not clear whether the p38 pathway is involved in neuronal apoptosis *in vivo*. Mice lacking the p38α gene, the only gene reported to be knocked out among the p38 isoforms, resulted in embryonic lethality (Adams et al., 2000; Tamura et al., 2000). Besides its apopotic role in certain cell types, there is some evidence showing that p38 activation promotes cell survival (Nemoto et al., 1998; Roulston et al., 1998; Mao et al., 1999). For example, activation of transcription factor MEF2 has been shown to be required for the survival of developing neurons. Expression of dominant-negative p38 reduces MEF2 dependent transcription and induces apoptosis (Mao et al., 1999). p38α appears to induce apoptosis, while p38β enhances survival in certain cell types and certain stimuli (Guo et al., 2001; Li et al., 2004).

The pyridinylimidazole compounds SB203580 and SB202190 are believed to selectively inhibit only p38α and p38β, but not p38γ and p38δ. It inhibits catalytic activity of p38 by competitive binding in its ATP pocket, but it has no effect on Thr (180) and Tyr (182) phosphorylation of p38 by upstream MKKs. By this way, it inhibits the phosphorylation of p38 substrates and prevent cell death (Kumar et al., 1999; Lee et al., 2000). SB203580 also can inhibit JNKs (Whitmarsh et al., 1997; Lisnock et al., 2000; Coffey et al., 2002). SB203580 at 10 μM blocks most of the neuronal JNK2 and 3 activity, *in vivo* c-Jun phosphorylation and transcriptional activity, and neuronal death (Coffey et al., 2002). However, 1 μM SB203580 is sufficient to block p38 activity *in vivo* (Coffey et al., 2002).

2.4 CELL DEATH

2.4.1 FORMS OF CELL DEATH

Apoptosis is an essential physiological process. When cells are no longer needed or have become seriously damaged, they have the ability to self-destruct by activation of an intrinsic cellular suicide program which is required for normal development and maintenance of tissue homeostasis (Vaux and Korsmeyer, 1999). The term of apoptosis was coined by Kerr et al. (1972) to describe the morphological characteristics of a certain type of cell death including cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation. These changes are in contrast to another process of cell death called necrosis, in which cells and their organelles tend to swell and rupture due to toxic substances, trauma, and ischemia. The apoptotic cell plasma membranes do not rupture and organelles retain their integrity, preventing the release of cellular components into the extracellular medium. *In vivo*, apoptotic cells are rapidly removed by phagocytosis without eliciting an inflammatory reaction. In contrast, necrosis usually affects large numbers of contiguous cells, which undergo swelling of the cytoplasm and of the

mitochondria and other organelles. It leads to the rupture of plasma membranes and lysis of the cells causing an inflammatory reaction.

Programmed cell death (PCD), which gives a mechanistic meaning to the original term of apoptosis, generally denotes any cell death mediated by the intracellular death program, no matter what initiates it and whether or not it displays all of the characteristic features of apoptosis. Accordingly, Jäättelä and colleagues brought out a more detailed categorisation of cell death, based on the morphology and fate of dying cells, rather than the activation of caspases (Table2).

Table 2. Four patterns of cell death. (Leist and Jaattela, 2001)

Apoptosis	**Apoptosis-like PCD**	**Necrosis-like PCD**	**Necrosis/cell lysis**
Chromatin condenses to compact form; phosphatidylserine exposure; cytoplasmic shrinkage; zeiosis (dynamic plasma membrane blebbing of a dying cell); formation of apoptotic bodies	Chromatin condensation is less compact (lumpier shapes), with display of phagocytosis-recognition molecules before lysis of the plasma membrane. Any degree and combination of other apoptotic features.	No chromatin condensation. Varying degrees of other apoptosis-like features.	Conceptual counterpart to PCD. It is prevented only by removal of stimulus, e.g. exposure to high concentrations of detergents, oxidants ionophores or high intensities of pathologic insults.
Caspases, particularly caspase-3, are activated.	Forms of caspase-independent apoptosis.	Caspase-independent signaling pathway. Caspase 8 and caspase 1 may be involved.	No caspases are activated.

In the last decade, much of the attention in the study of PCD was focused on caspases (cysteinyl aspartate-specific proteases), one of the biochemical hallmark of apoptosis. Caspases are a family of cysteine proteases which cleave substrates after a conserved aspartate residue, leading to apoptosis. They normally are inactive in a zymogen form and are activated by proteolytic cleavage into a small and a large subunit to reconstitute as a heterodimer in the active form (Thornberry and Lazebnik, 1998). The cleaved caspase can induce a dramatic conformational change, which exposes the enzyme catalytic pocket resulting in its activation. Once activated, initiator caspases (such as caspase-8, -9 -10) activate downstream executioner caspases (such as caspase-3 -7), which in turn amplify the caspase cascade, resulting in apoptosis (Thornberry and Lazebnik, 1998; Budihardjo et al., 1999). Various strategies for cell disassembly by caspases have been suggested, including direct disassembly of cell structure (such as lamina) (Orth et al., 1996), cleavage of proteins involved in cytoskeletal regulation (such

as gelsolin) (Kothakota et al., 1997), and inactivation of apoptosis inhibitor proteins (such as the cleavage of ICAD) (Enari et al., 1998).

2.4.2 CASPASE-DEPENDENT CELL DEATH

The origin of the cell death stimulus determines which caspases are involved, and can be classified as being either extrinsic or intrinsic. The extrinsic pathway of cell death is responsible for the elimination of unwanted cells during development and immune system mediated tumor removal. One of the caspase activation pathways is the cell surface death receptor-mediated pathway (Ashkenazi and Dixit, 1998). In this pathway, extracellular hormones or agonists belonging to the tumor necrosis factor (TNF) superfamily recognize and activate their corresponding receptors. By protein-protein domain interaction, the receptors recruit specific adaptor proteins, e.g. FADD (Fas-associating protein with death domain) to form a complex called the death-inducing signaling complex (DISC). Activated caspase-8, following its recruitment to the DISC, can activate downstream caspases leading to cell death (Ashkenazi and Dixit, 1998; Luo et al., 1998; Budihardjo et al., 1999).

The intrinsic pathway contributes to cell elimination in response to chemotherapeutic drugs, mitochondrial damage and ionizing radiation. This pathway involving the caspase activation cascade is triggered by cytochrome c released from the intermembrane space of mitochondria (Budihardjo et al., 1999). Caspases with caspase-activating recruitment domains (CARDs), such as caspase-9, are most probably activated through this intracellular activating complex (cytochrome c/Apaf-1/caspase-9) (Li et al., 1997). Once receiving apoptotic stimuli, the outer membrane of mitochondria becomes permeable to cytochrome c (Budihardjo et al., 1999). Once it is released to the cytosol, cytochrome c binds to Apaf-1 in a 2:1 ratio forming an oligomeric Apaf-1/cytochrome c complex (apoptosome) (Zou et al., 1999). In the presence of dATP or ATP, this complex recruits procaspase-9 and induces its autoactivation (Zou et al., 1999). In turn caspase-9 activates downstream caspases, including caspase-3,-6,-7 (Budihardjo et al., 1999).

The Bcl-2 family of cytoplasmic proteins is thought to be the major regulator of the mitochondrion-initiated caspase activation pathway via controlling cytochrome c release (Adams and Cory, 1998). Bcl-2 family members share at least one of the four conserved Bcl-2 homology (BH) domains and are divided into two main groups according to their antiapoptotic or proapoptotic functions. Antiapoptotic Bcl-2 members, such as Bcl-2 and Bcl-$X_{L,}$ harbor BH domains (1-4). The proapoptotic family can be further classified according to whether they contain BH1, BH2 and BH3 (e.g. Bax, Bak) or only possess a BH3 domain, know as BH3-only proteins (e.g. Bid, Bad) (Adams and Cory, 2001). Most members possess a hydrophobic C-terminus and presumably can be inserted into a membrane, such as the endoplasmic reticulum and the outer mitochondrial membrane where the antiapoptotic members normally reside and proapoptotic members assemble during apoptosis. In response to stimuli, the proapoptotic family translocates to the mitochondria from other cellular compartments and promotes the release of cytochrome c. For instance, Bax translocates from the cytosol to the mitochondria in response to the apoptotic signal. A Bid-induced conformation change and subsequent oligomerization allows it to insert into the outer mitochondria membrane (OMM), leading to the release

of cytochrome c (Gross et al., 1998; Jurgensmeier et al., 1998; Finucane et al., 1999; Eskes et al., 2000). However, caspase inhibitors can not prevent Bax-induced cytochrome c release, but Bcl-2 and Bcl-X_L can, suggesting that Bax may kill cells without activating caspases (Gross et al., 1998; Jurgensmeier et al., 1998; Rosse et al., 1998; Finucane et al., 1999; Gross et al., 1999). Other proapoptotic proteins, BH3-only proteins, are thought to kill entirely by activating Bax or Bak directly or locking anti-apoptotic Bcl-2 members into an inactivate conformation by binding them (Kelekar and Thompson, 1998). The anti-apoptotic proteins Bcl-2 or Bcl-X_L interact with and inhibit Bak, Bax and BH3-only proteins to prevent cytochrome c release from mitochondria (Adams and Cory, 1998).

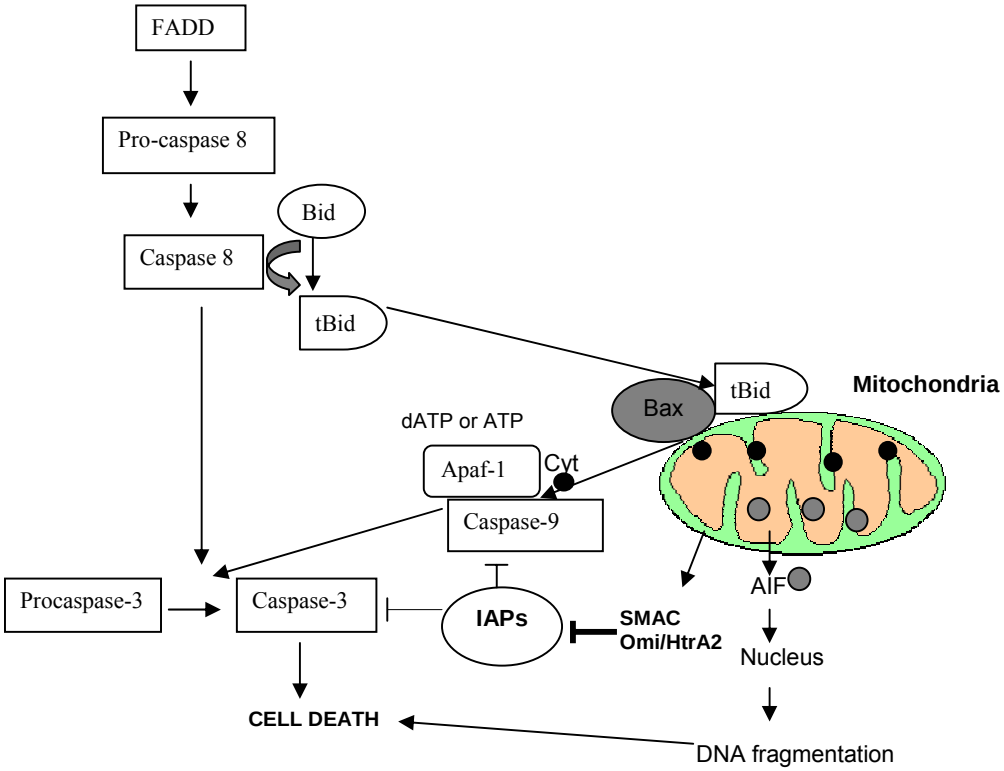

Figure 2. Regulation of the mitochondria apoptotic pathway.
Abbreviations: cyt, cytochrome c; FADD, Fas-associating protein with death domain; SMAC, second mitochondria-derived activator of caspase; IAPs, inhibitors of apoptosis proteins.

Another family of proteins mediating cytochrome c/Apaf-1 caspase cascades are inhibitors of apoptosis proteins (IAPs), which inhibit caspase activity by binding directly to active caspases or bind to procaspase-9 (Deveraux et al., 1998; Deveraux and Reed, 1999; Bratton et al., 2001). A mitochondrial protein called the second mitochondria-derived activator of caspase (SMAC)/direct IAP binding protein with low pI (DIABLO) was found to promote cytochrome c/Apaf-1-dependent caspase activation by antagonizing IAP function (Du et al., 2000; Verhagen et al., 2000). Like cytochrome c, it is normally localized in mitochondria and released into the cytosol in response to

apoptotic stimuli (Du et al., 2000; Verhagen et al., 2000). Furthermore, another mitochondrial protein, Omi/HtrA2, can also inhibit the function of IAPs (Suzuki et al., 2001; Martins et al., 2002).

Currently two mechanisms for outer mitochondrial membrane (OMM) permeabilization leading to release of cytochrome c have been recognized. One is the opening of permeability transition (PT) pore followed by osmotic swelling of the mitochondrial matrix, rupture of the OMM, and the release of cytochrome c (Crompton, 1999; Gogvadze et al., 2001). The second one involves Bcl-2 family members of proteins, where the activation of Bax or Bax-like protein may play an important role (Wei et al., 2001; Zong et al., 2001; Kuwana et al., 2002). Cytochrome c is normally bound to the inner mitochondrial membrane (IMM) through the anionic phospholipid cardiolipin, it is reported that cardiolipin has an essential role in retaining cytochrome c within the intermembrane space and oligomeric Bax alone is not sufficient for cytochrome c release (Ostrander et al., 2001; Ott et al., 2002).

2.4.3 CASPASE-INDEPENDENT CELL DEATH

While much attention in the study of cell death focuses on caspases, cell death still occurs when caspases are blocked, which suggests an alternative pathway defined as caspase-independent (Lockshin and Zakeri, 2002; Jaattela and Tschopp, 2003). Caspase-independent neuronal apoptosis has already been found after ischemia (Zhan et al., 2001) and excitotoxicity (Miller et al., 1997). This way of cell death revealed that mitochondria play an important role via the release of proapoptotic proteins, such as apoptosis-inducing factor (AIF) and endonuclease G (Endo G) involving in DNA fragmentation and subsequent chromosomal condensation (Susin et al., 1999; Li et al., 2001). Mammalian AIF is expressed as a precursor of a 67-kDa protein which contains an N-terminal mitochondrial localization sequence (MLS, residues 1-100), a flavin adenine dinucleotide (FAD)-binding domain, a NADH- binding domain and a C-terminal domain (Susin et al., 1999; Mate et al., 2002). Mutational analysis reveals that the oxidoreductase activity of AIF is not required for its apoptogenic property (Miramar et al., 2001; Mate et al., 2002), while the C-terminal domain plays an essential role in death function (Susin et al., 1999). The mature form of AIF is generated by the cleavage of the MLS, after being imported into the mitochondrial intermembrane space (Susin et al., 1999). In response to apoptotic insult, the permeabilized outer mitochondrial membrane allows AIF to translocate to the nucleus inducing chromatin condensation as well as high molecular weight (50kp) DNA fragmentation, possibly by binding to DNA (Susin et al., 1999; Cande et al., 2002; Ye et al., 2002). The translocation of AIF has been reported in several models of neuronal apoptosis, including the death of photoreceptors induced by retinal detachment (Hisatomi et al., 2001), neuronal cell death induced *in vivo* by brain trauma (Zhang et al., 2002) and cerebral ischemia (Zhu et al., 2003), hydrogen peroxide, peroxynitrite (Zhang et al., 2002) and the excitotoxin NMDA (Yu et al., 2002).

2.5 RHO GTPase PROTEINS

2.5.1 THE REGULATION OF RHO GTPase PROTEIN ACTIVITY

Small GTP-binding proteins (G-proteins) are monomeric G proteins with molecular masses of 20-30 kDa. More than 100 small G-proteins have been identified in eukaryotes from yeast to human. The members of this superfamily are structurally subdivided into at least five families: Ras (eg. Ras, Rap and Ral), Rho (Rho, Rac and Cdc42), Rab (more than 60 members), Arf (Arf1-Arf6, Arl1-Arl7 and Sar), and Ran family GTPases (Takai et al., 2001).

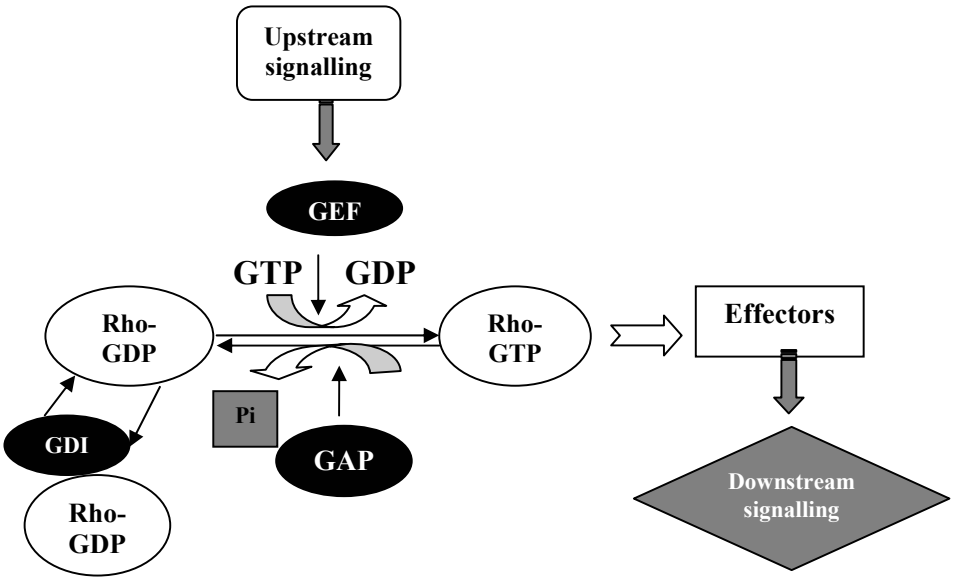

Figure 3. The Rho GTPase cycle.
Abbreviations: GEF, guanine nucleotide exchange factor; GDI, GDP dissociation inhibitor; GAP, GTPase-activating protein; GTP, guanosine triphosphate; GDP, guanosine diphophate (Modified from (Takai et al., 2001)).

Rho GTPases (Rho/Rac/Cdc42) are regulators of several signaling networks activated by a wide variety of receptors. They can affect actin cytoskeletal dynamics, transcriptional regulation, cell cycle progression and membrane trafficking. Like other small GTP-binding proteins, Rho GTPases are guanine nucleotide binding proteins that cycle between two conformational states: GDP-bound inactive and GTP-bound active forms. Once stimulated by an upstream signal, GDP dissociates from the GDP-bound form and is replacecd by GTP, leading to a conformational change of the downstream effector-binding regions, which eventually interacts with an effector or target molecule to initiate a downstream response. The dissociation of GDP from the GDP-bound form is the rate-limiting step of the GDP/GTP exchange which is controlled by guanine nucleotide exchange factors (GEFs) and GDP dissociation inhibitors (GDIs) (Van Aelst and D'Souza-Schorey, 1997; Kjoller and Hall, 1999). GEFs promote the exchange of GDP for GTP, while GDIs can be in complex with the GDP-bound form to keep Rho GTPases in cells in the inactive form (Olofsson, 1999). All Rho-GEFs contain a Dbl (diffuse B-cell lymphoma)-homology (DH) domain, which provides the catalytic activity (Hart et al.,

1994; Cherfils and Chardin, 1999), and an adjacent pleckstrin homology (PH) domain, which mediates membrane localization through lipid binding and also acts as a regulator of the DH domain (Zheng et al., 1996; Rameh et al., 1997; Liu et al., 1998). The GTP-bound form can be converted into the GDP-bound form to release the bound downstream effectors. GTPase-activating proteins (GAPs) enhance the intrinsic rate of hydrolysis of bound GTP to GDP leading to inactivation (Lamarche and Hall, 1994).

The Rho GTPase family links plasma membrane receptors to reorganization of the actin cytoskeleton. In fibroblasts, addition of lysophosphatidic acid (LPA) induces the formation of actin stress fibers, which is regulated by Rho proteins. Growth factors, for instance insulin and PDGF, stimulate polymerization of actin at the plasma membrane to induce ruffling and lamellipodia formation regulated by Rac proteins (Ridley et al., 1992). Bradykinin induces activation of Cdc42 resulting in filopodium formation (Kozma et al., 1995; Nobes and Hall, 1995). LPA-induced Rho activation is mediated by Gα13, one of Gα subunits of guanine nucleotide-binding proteins (Gohla et al., 1998). Gα13-mediated RhoA activation involves direct interaction with RhoGEF proteins, such as p115-RhoGEF (Vogt et al., 2003). LPA-induce Rho activation can be completely blocked with overexpression of C3 transferase (*Clostridium botulinum*), a bacterial exoenzyme that specifically ADP-ribosylates the effector region of RhoA, RhoB and RhoC and inhibits their function by preventing interaction with downstream effectors (Aktories et al., 1989; Chardin et al., 1989; Ridley and Hall, 1992).

2.5.2 DOWNSTREAM EFFECTORS OF RHO GTPase PROTEINS

The diverse activities of Rho GTPases result from the interactions between Rho GTPases and numerous downstream effectors. They include serine/threonine PKN-related kinase (PRK family) (Amano et al., 1996; Watanabe et al., 1996), ROCK I and ROCK II kinase (also known as ROKβ and α) (Leung et al., 1995; Ishizaki et al., 1997), two non-kinase molecules Rhotekin (Reid et al., 1996) and Rhophilin (Watanabe et al., 1996), and Citron (Madaule et al., 1995). Rho proteins also interact with the myosin binding subunit (MBS) of myosin phosphatase (Kimura et al., 1996); p140mDia and mDia2, which are mammalian homolog of *Drosophila* diaphanous (Watanabe et al., 1997; Alberts et al., 1998), and Kinectin (Hotta et al., 1996).

Rho-ROCK-myosin phosphatase pathway is one of the downstream cascades of Rho GTPase. p160ROCK (ROKβ/Rho kinase or ROCK I), which is activated by GTP-Rho, has been shown to phosphorylate and to inhibit myosin light chain phosphatase, causing sustained smooth muscle contraction even after a decrease of Ca^{2+} concentrations (Kimura et al., 1996). ROCK is also involved in another pathway, the Rho-ROCK-LIM kinase pathway. ROCK directly phosphorylates LIM kinase, which in turn posphorylates the actin depolymerizing factor cofilin, resulting in Rho-induced reorganization of the actin cytoskeleton (Maekawa et al., 1999). ROCK I (ROKβ), but not ROCK II, has been shown to be cleaved during apoptosis by activated caspases to generate a truncated active form. The cleaved and active ROCK proteins thus phosphorylate downstream targets to promote cell contractility, subsequent membrane blebbing, and formation of apoptotic bodies, which can be inhibited by the ROCK inhibitor Y-27632 (Coleman et al., 2001).

Recently, ROCK was found to represent a point of signal divergence downstream from RhoA, as it not only regulates the reorganization of actin and expression from the *c-fos* serum response element (SRE), but also has another independent pathway connecting JNK to stimulation of c-Jun expression (Marinissen et al., 2004).

Several potential effectors of Cdc42 and Rac proteins have also been identified. Some of these proteins are common effectors for both Rac and Cdc42 proteins. Among them, the family of serine/threonine protein kinases known as PAKs (p21-activated kinases) is especially interesting. The PAKs were proposed to act as the upstream kinases of JNK and p38 as mediators of Rho GTPases (Bagrodia et al., 1995; Knaus et al., 1995; Manser et al., 1995). There are at least six mammalian PAK isoforms known as PAK1-6 (Jaffer and Chernoff, 2002). All PAKs share a similar 18-amino acid CRIB (Cdc42/Rac interactive binding) motif that mediates the interaction with Rac/Cdc42 proteins. PAK1 contains the G-protein binding domain and an overlapping autoinhibitory domain (Frost et al., 1998; Zhao et al., 1998). In the absence of small G proteins, PAK kinase activity is suppressed by an intermolecular interaction between the regulatory and catalytic domains. The binding of Rac and Cdc42 disrupts this interaction, thus releasing the kinase activity from autoinhibition (Frost et al., 1998; Zhao et al., 1998; Tu and Wigler, 1999). PAKs also play a role in regulating the reorganization of the actin cytoskeleton. PAK1 can induce two types of morphological changes: one is essencial for the disassembly of focal adhesions; the other promotes lamellipodia formation and membrane ruffling (Frost et al., 1998).

2.5.3 REGULATION OF TRANSCRIPTION FACTORS AND CELL DEATH

Rho GTPases are involved in regulating nuclear signaling. They activate JNK and p38 in certain type of cells, which regulate the activity of transcription factors such as *c*-Jun (Coso et al., 1995; Minden et al., 1995). MLK3 and MEKK4, which contain CRIB motif, bind to Rac and Cdc42, mediating activation of the JNK signaling pathway (Teramoto et al., 1996a; Gerwins et al., 1997). RhoA regulation of the expression of c-Jun involves PKN, which activates p38γ (Marinissen et al., 2001). In addition, Rho GTPases stimulate the activity of the serum response factor (SRF), a transcription factor regulating a number of genes including *c-fos* (Hill et al., 1995). The expression of *c-fos* is controlled by serum response element (SRE) which can bind several regulatory molecules such as SRF. Rho proteins are required for lysophosphatidic acid (LPA) - and serum-induced transcriptional activation by SRF. The expression of constitutively active RhoA protein stimulated the transcriptional activity of *c-fos* SRE independently from the activity of MAPKs (Chihara et al., 1997).

Rho GTPases are also implicated in the regulation of programmed cell death. Introduction of human rho gene induces apoptosis upon serum deprivation in fibroblasts (Jimenez et al., 1995) and Rac2 transgenic mice showed increased apoptosis in the thymus (Lores et al., 1997). Overexpression of Rac1 induces an increase of ceramide levels and Fas ligand (FasL) gene expression, leading to cell death. This requires protein synthesis and caspase-3 activity, but is independent of the release of cytochrome c from mitochondria (Embade et al., 2000).

3. AIM OF THIS STUDY

Stress-activated protein kinases (SAPKs) have been shown to play a key role in neuronal cell death; however, it has been unclear about the relative roles of the JNK and p38 kinases in it. In our present study, we distinguish the role of p38α and JNK (also JNK isoforms) in cerebellar granule neurons challenged by two different stimuli (glutamate or trophic support withdrawal). Furthermore, we aim to reveal novel targets for future neuroprotective agents by elucidating pathways regulating stress-activated protein kinase pathways in glutamate-induced neuronal death.

1. To investigate the mechanisms regulating c-Jun by JNK isoforms (JNK1 and JNK2/3) stressed by withdrawal of trophic support.

2. To distinguish the different requirements of stress-activated protein kinases (p38 and JNK) and different modes of neuronal death in different stress stimuli.

3. To explore the relationship between p38 activation, nitric oxide and the PSD95-nNOS interaction in glutamate-induced neuronal death and to determine the neuroprotective possibility of the PSD95-nNOS binding domain.

4. To study the possible involvement of Rho GTPase (Rho GTPase>>p38) in glutamate induced neuronal death.

4. EXPERIMENTAL PROCEDURES

4.1 CELL CULTURES AND TRANSFECTION

4.1.1 CEREBELLAR GRANULE NEURON CULTURES

Cerebellar granule neurons were prepared from 7-d old rats. This preparation is well characterized and is reported to contain 95% small interneurons, predominantly granule neurons (Thangnipon et al., 1983; Courtney et al., 1997). Cells were cultured in minimal essential medium (Life Technologies, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum (Life Technologies), 33 mM glucose, 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 mM supplementary KCl (final 25.4 mM KCl). Cells were plated at 250,000/ cm^2 onto culture surfaces coated with poly-L-lysine (15µg/ml): 35 mm dishes or wells of 12- or 24-well plates (Costar, Corning, NY, and Greiner GmbH, Solingen, Germany) or 10.5 mm x 10.5 mm coverslips for different experiments. Culture medium was replaced after 24 hours with the inclusion of 10 µM cytosine arabinofuranoside (Sigma) to reduce non-neuronal cell proliferation. After this time, fresh culture medium was not re-added to the cells, to avoid serum glutamate-associated toxicity. Cells were cultured in a humidified 5% CO2 atmosphere at 37°C. Neurons used were between 7 and 10 days *in vitro.* Neurons were transfected according the calcium phosphate based method with plasmids as indicated. When tested, co-transfection efficiency was near 100% (Coffey et al., 2002; Hongisto et al., 2003).

4.1.2 COS-7 CELL CULTURES

COS-7 cells were cultured in minimal essential medium (MEM) containing 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, and penicillin and streptomycin as above. Cells were cultured in a humidified 5% CO2 atmosphere at 37°C. 24 hours after replating, cells were ready to be transfected with plasmids as indicated, using Polyfect (Qiagen) according to the manufacturer's protocol.

4.1.3 NEURO 2A CELL CULTURES

Neuro 2A cells were maintained in minimal essential medium (MEM) containing 10% (*v/v*) fetal calf serum (FCS), 2 mM glutamine, and penicillin and streptomycin as above and 1:100 nonessential amino acids (Life Technologies BRL). Cells were cultured in a humidified 5% CO2 atmosphere at 37°C. 24 hours after replating, cells were ready to be transfected with plasmids as indicated, using Polyfect (Qiagen) according to the manufacturer's protocol.

4.2 CELL TREATMENT EXPERIMENTS

4.2.1 GLUTAMATE TREATMENT

Glutamate treatment was carried out by rinsing cells in Mg-free Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 5.6 mM D-glucose, 5 mM Hepes, pH 7.4) and placing in the same buffer with glutamate (50 μM or as shown) 30 minutes, or as shown; 10 μM glycine was routinely included with glutamate as it is an essential co-agonist for the NMDA receptor (Courtney et al., 1990a). Subsequently cells were rinsed in Locke's buffer with 1 mM $MgCl_2$ and conditioned medium was replaced for the time indicated.

4.2.2 WITHDRAWAL OF TROPHIC SUPPORT TREATMENT

Cerebellar granule neurons were deprived of trophic support by incubating the cells with medium free of serum and without additional KCl for the times indicated.

4.2.3 NITRIC OXIDE TREATMENT

Nitric oxide treatment was carried out by adding 10 μM or 250 μM nitric oxide donor Dea/NO (DeaNONOate) or 300 μM peroxynitrite to the cell culture media directly and they were maintained throughout the whole experiment. The concentration of peroxynitrite ($ONOO^-$) was determined by measuring its absorbance at 302 nm ($\varepsilon = 1670$ $M^{-1}cm^{-1}$). The concentration of $ONOO^-$ was measured for every experiment just before it was added to the samples.

4.2.4 LPA TREATMENT

Cultures were rinsed twice with serum-free culture medium (without serum, antibiotics or glutamine), and 10 μM LPA was added to the same medium.

4.2.5 KCl TREATMENT

Cultures were rinsed twice in Locke's buffer and placed in the same buffer with 1 mM $MgCl_2$ and 30 mM KCl.

4.2.6 DRUG TREATMENT

In **Publication I**, pharmacological agents (50 μM of the selective MEK1/2 inhibitor PD98059, SB203580 1 μM, 10 μM or 100 μM) were added 60 min before withdrawal

stress or 30 min before withdrawal stress or 30 min before anisomycin (10 μg/ml) treatment; In **Publication II**, pharmacological agents (zVAD-fmk 100 μM, MK-801 2 μM, SB203580 1 μM, 1, 9-anthrapyrazolone "SP600125" 1 or 3 μM) were added 60 minutes before withdrawal stress or glutamate treatment; In **Publication III,** SB203580 1 μM were added 30 minutes before or after glutamate treatment. 7-nitroindazole (3 μM, "7-NI") and *N*-ω-propyl-L-Arginine (1 μM, "N^{w}-PLA") were added 60 minutes before nitric oxide treatment respectively. The agents were also present in all media in which the cells were subsequently incubated. The NOS inhibitors are competitive with arginine; therefore cells were placed in arginine-free conditions during the period of incubation with these inhibitors (or incubation with carrier in the case of controls). Arginine (1 mM) was added to the preincubation and stimulation solutions, where indicated.

4.3 ASSESSMENT OF CELL DEATH

4.3.1 VIABILITY ASSAY

7 days after plating, neurons were co-transfected with GFP marker plasmids and either empty vector (pCMV), pEBS7-Bcl2, pcDNA3-p38α-AF, or pMT3-MKK3a-kinase dead "MKK3a-KD" as shown. 24 hours after transfection, cells were treated with or without glutamate treatment as described above and culture medium were replaced for 24 hours. i) **Immunoblotting-based viability assay:** 24 hours after stimulation with or without glutamate, cells were rinsed in ice-cold PBS, lysed in 75 μl Laemmli sample buffer, and processed for immunoblotting as described below; ii) **Cell counting-based viability assay:** cerebellar granule neurons were transfected as above. After 24 hours incubation they were treated with or without glutamate as described above. Transfected cells in 4 evenly spaced fields per coverslip were counted by epifluorescence using 450-490 nm excitation light and a 20 x air objective. After a further 24 hours incubation, the transfected cells were counted again as above. Viability was calculated as the proportion of GFP-positive cells remaining after stimulation.

4.3.2 PYKNOSIS ASSAY

Cerebellar granule cultures at 6-7 DIV were transfected as described. Neurons were co-transfected with GFP marker plasmids and either empty vector (pCMV), or pEBS7-Bcl2 (in **Publication II**) or pEF-toxin-C3, or pRK5-Rac-T17N (in **Publication IV**). 24 hours after transfection, cells were treated with or without glutamate treatment as described above and culture medium were replaced for 3 hours. In **Publication III,** neurons were co-transfected with GFP marker plasmids and either empty vector (pCMV), GFP-nNOS-PBD, GFP-PSD95-PDZ1, GFP-PSD95-PDZ2, or GFP-PSD95-PDZ3. 24 hours after transfection, cells were treated with or without glutamate or nitric oxide donor Dea/NO as described above. After treatments (typically after 3 hours or as indicated), both transfected and untransfected cells were fixed with 4% paraformaldehyde, rinsed with ice-cold PBS and stained with Hoechst 33342. For transfected neurons, fluorescence

image fields of GFP emission using 450-490 nm excitation light and a 20x air objective are taken to locate transfected cells, and the corresponding image of Hoechst fluorescence is examined to determine whether transfected cells had pyknotic nuclei. Four evenly spaced fields were counted per coverslip. Imaging of DNA dyes and transfected fluorescent proteins was carried out with a cooled Apogee KX85 CCD camera under control of MicroCCD software (Diffraction Limited, Ontario, Canada) and IX70 Olympus microscope and 20 x air objective (N.A. 0.4) with appropriate filter cubes. For untransfect cells, they were scored on the basis of nuclear morphology. A pyknotic nucleus was taken to indicate death of the cell.

4.3.3 CASPASE ASSAYS

Caspase 3-like (DEVDase) activity in cell extracts was assayed using fluorogenic substrate DEVD-AMC (Pharmingen) using amounts of extract and substrate according to the manufacturer's protocol. Cell extract was prepared by the treatment of cells with ice-cold cell extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 130 mM NaCl, 2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100). DEVDase activity was measured after incubation of extract with 20 μM DEVD-AMC for 1 hour at 37 ^{o}C.

4.3.4 ANALYSIS OF DNA INTEGRITY

This was performed essentially as described earlier (Solovyan et al., 2002). Briefly, cells were embedded into low-melting point agarose drops followed by incubation for 1 hour at 37°C in lysis buffer (20 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.5% SDS) containing 100 μg/ml Proteinase K and 100 μg/ml RNase A. Agarose drops containing deproteinated DNA samples were loaded quantitatively to the wells of 1% agarose gel and subjected to either conventional or field inversion gel electrophoresis (FIGE). Conventional gel electrophoresis was carried out at 70 V for 3-4 hours using 0.5 x TBE buffer (0.089 M Tris, pH 8.5, 0.089 M boric acid, 0.002 M EDTA). FIGE was performed at 85 V for 18 hours in 0.5 x TBE buffer under constant pulses of electric field (24 seconds forward and 8 seconds backward) allowing resolution of DNA molecules of size up to 500 kb (Heller and Pohl, 1989).

4.3.5 DETERMINATION OF LACTATE DEHYDROGENASE (LDH) RELEASE

LDH release to the extracellular medium was measured at time points from 3-24 hours after glutamate exposure. Cell culture media were collected and cells were lysed by Promega cytotoxicity assay kit. Released LDH in culture supernatants was measured with a 30-minute coupled enzymatic assay, which resulted in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed was proportional to the number of lysed cells. Data were collected at the wavelength of 492 nm.

4.3.6 ELECTRON MICROSCOPY

Cells were fixed for 30 minutes with 2.5 % glutaraldehyde in 0.1 M sodium-phosphate buffer (PBS) pH 7.4, washed three times with the same buffer and then post-fixed with 1 % osmic acid in PBS, dehydration and embedding with LX-112 resin (Ladd Research Industries, Inc.). Sections (50 to 70 nm thick) were mounted on copper grids and then examined by transmission electron microscopy using a JEM-1200 EX microscope (JEOL, Tokyo, Japan).

4.4 EVALUATION OF KINASE ACTIVITIES

4.4.1 AFFINITY PURIFICATION ASSAY

4.4.1.1 ACTIVATION OF TRANSFECTED P38α ASSAY

Cerebellar granule neurons were plated in 35 mm dishes for 7 days. In **Publication II**, neurons were co-transfected with EBG-p38α and either empty vector (pCMV) or pMT3-MKK3a-kinase dead "MKK3a-KD". In **Publication III,** neurons were co-transfected with EBG-p38α and either empty vector (pCMV) or GFP-nNOS-PBD. In **Publication IV**, neurons were co-transfected with the plasmids pEBG-p38α together with empty vector pCMV, pEF-toxin-C3, pRK5-Rac1-T17N, or pRK5-RhoQ63L as indicated. 24 hours after transfection, cells were treated with glutamate as described for 5 minutes, rinsed once with ice-cold PBS and lysed in 500 µl of lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM benzamidine, 1 µg/ml aprotinin, leupeptin, and pepstatin A, and 100 µg/ml PMSF) used for MKK3a-KD (in **Publication II**), nNOS-PBD (in **Publication III**) or pEF-toxin-C3, pRK5-Rac1-T17N, or pRK5-RhoQ63L (in **Publication IV**) pull-downs. Homogenized and precleared supernatants were incubated with 10 µl (bed volume) pre-washed S-Hexylglutathione-agarose beads (Sigma, MO) for 3 hours at 4°C. Beads were spun out and washed 3 times in lysis buffer followed by boiling aspirated pellet in 40 µl 1 x Laemmli sample buffer and immunoblotting. EBG-p38α ran at about 65kD, and anti-phospho-p38 and anti-pan-p38 immunoreactive bands shown migrate at this molecular weight.

4.4.1.2 DETECTION OF nNOS-PSD95 INTERACTION (in Publication III)

One day after plating in 10 cm dishes, COS7 cell cultures were co-transfected with GST tagged nNOS-PBD and either empty plasmid (pCMV), GFP-PDZ1, GFP-PDZ2, GFP-PDZ3 or GFP-C1 as indicated. After 48 hours of transfection, cells were rinsed once with ice-cold PBS and lysed in 800 µl low-salt buffer (20 mM Na_2 β-glycerophosphate, 30 mM NaF, 2 mM EDTA, 2 mM $Na_4P_2O_7$, 1 mM DTT, 10 µg/ml aprotinin, leupeptin, pepstatin A, 0.5 mM AEBSF, 0.5 % igepal). Homogenized and precleared supernatants

were rotated for 3 hours at 4°C with 15 µl (bed volume) S-Hexylglutathione-agarose beads (Sigma, MO) pre-equilibrated in low-salt buffer. Beads were spun out and washed 3 times in the same buffer followed by boiling the aspirated pellet in 50 µl 1 x Laemmli sample buffer for immunoblotting.

4.4.2 RHO AND RAC1 PULL DOWN ASSAY (in Publication IV)

Production of immobilised GST, GST-PAK-CD and GST-Rhotekin fusion proteins was as follows: *Escherichia coli* BL21(DE3) cells transformed with pGEX-KG (generous gift of John Kyriakis), pGEX-PAK-CRIB-domain (amino acids 56-272) or pGEX-Rhotekin-Rho-binding domain (amino acids 7–89) (generous gifts of Alan Hall) constructs was grown at 37°C to an absorbance of 0.7 OD_{595nm}. Expression of the recombinant proteins was induced by 0.3 mM isopropylthiogalactoside for 4 hours at 25°C, and purified by affinity chromatography on glutathione sepharose.

Rho and Rac activity were measured by the previously described Rhotekin and PAK-CRIB pull-down assays (Sander et al., 1998; Ren et al., 1999). 8 days after plating in 10-cm dishes, cerebellar granule neuron cultures were treated with or without glutamate 50 µM as above. After 3 minutes of glutamate addition, cells were rinsed with ice-cold PBS once and lysed in 800 µl lysis buffer (50 mM Tris pH 7.2, 500 mM NaCl, 1% (*v*/*v*) Triton X-100, 5 mM $MgCl_2$, 1 mM DTT and protease inhibitors). Homogenized lysates were precleared and supernatants were incubated with 10 µg immobilised GST bound to glutathione-sepharose to remove non-specific binding. Beads were spun out and lysates were rotated with 10 µg of immobilised GST-Rhotekin at 4°C for 30 minutes followed by two washes in 50 mM Tris pH 7.2, 150 mM NaCl, 1% (*v*/*v*) Triton X-100, 5 mM $MgCl_2$ and 1 mM DTT. The aspirated pellets were boiled at 95°C in Laemmli sample buffer, and active RhoA was detected by immunoblotting with monoclonal anti-RhoA antibody. Supernatants precleared with immobilised GST and GST-Rhotekin and adjusted to PAK-binding buffer (50 mM Tris pH 7.2, 100 mM NaCl, 1% (*v*/*v*) Triton X-100, 2 mM $MgCl_2$, 1 mM DTT and protease inhibitors) were incubated with 20 µg of immobilised GST-PAK-CRIB-domain fusion protein at 4°C for 30 minutes and washed 2 times with lysis buffer. Protein was eluted in sample buffer and blotted with anti-Rac1 monoclonal antibody.

4.4.3 PROTEIN KINASE ASSAY

Cerebellar granule neurons were stimulated as described, washed twice with PBS, and lysed in 500 µl of lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM benzamidine, 1 µg/ml aprotonin, leupeptin, and pepstatin A, and 100 µg/ml PMSF). Homogenized and precleared supernatants for immunecomplex kinase assay were incubated with antibodies specific for JNK1 (G151–333; PharMingen) or JNK2/3 (14–258; Upstate Biotechnology) followed by protein G and protein A Sepharose, respectively. Immobilized kinases were washed as previously described (Coffey et al., 2000). Active recombinant JNKs (see above) were sequestered from COS-7 cell lysates

using S-hexylglutathione agarose. Kinase activity toward GST-c-Jun (5–89) was measured as previously described (Coffey et al., 2000), with the addition of SB203580 as indicated.

4.4.4 REPORTER ASSAY

Luciferase reporter assays were carried out in primary cultured cerebellar granule neurons as previously described (Coffey et al., 2000). In **Publication I and II**, cells were transfected with a firefly luciferase reporter plasmid driven by five GAL4 elements in tandem, pGL3-G5E4Δ38, a plasmid expressing a fusion protein of the p38α-specific substrate MEF2A with the DNA binding domain of GAL4, and pRL-CMV expressing sea pansy luciferase as an internal standard against which signals were normalized, and either pcDNA3-MKK6E expressing a constitutively active MKK6 and pEBGp38α or empty vector (pCMV) as indicated. In **Publication I**, neurons were transfected with c-Jun promoter-driven luciferase reporter plasmid (pGL3-JC6), GAL4-luciferase reporter, GAL4-c-Jun(6–89) fusion construct, GAL4 fused to c-Jun (5–105) wild-type "wt"or Ser/Thr--Ala point mutants, Renilla luciferase internal control, empty vector (pCMV), and JIP-JBD. In **Publication II,** for withdrawal-induced c-Jun reporter assays, GAL4-Mef2 was replaced with GAL4-cJun (6-89) and MKK6E/p38α wild-type plasmids were not used. Dominant negative p38αAF and MKK3-KD plasmids were co-transfected where indicated. Empty vector (pCMV) was added to equalize transfections to 2 μg total DNA/24 well plate well. The following day cells were lysed in passive lysis buffer (Promega). Firefly (reporter) and Renilla (internal standard) luciferase activities were assayed with the dual luciferase assay kit (Promega) according to the manufacturer's instructions.

4.5 IMMUNOBLOTTING

Prepared samples in 1 x Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS (w/v), 5% 2-mercaptoethanol, 10% glycerol (v/ v), and 0.001% bromophenol blue (w/v)) were resolved by 10% or 15% of SDS-PAGE and transferred by semidry transfer onto nitrocellulose. Nitrocellulose was blocked with 5% milk in either TBS–Tween 20 (0.1%) or PBS–Tween 20 (0.05%).

Primary antibodies used as follows were incubated at 4^{o}C overnight. In **Publication I,** rabbit anti phospho-JNK (NEB, Beverly, MA; 1:1000)**,** rabbit anti-phospho- extracellular signal regulated kinase (ERK) (Promega; 1:20,000), rabbit anti-JNK2/3 ("SAPK1a"; Upstate Biotechnology, Lake Placid, NY; 14–258, 0.2μg/ml), rabbit anti-phosphoserine 73 c-Jun ((New England Biolabs, Beverly, MA; 1:1000), rabbit pan-JNK antibody ("JNK3"; Upstate Biotechnology; 1μg/ml), mouse anti-pan-ERK (Transduction Laboratories, 1:5000), mouse anti-flag (M2; Sigma, St. Louis, MO; 0.3μg/ml), mouse anti c-Jun (Transduction Labs, Lexington, KY; 0.5μg/ml), mouse anti-phosphoserine 63 c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA; 1μg/ml), mouse anti-GFP (Clontech; 0.2μg/ml), mouse anti-JNK1 (PharMingen, San Diego, CA; G151–333, 0.5μg/ml) were

used. Besides the above antibodies, rabbit anti cleaved caspase-3 (Trevigen, Gaithersburg, MD; 1:2000 (**Publication II),** rabbit anti phospho-p38 (NEB, Beverly, MA; 1:1000), mouse anti-pan-p38 (Transduction Labs, Lexington, KY; 1:5000) **(Publication II, III, IV),** mouse anti-actin (generous gift of Brigitta Jockusch, Braunschweig, Germany; 1:1000) **(Publication II),** mouse anti RhoA (Santa Cruz Biotechnology, Santa Cruz, CA; 1μg/ml), mouse anti Rac1 (Transduction Labs, Lexington, KY;1:1000) **(Publication IV)**, mouse monoclonal anti-cytochrome c (Biomol, SA-226; 1μg/ml), mouse anti-cytochrome oxidase (COX) subunit IV (Molecular Probes, A21348; 0.2μg/ml) **(Publication II)** were used. Rabbit anti-p38α and p38β were antisera raised in rabbit against unique peptides from the sequences of these proteins (1:20000 and 1:16000 respectively) **(Publication II).** Secondary reagents from various sources (Santa Cruz, UBI, Sigma, Molecular Probes) were incubated at 1:50000 at room temperature.

Blots were then developed using the enhanced chemiluminescence detection method. Films were preflashed with a "sensitize" preflash unit according to manufacturer's instructions (Amersham Biosciences, Arlington Heights, IL) so that signals were recorded from the film above the nonlinear response range. Multiple exposures were taken at different times, so that nonsaturated ECL film (Amersham) was used for quantitation. Films were digitized by densitometry with a flatbed transparency scanner and quantified by image analysis software developed by the authors.

4.6 IMMUNOCYTOCHEMISTRY

Coverslips with neurons at 7 DIV were fixed with 4% paraformaldehyde for 20 minutes followed by permeabilization in PBS/Triton X-100 (1%) for 3 minutes. After washing with PBS, cells were blocked with 10% serum/0.2% Tween-20/PBS. Primary antibodies mouse anti-JNK1, rabbit anti-JNK2/3 or without primary antibody were incubated overnight at 4°C followed by 1 hour incubation with Alexa 488 for JNK1 or 1 hour incubation with biotin-conjugated goat anti-rabbit IgG and then streptavidin Alexa-488 for JNK2/3 (Molecular Probes, Leiden, The Netherlands).

4.7 CALCIUM IMAGING

Fura-2 calcium imaging was performed with an Incyte imaging system, with alternating narrow band 340 and 380 nm excitation filters and a 500 nm dichroic cube with 515/30 nm emission filter. Excitation and emission times were optimized to reduce the 340 signal and increase the 380 signal to maximize the dynamic range and avoid camera saturation. Background-corrected ratios were calculated with image analysis software described (Lindqvist et al., 1995). GFP was imaged with 450-490 nm excitation filter and the same dichroic cube.

4.8 ELECTROPHYSIOLOGICAL RECORDING

Whole-cell currents were recorded from single cultured cerebellar granule cells (at 7 DIV) and transfected with either pEGFP-C1 (empty vector) or the PSD95 PDZ2-binding region of nNOS (pEGFP-nNOS), using an Axopatch 200B amplifier (Axon Instruments). Recorded membrane currents were digitized using a Digidata 1320A interface (Axon Instruments) and analysed using pCLAMP software. Patch pipettes were pulled from thin-wall borosilicate glass (outer diameter 1.5 mm, inner diameter 1.17 mm; Clarke Electromedical, Pangbourne, UK) and fire polished to give a final resistance of ~5 MΩ when filled. The pipette-filling solution contained (mM): 145 K gluconate, 10 HEPES, 5 EGTA, 5 $MgCl_2$, 5 Na_2ATP, 0.2 GTP; adjusted to pH 7.2. The extracellular solution contained (mM): 145 NaCl, 5 KCl, $1CaCl_2$, 5 HEPES, 5 glucose, 20 sucrose; adjusted to pH 7.4. NMDAR currents were elicited at a holding potential of –60 mV by the rapid (U-tube) application of 200 μM NMDA in extracellular solution containing 20 μM D-serine, 50 μM bicuculline, 1 μM tetrodotoxin, and 5 μM NBQX.

4.9 FRET-BASED IMAGING OF CYTOPLASMIC FREE CALCIUM

Cerebellar granule neurons were cultured on 10-mm square glass coverslips and cotransfected at 6-7 DIV with "precocious cameleon" YC2.12 (Nagai et al., 2002) and pEBG vector or pEBG-nNOS-PBD as indicated. This calcium probe is based on the Venus YFP variant which is most resistant to changes in pH and chloride, and matures quickly, thereby avoiding artifactually low FRET caused by retarded maturation of the acceptor fluorophore. The following day, the coverslips were washed once in Locke's buffer with 1 mM $MgCl_2$, twice more in Locke's buffer without $MgCl_2$, and placed in 1 ml of Locke's buffer without $MgCl_2$ in a chamber on a microscope (model IX70; Olympus). Cells were illuminated with a mercury lamp, a neutral density filter, and a 440 nm/21 nm excitation filter. CFP and YFP emission image pairs were acquired (2-second integration time per channel and 13 seconds between image pairs) through a 455 nm with a dichroic mirror and 480 nm/30 nm and 530 nm/26 nm filters in a filter wheel (CVI laser; Apogee Instruments, Inc.) mounted parfocally in front of a cooled CCD (model KX85; Apogee Instruments Inc.). The acquired CFP emission images represent CFP signals quenched by FRET, and are referred to hereafter as CC images. The acquired Venus-YFP emission images represent raw FRET signals, and are referred to hereafter as CY images. As calcium rises, it causes the probe to fold to a more compact conformation, leading to increased CY signal and quenching the CC signal. The ratio of background-corrected CY and CC signals was calculated and is directly related to calcium changes. NMDA and glycine (at final concentrations of 100 μM and 10 μM, respectively) were added at time 0. FRET time courses (CY/CC ratio) were calculated from the acquired image series with image analysis software developed by the authors (Lindqvist et al., 1995) that had been modified to permit the processing of these image datasets as described above. Background-corrected fluorescence ratio time courses were normalized to the average prestimulation CY/CC ratio values for each cell, the means of cells within each field were calculated, and the data presented as mean ± S.E.M of independent experiments.

4.10 SUBCELLULAR FRACTIONATION

8 days after plating in 6 cm dishes, cerebellar granule neuron cultures were treated with glutamate 50 μM or trophic support was withdrawn as described above. 60 minutes after beginning the 30-minute glutamate treatment or after 7 hours of trophic support withdrawal, cells were rinsed with ice cold PBS once and lysed in 700 ul homogenization buffer (0.32 M sucrose, 1 mM EDTA, 2 mM $MgCl_2$, 20 mM Tricine-NaOH, pH 7.8, 10 μg/ml aprotinin, leupeptin, and pepstatin A, and 100 μg/ml PMSF). Homogenized lysates were cleared of nuclei and unbroken cells by centrifugation at 100 g for 5 minutes, followed by collection of crude mitochondrial pellet by centrifugation at 16000 g for 20 minutes. The location of nuclei and mitochondria were checked in parallel by staining DNA with Hoechst 33342 and mitochondria with mitotracker (Molecular Probes, Eugene, OR). Crude mitochondrial pellets were lysed in Laemmli sample buffer. Protein was precipitated from 250 μl of supernatants by adding 1250 μl acetone, incubating at -20 °C for 10 minutes and centrifugation at 12000 g for 5 minutes. The precipitated cytosolic protein was dried and lysed in Laemmli sample buffer. The location of cytochrome c was assessed by immunoblotting crude mitochondria and cytosol samples with monoclonal anti-cytochrome c (Biomol, SA-226). Equal loading of mitochondria and cytosol was assessed by immunoblotting with mouse anti-cytochrome oxidase (COX) subunit IV (Molecular Probes, A21348) and by coomassie staining respectively.

5. RESULTS

5.1 DISTINCT REQUIREMENTS FOR P38α AND JNK

Table 3. Distinct requirements for p38α and JNK in different forms of apoptotic neuronal death.

INVESTIGATIONS		WITHDRAWAL OF TROPHIC SUPPORT	GLUTAMATE
Selective activation of stress-activated protein kinases	**JNK and p38**	JNK (by monitoring the phosphorylation of c-Jun)	p38 (by monitoring the phosphorylation of p38) peaking at 5 minutes
Activation of caspases	**Cleaved caspase 3,7 and DEVDase**	Strongly induces cleaved caspase 3,7 and DEVDase activity	Does not induce cleaved caspase 3 or significant DEVDase activity
	Expression of CrmA (inhibit caspase 1, 8) and CrmA-DQMD (inhibit caspase 3)	CrmA-DQMD significantly reduces trophic support withdrawal-induced neuronal cell death	Neither of them reduce glutamate-induced neuronal cell pyknosis
Internucleosomal DNA fragmentation profiles	**Field inversion agarose gel electophoresis**	Evokes formation of DNA fragments smaller than 50-kb sensitive to zVAD-fmk	Evokes formation of 50-kb fragments prevented by MK-801 or 1 μM SB203580
	Conventional gel electrophoresis	Reveals 200bp and larger oligonucleosomal	No smaller fragments were observed
Nuclear morphology	**Chromatin morphology (electron microscopy)**	Condensed chromatin morphology	Lumpy morphology
Mitochondrial protein	**Release of cytochrome c from mitochondrial to cytosol**	Easily detectable cytosolic cytochrome c	Lower level of cytosolic cytochrome c
Effects of inhibitors on neuronal cell death	**1,9-anthropyrazolone (SP600125) (inhibitor of JNKs)**	1-3μM inhibits JNK activity and subsequent neuronal death	1-3 μM does not inhibit p38 activity or subsequent neuronal death
	SB203580 (inhibitor of p38)	1 μM does not inhibit JNK activity or neuronal death	1 μM inhibits p38α activity and neuronal death
	NMDA receptor blocker (MK801)	No effects on trophic support withdrawal-induced neuronal cell death	Strongly prevents glutamate-induced neuronal cell death
	Pancaspase inhibitor (zVAD-fmk)	Strongly prevents trophic support withdrawal-induced neuronal cell death	No effects on glutamate-induced neuronal cell death
Expression of dominant negative constructs	**p38αAF and MKK3KD**	Neither of them reduces JNK activation	Both of them reduce p38 activation and glutamate-induced loss of membrane integrity

5.2 REGULATION OF TROPHIC SUPPORT WITHDRAWAL INDUCED JNK PATHWAY

The transcription factor c-Jun is a major stress-activated protein in neuronal cells and is likely to coordinate transcriptional programs in response to stress. Our immunoblotting reveals rapid shift of c-Jun protein mobility within 15 minutes after trophic support withdrawal, which is confirmed by parallel detection of phospho-c-Jun species by immunoblotting with phosphoserine 63 c-Jun and phosphoserine 73 c-Jun antibodies. The changes in phosphorylation and mobility precede the increase in total c-Jun protein that begins after 60 minutes, without detectable change in JNK activation. It indicates total JNK activity correlates poorly with c-Jun regulation after withdrawal of trophic support. In addition, neurons from wild type mice showed negligible activation of total JNK in response to withdrawal of trophic support, while neurons from Jnk1$^{-/-}$ mice had lower basal activation, and a trophic withdrawal-evoked activation of total JNK was revealed. Both of them showed the same level of c-Jun at 4 hours of treatment. These data show that cerebellar neurons possess a high basal JNK1 activity which does not phosphorylate c-Jun in response to withdrawal of trophic support.

Furthermore, immunofluorescence staining indicates that JNK1 is retained in the cytoplasm in punctuate structures, especially visible along processes, whereas JNK2/3 is more diffuse and more localized to the nucleus, where c-Jun is expected to be located. There is no overall nuclear translocation of JNK1 and JNK2/3 in response to this stress. SB203580 is a p38 inhibitor, which at high concentration can also inhibit JNKs (Whitmarsh et al., 1997; Coffey et al., 2000). We found that 10 μM SB203580 was able to significantly inhibit neuronal JNK2/3 activity, mobility shifts of c-Jun phosphorylation and increase of total levels of c-Jun, and partially prevented neuronal death, but was unable to inhibit total JNK and JNK1 in response to stress. 1 μM SB203580, which is sufficient to inhibit activation of p38, cannot prevent trophic withdrawal-induced cell death. These data support that JNK2/3 specifically targets c-Jun during neuronal stress in the presence of constitutively elevated JNK1 activity. Inhibition of JNK2/3 but not p38α may prevent this form of neuronal cell death.

In addition, increased levels of c-Jun PCR products in response to stress can be partially inhibited by 10 μM SB203580. Together with the finding that the c-Jun promoter activated by trophic support withdrawal can be inhibited by JIP-JBD, a construct that inhibits JNK, it suggests that JNK is required for transcriptional upregulation of c-jun mRNA at the promoter level by withdrawal of trophic support from cerebellar granule neurons.

5.3 GLUTAMATE-INDUCED REGULATION OF P38 PATHWAY AND SUBSEQUENT NEURONAL CELL DEATH

5.3.1 GLUTAMATE INCREASES INTRACELLULLAR CALCIUM LEVELS AND P38 ACTIVATION

Glutamate is believed to underlie the neuronal death in which JNK and p38 are implicated. In our observation, addition of glutamate to cerebellar granule neuron cultures leads to a transient but strong activation of p38 peaking at 5 minutes, with little elevation at 30 minutes. Glutamate-induced neuronal pyknosis was already detectable after 60 minutes, and was completed by 3 hours preceeding loss of membrane integrity by 6 hours. Addition of 1 μM SB203580 30 minutes before the glutamate treatment strongly prevents the pyknosis, while adding the inhibitor 30 minutes after the glutamate exposure shows no protection. Furthmore, glutamate induces a large calcium spike preceding the peak of p38 phosphorylation, followed by a sustained elevated level of neuronal cytoplasmic free calcium. The p38 response can be reduced by NMDA receptor antagonist MK-801; while inhibition of p38 can not prevent the calcium response. These suggest that the early transient increase of p38 activity is important for glutamate-induced pyknosis and the p38 activation was entirely dependent on extracellular calcium.

Bcl-2 promotes the integrity of the mitochondrial membrane and inhibits release of proapoptotic factors (Adams and Cory, 1998). We found that expression of Bcl-2 significantly reduced neuronal pyknosis and was able to maintain long term cell viability subsequent to glutamate. However, expression of Bcl-2 had no significant effects on either peak or sustained increase of the glutamate-evoked calcium response. Therefore it suggests that inhibition of glutamate-induced death by Bcl-2 cannot be ascribed to an influence on the cytoplasmic free calcium response.

5.3.2 REGULATION OF PSD95-nNOS INTERACTION IN RESPONSE TO GLUTAMATE

NO has been shown to contribute to excitotoxic neuronal cell death (Dawson et al., 1996). Our data suggested an essential role for p38 in glutamate-evoked neuronal cell death. In ordr to investigate the possible relationship between NO and p38, we first examined the effect of nNOS inhibitors on p38 activation. 7-nitroindazole ("7-NI"), which does not discriminate between NOS isoforms and N-ω-propyl-L-Arginine ("N^{w}-PLA"), which selectively targets nNOS (Zhang et al., 1997), both of them significantly reduce the glutamate-induced p38 activation at 5 minutes and the following neuronal pyknosis. Additionally, we found that NONOate diethylamine/NO adduct (Dea/NO), which degrades with a t½ of 2.1 minutes (37 °C) to release NO, at 250 μM or 10 μM induces a substantial increase in phospho-p38 level at 5 minutes, which cannot be prevented by MK801. The concentration of peroxynitrite ($ONOO^{-}$) reported to activate p38 in 293 cells (300 μM, (Ge et al., 2002)) also induces a rapid p38 phosphorylation. Both of them induce rapid pyknosis, in a similar manner to glutamate, which zVAD-fmk fails to

prevent. We concluded that glutamate-induced activation of p38 and subsequent cell death require activity of nNOS and can be reproduced with NO donors.

It has been shown that PSD95 can recruit the calcium-dependent nNOS to the mouth of the calcium-permeable NMDA receptor, and depletion of PSD95 inhibits excitotoxicity (Sattler et al., 1999; Aarts et al., 2002). We developed a construct we named nNOS-PBD (PSD95-Binding Domain) expected to bind PSD95 in a manner identical to, and therefore competitive with, endogenous nNOS. Compared with empty vector, co-transfection of nNOS-PBD substantially reduced the p38 activation in response to glutamate. Similarly, nNOS-PBD greatly reduces glutamate-induced pyknosis. However, nNOS-PBD is unable to inhibit the p38 activation and subsequent pyknosis challenged with NO donors. Furthermore, patch-clamp recordings of whole cell currents induced by the rapid application of NMDA and cotransfection of a FRET-based calcium reporter "precocious cameleon" or YC2.12 (Nagai et al., 2002) demonstrated that cells transfected with nNOS-PBD or vector control were indistinguishable, showing that neither NMDA currents nor the evoked calcium responses were affected by nNOS-PBD. Together, these data support that the nNOS-PBD construct reduces glutamate-induced p38 activation and pyknosis by acting upstream of NO production, without causing general perturbation of either the electrophysiological characteristics of the NMDA receptor or the downstream signaling pathways.

In addition, we found that both PDZ2 and PDZ1 conferred neuroprotection, but PDZ3 failed to protect. It suggests that construct of PSD95 PDZ2 domain binding to nNOS is also neuroprotective, while PDZ1 domain may confer its neuroprotection by interaction with the C-terminus of NMDA receptor subunits, thereby dissociating the entire PSD95 from NMDA receptors (Aarts and Tymianski, 2003).

5.3.3 REGULATION OF RHO GTPase IN RESPONSE TO GLUTAMATE

Rho has been reported to be regulated by calcium in Xenopus brain (Li et al., 2002). Our data have shown that glutamate-induced activation of p38α in cultured cerebellar granule neurons is dependent on extracellullar calcium and the calcium-permeable NMDA receptor channel. We investigated whether Rho may contribute to this response. Transfection of neurons with C3 toxin, which ADP-ribosylates Rho and prevents it from interacting with effectors (Genth et al., 2003), prevented glutamate-evoked activation of co-transfected p38α and the excitotoxic cell death. Using pull-down assay with recombinant rhotekin-RBD immobilised on beads (Ren et al., 1999), we could detect a significant increase in Rho activity 3 minutes after glutamate stimulation preceding the peak of p38 activity. In addition, we applied fluorescence-resonance energy transfer (FRET) probe of Rho activation, Raichu-RBD (Yoshizaki et al., 2003). This probe consists of Venus-YFP and CFP moieties separated by rhotekin-RBD. Rhotekin-RBD specifically interacts with activated forms of RhoA, RhoC and to a lesser extent RhoB (Reid et al., 1996). Thus activation of Rho leads to binding to rhotekin-RBD and increased separation of the fluorophores and consequent loss of FRET. Cells transfected with this probe were monitored by emission of CFP and Venus-YFP fluorescence, and Rho activation leads to a loss in FRET observed as an increase in CFP/YFP emission

ratio (FRET ratio). Addition of glutamate induced a rapid rise in FRET ratio in more central regions of the cell body. Furthermore, compared with empty vector, constitutively active Rho mutant RhoA-Q63L cotransfected with p38α revealed a strong activation of p38α in cerebellar granule neurons, but was undetectable in neuro 2A cell lines. These data indicate that active Rho is sufficient to activate p38α, which is restricted to the primary cultured neurons.

The activation timecourse of FRET is rapid and consistent with being upstream of the p38 response. The presence of the NMDA channel blocker MK-801 as well as the cotransfection of C3 substantially reduced the glutamate-evoked FRET response. This suggests that the glutamate-evoked FRET response is dependent on not only NMDA receptor activity but also Rho activity. Additionally, we utilised this highly sensitive FRET Rho probe to investigate if the elevation of intracellular free calcium is sufficient for activation of Rho. Depolarisation of the plasma membrane of cerebellar neurons by addition of 30 mM KCl to the extracellular medium induces a characteristic calcium response (Courtney et al., 1990b). Addition of 30 mM KCl induced an increase in FRET ratio (+KCl +Ca trace). Addition of KCl in the absence of extracellular calcium also induced a small response above baseline, possibly due to the minor osmotic stress (+ KCl –Ca). However, a substantial proportion of the KCl response was dependent on the presence of calcium. Addition of KCl or LPA induced a rapid increase in p38 activation similar to that induced by glutamate, but without neuronal death. These data support our proposal that p38 activation by Rho is a necessary but not sufficient requirement for glutamate-induced cell death.

6. DISCUSSION

6.1 DISTINCT ROLE OF p38α AND JNK IN DIFFERENT FORMS OF APOPTOTIC NEURONAL DEATH

Stress-activated protein kinases (SAPKs) are believed to play an obligatory role in neuronal cell death. Early observations in PC12 cells suggested a contribution of both p38 and JNK families in caspase-dependent apoptosis induced by withdrawal of nerve growth factor (Xia et al., 1995). However, there is no study to explain any differential roles of these kinases in neuronal cell death. The use of SB203580 has implicated a p38 contribution to axotomy-induced apoptosis of retinal ganglion cells (Kikuchi et al., 2000), glutamate-induced apoptosis of cerebellar granule neurons (Kawasaki et al., 1997) and ceramide-induced death of cortical neurons (Willaime et al., 2001). However, we found that 10 μM SB203580 typically used to target p38 also can inhibit JNK-dependent death and there is a role of the p38 kinase in survival pathways (Mao et al., 1999; Okamoto et al., 2000). This led us to reconsider the conclusions of p38 relying on SB203580. Therefore we compared cerebellar neuronal death induced by glutamate or by withdrawal of trophic support and the contributions of JNK and p38 stress-activated protein kinases to corresponding form of death.

Withdrawal of trophic support from cerebellar granule neurons activated both JNK and p38. Phosphorylation of c-Jun was increased before the subsequent increase of c-Jun protein, while only a very minor change was detected in total JNK activity in response to this stress, consistent with previous negative results (Gunn-Moore and Tavare, 1998; Watson et al., 1998). This indicates either a special increase in phosphorylation or decrease in dephosphrylation of c-Jun during this early stage of withdrawal-induced signaling. Cerebellar granule neurons possess a high basal JNK1 activity that does not phosphorylate c-Jun in response to the stress; rather it masks the lower JNK2/3 activity which is activated by this stimulus. This confirms the hypothesis that the stress-activated pool of JNK in cerebellar granule neurons corresponds to specific isoforms.

The regulation of the p38 MAPK in response to trophic support withdrawal is controversial. One report found no response, by using antibody raised against a Xenopus sequence (Rouse et al., 1994) similar to mammalian p38α and β, but quite different from γ and δ isoforms. Another report using a commercial antibody of undefined specificity showed an increase in activity (Yamagishi et al., 2001). We dectect increased phosphorylation of p38α and/or δ after trophic withdrawal. 1 μM SB203580 strongly inhibits p38α activity against multiple substrates in cerebellar granule neurons, in HeLa cells (Coffey et al., 2000) and *in vitro*, while 10 μM is required to block recombinant JNK2 isoforms (Whitmarsh et al., 1997) and stress-activated endogenous JNK2/3 from neurons. The ability of 10 μM but not 1 μM of SB203580 to block not only c-Jun phosphorylation in response to different stresses but also to block increased c-Jun transactivation capacity strongly suggests that JNK2/3 are good candidates responsible for the c-Jun activation in neurons, but p38α is not.

Glutamate-induced neuronal death has been considered a model of neuronal death during stroke and is also implicated in other disorders. Different forms of cell death are reported

in ischaemic brains (Didenko et al., 2002). There is evidence that JNK inhibition reduces neuronal death after cerebral ischaemia, although the protection is incomplete (Borsello et al., 2003). We found that the responses of p38, JNK and c-Jun to glutamate, at concentrations in the range recorded in stroke models (Lipton, 1999), are quite similar to those observed after trophic support withdrawal (Coffey et al., 2002). One difference is that the glutamate-induced p38 response is more transient without consistent detectable induction of c-Jun protein. This raises the possibility that glutamate does not induce a gene responsible for the death in this model, which can be supported by our unpublished data that the glutamate-induced death is not blocked by inhibitors of transcription and translation. In contrast, our data and previous studies show that JNK phosphorylates c-Jun, mediates expression of death genes and neuronal death in response to withdrawal of trophic support (Watson et al., 1998)).

The SAPK dependence of the ensuing cell death, measured by pyknosis, differs strikingly between the two models. Glutamate-induced pyknosis is potently blocked by 1 μM of SB203580 that selectively inhibits p38 and have no effect on the death induced by withdrawal of trophic support. In contrast, the JNK inhibitor 1, 9-pyrazoloanthrone (SP600125) eliminates death induced by withdrawal of trophic support without effect on glutamate-induced death. SB203580 inhibits p38α and β, but not γ or the δ isoform (English and Cobb, 2002). However, it is unclear which of the α/β isoforms are expressed in cerebellar granule neurons. Lysates of 293 cells transfected with either FLAG-p38α or FLAG-p38β permitted us to test the sensitivity of p38α and β antiserum. Neurons blotted with the same isoform-specific p38α and β antiserum do not express detactable p38β, which indicates that p38α is responsible for p38 responses that are SB203580-sensitive. In addition, MKK3 dominant negative isoform used does not interact with p38β (Enslen et al., 1998). This may suggest that it is p38α that mediates the glutamate-induced death.

Caspases typically confer specific chromatin cleavage patterns and chromatin morphologies to different forms of cell death (Leist and Jaattela, 2001). JNK-dependent death exhibited internucleosomal DNA fragmentation and uniformly condensed chromatin that did not fragment to multiple bodies, which is consistent with caspase-dependent death (Kerr et al., 1972; Thornberry and Lazebnik, 1998). In contrast, p38-dependent death induces no caspases, leading to accumulation of high-molecular weight DNA fragments and “lumpy” chromatin morphology which has been reported in caspase-independent death mechanisms and in ischaemic brain (Fukuda et al., 1999; Leist and Jaattela, 2001; Sheldon et al., 2001; Didenko et al., 2002). Furthermore, inhibition of caspases with Crm-A based plasmids or with cell permeable pan-caspase inhibitor zVAD had no effect on the p38-dependent death, whereas JNK-dependent death was blocked by either zVAD or caspase 3-inhibiting CrmA, consistent with previous observations that peptide inhibitors of caspases inhibit death induced by withdrawal of trophic support (Gerhardt et al., 2001). Interestingly, both forms of cell death were prevented by Bcl-2 overexpression, which may suggest that mitochondria probably play a central role in both forms of cell death. This is supported by our observation that we did observe a small but detectable release of cytochrome c into the cytoplasm after glutamate treatment, although it was not as clear as the response to withdrawal of trophic support. There are a number of possibilities that might explain why this may not result in activation of caspases. It is possible that an insufficient amount of cytochrome c is released in order to activate

caspase. Alternatively, other pathways may prevent the activation of cytochrome c after glutamate treatment. For example it has been reported that calpain cleaves apaf-1 and can prevent caspases activation in response to excitotoxic stimulus (Lankiewicz et al., 2000; Reimertz et al., 2001).

The differences between these two forms of neuronal death are evident, but there is also some evidence for a role of JNK and/or of caspases in excitotoxicity in other models (Yang et al., 1997; Le et al., 2002; Borsello et al., 2003). In addition, glutamate has been reported to induce different forms of cell death even in the same cell type (Armstrong et al., 1997; Kawasaki et al., 1997). In conclusion, we find that exposure of cerebellar granule neurons to two different neurodegenerative stimuli - withdrawal of trophic support and glutamate induces distinguishable forms of death on multiple levels.

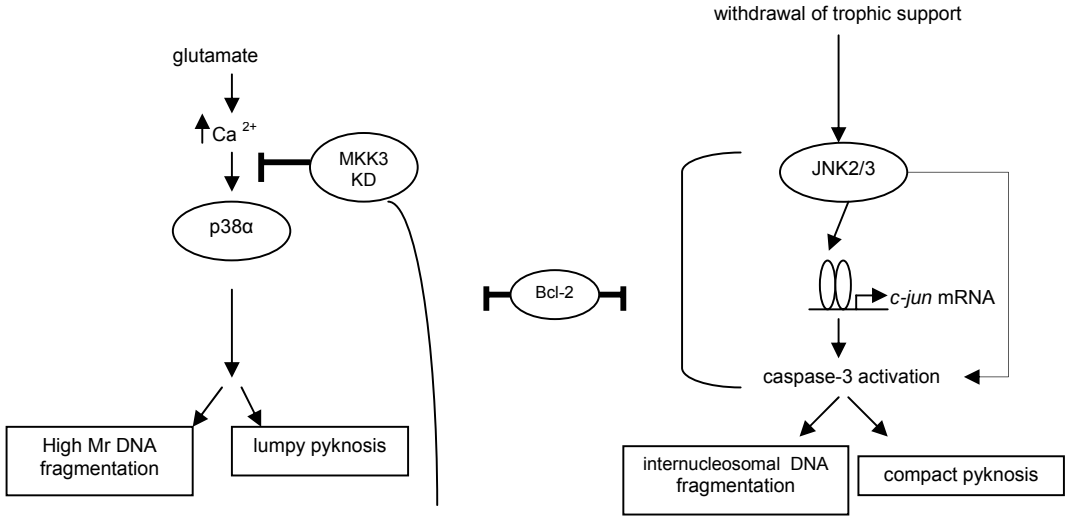

Figure 4. The selective contribution of p38 and JNK to different forms of neuronal cell death.

The model of glutamate-induced neuronal death has been established and characterized, but there are still gaps in our knowledge of what occurs between glutamate-induced calcium increase and the activation of p38α. Therefore based on the above model, we continued to investigate the upstream regulators of p38α pathway.

6.2 THE REGULATORS OF GLUTAMATE-INDUCED P38 PATHWAY

6.2.1 nNOS-PSD95 INTERFACE MEDIATES THE GLUTAMTE-INDUCED P38 PATHWAY

Our studies and other recent evidence suggest an essential role for the stress-activated protein kinase p38 in excitotoxic neuronal cell death in cultured cerebellar neurons (Kawasaki et al., 1997), hippocampal neurons (Legos et al., 2002) and cerebral ischemia *in vivo* (Legos et al., 2001). NO contributes to excitotoxic neuronal cell death (Huang et

al., 1994; Dawson et al., 1996). NO species are also known to activate p38 in non-neuronal cells, but this depends on a TAB1-mediated p38 autophosphorylation mechanism (Ge et al., 2002) that is not required for glutamate-evoked neuronal p38 activation (unpublished data). In cerebellar granule neurons, an activation of p38 after 3 hours of incubation with NO donor has been reported (Lin et al., 2001). However, there is little information of the possible relationship between NO and p38 in glutamate-induced cerebellar neuronal death. Previous studies showed that nNOSα knockout mice exhibit a reduced sensitivity to ischaemic neuronal death (Huang et al., 1994) and reduced NMDA-induced death of cortical cultures (Dawson et al., 1996). In addition, small molecule nNOS inhibitors are neuroprotective (Chabrier et al., 1999). In our present studies, both the p38 activation and cell death were inhibited by NOS and nNOS inhibitors after glutamate treatment, suggesting that nitric oxide synthase activity was necessary for the activation of p38 which is critical for glutamate-evoked cell death. Furthermore, NO donor was capable of activating p38 and cell death, in a manner strikingly similar to death induced by glutamate, which is consistent with the proposal that glutamate-induced cell death involves generation of nitric oxide. This provided a necessary prerequisite for further investigations of possible neuroprotective value of targeting the nNOS-PSD95 interaction which has been unexplored. The source specificity hypothesis is another possible prerequisite. It suggests that Ca^{2+} dependent toxicity is not simply a function of increased Ca^{2+} influx; rather it is regulated through distinct Ca^{2+}-signaling pathways linked to specific routes of Ca^{2+} influx (Tymianski et al., 1993), for example, calcium influx through NMDA receptors is especially neurotoxic. If neurotoxicity is dependent merely on calcium load not on localization, then nNOS would continue to contribute to neurotoxicity even if no longer physically associated with the NMDA receptor. The activity of nitric oxide synthase also appear to be necessary (and sufficient) for the activation of p38 which is critical for glutamate-induced cell death. Therefore any neuroprotection that is the result of disrupting the nNOS-PSD95 interface should inhibit glutamate-evoked p38 activation as well as cell death.

It has been shown that the PDZ domain of neuronal nitric oxide synthase (nNOS) binds to the second PDZ domain of PSD95, and the C-terminal domain of the NR2 subunit is able to bind the first and second PDZ domains in PSD95 (Brenman et al., 1996); (Niethammer et al., 1996). Recent evidence also showed that ablation of PSD95 or uncoupling it from the NMDA receptor provides substantial neuroprotection in an ischaemia model (Sattler et al., 1999; Aarts et al., 2002). However, it remains unclear whether the neuroprotective effects are the result of uncoupling nNOS from glutamate-evoked calcium influx or the results of other signaling pathways attached to glutamate receptors (Aarts and Tymianski, 2003), as PSD95 is a multi-domain protein believed to be a central mediator of assembly of the post-synaptic density complex. It consists of a vast array of signaling and structural molecules coupled to glutamate receptors in these specialized regions of neuronal cells (Husi et al., 2000). Thus in addition to dissociation of nNOS from the NMDA receptor, complete removal of PSD95 in the above reports can be anticipated to bring the consequences which may potentially have the undesirable side effects (Aarts and Tymianski, 2003). So the disruption of the interaction between nNOS and PSD95 interface may give the possibility to increase the specificity of inhibition. PSD95 mediates coupling of NMDA receptors to nNOS, so that it can be efficiently activated by glutamate-mediated calcium influx (Sattler and Tymianski, 2000). Based on

this mechanism, we used the NH_2-terminal fragment of nNOSα to develop a construct which was expected to bind PSD95, such that it competes with endogenous nNOS. nNOSα possesses a PDZ domain which can interact with the second PDZ domain of PSD95 (Brenman et al., 1996). This domain is absent in nNOSβ and γ, but the catalytic domains are identical in all these isoforms (Alderton et al., 2001). The fragment contains the nNOS PDZ domain and adjacent β finger, both of which are required for binding PSD95 PDZ2 domain (Christopherson et al., 1999; Tochio et al., 2000). Expression of this construct in neurons inhibited p38α activation and reduced the subsequent cell death induced by glutamate. Moreover, the normal electrophysiological characteristics of the NMDA receptors and the cells' calcium response to NMDA were unaffected, which suggests that the action of nNOS-PBD was specific to the NMDA receptor-p38 pathway. The possible target of nNOS-PBD may be PDZ2 of PSD95 (or related molecules such as PSD93 (Brenman et al., 1996), leading to inhibition of the nNOS-PSD95 interaction. Overexpression of nNOS-PBD had no effect on NO-induced activation of p38 or the subsequent neuronal death. This further confirms our hypothesis that if the nNOS-PBD acts by preventing interaction of PSD95 (or a related molecule) with nNOS, thereby preventing NO production and the downstream consequences, then it should be possible to bypass this effect by exogenously supplying NO. Additional evidence for the role of this interaction is that expression of PDZ2 competing with endogenous PSD95 in the nNOS interaction again inhibited glutamate-induced neuronal cell death, wheares PDZ3 had no effect. PDZ1 was also somewhat protective as it can interact with the C-termini of subunits of the NMDA receptor complex (while, simultaneously, PDZ2 can bind nNOS) (Christopherson et al., 1999), thereby dissociating the entire PSD95 molecule from NMDA receptors (Aarts and Tymianski, 2003).

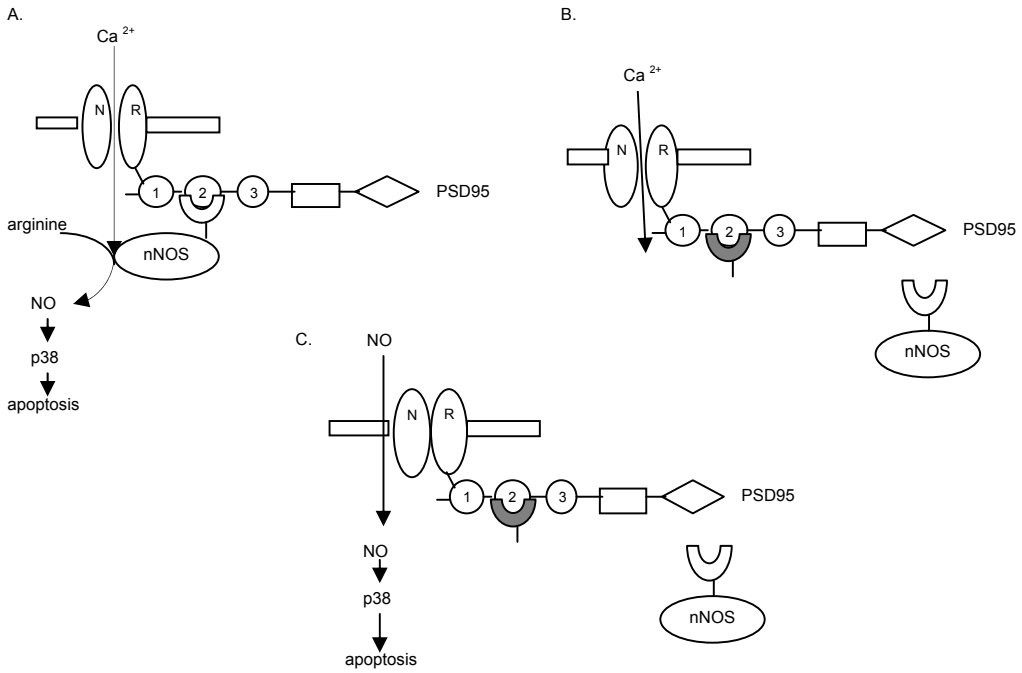

Figure 5. A hypothetical model proposed to describe the neuronal death signaling revealed by the construct investigated.

(A) In an untransfected neuron, PSD95 has reported to be able to bring nNOS in close proximity to the calcium pore of the NMDA receptor. As a result, glutamate induces activation of nNOS and, subsequently, of p38.
(B) When nNOS-PBD was transfected in neurons (in excess of endogeous PSD95), it competes with and prevents interaction of endogenous nNOS with PSD95, resulting in a reduced ability of glutamate to activate p38 and pyknosis.
(C) However, the presence of nNOS-PBD has no effect on the responses to addition of exogenous NO as this bypasses the requirement for PSD95–nNOS interaction.

In conclusion, these results suggest that the interaction between nNOS and PSD95 (or a related molecule such as PSD93) is important for glutamate-induced activation of p38α stress-activated protein kinase and the ensuing cell death, and that the nNOS-PDZ2 interface is a potential target suitable for neuroprotective drug design.

6.2.2 RHO GTPase AS AN ESSENTIAL COMPONENT IN THE GLUTAMATE-INDUCED P38 PATHWAY

Rho-family GTPases are best known as regulators of the actin cytoskeleton. Rho regulates JNK in a cell-type dependent manner (Teramoto et al., 1996b; Marinissen et al., 2004). In Xenopus brain, Rho is also known to be regulated by calcium, but the relationship between Rho activity and the neuronal response to toxic levels of calcium has not been explored (Li et al., 2002). We have demonstrated that glutamate-induced activation of p38α in primary cultured neuron is dependent on extracellular calcium and the calcium-permeable NMDA receptor channel. FRET reporters permitted us to reveal that glutamate activates Rho in mammalian neurons with a rapid timecourse consistent with our proposal that Rho mediates the rapid activation of p38α by glutamate. This measurement is more direct and objective as the revealed activation of Rho by glutamate in mammalian systems in the previous studies has been mainly inferred from downstream responses. However, previous studies supported a role for Rho in activation of p38γ, but investigations of p38α have suggested that this isoform is not regulated by Rho (Marinissen et al., 2001). In our studies, the presence of active Rho failed to activate p38α in neuro 2A cell lines, but revealed a strong activation of p38α in neuronal cultures. This is consistent with the fact that all published results were carried out on cell lines and previous observations that even neuroblastoma and other neuron-like cell lines exhibit distinctly non-neuronal behaviour of their SAPKs (Coffey et al., 2000).

The Cdc42 and Rac members are potent activators of SAPKs and indirect evidence points to a role for these proteins in death induced by nerve factor growth withdrawal (Bazenet et al., 1998). However, Rac1 was not involved in glutamate-induced p38α pathway. Toxin-based inhibition of Rac in unchallenged neurons reportedly leads to apoptosis, whereas inhibition of Rho with C3 toxin was reported not to reduce cell viability (Linseman et al., 2001). This is consistent with our data that cells do have basal Rac activity and C3 toxin did not itself reduce viability (in fact we could not detect basal Rho activity), but restored neuronal survival after glutamate treatment. It can also be expected that inhibition of Rho will have an impact on the actin cytoskeleton, in turn indirectly and gradually affecting NMDA receptor localisation. This could potentially influence

responses to glutamate. However, regulation of the actin cytoskeleton has been reported to selectively affect synaptic glutamate receptor function without influence on responses to bath application of glutamate (Sattler et al., 2000) as used in the present study. Therefore the neuroprotection conferred by C3 is most likely not secondary to general structural rearrangements.

Lysophosphatidic acid (LPA) is a well known activator of Rho via a 7 transmembrane domain receptor-G_{13} pathway (Gohla et al., 1998). Depolarisation of the plasma membranes of cerebellar granule neurons by addition of 30 mM KCl to the extracellular medium induces a characteristic calcium response (Courtney et al., 1990b). Both LPA and KCl activate p38 within minutes without inducing apoptosis, indicating that the Rho-p38α pathway is required rather than sufficient for apoptosis. It may suggest that glutamate has an additional effect required for induction of apoptosis. Furthermore, glutamate, LPA and KCl activate Rho and lead to the activation of p38 within minutes, suggesting that the contribution of Rho activation to glutamate-evoked activation of p38 is rapid and not a gradual response that could be expected from receptor relocalisation driven by cytoskeletal rearrangements. In conclusion, the results showed the requirement of Rho GTPase in the pathway linking calcium influx with stimulation of neuronal p38α SAPK and subsequent apoptosis.

Altogether, our study proposed that mechanistically different forms of apoptosis have differing requirements for p38 and JNK activities in neurons. Furthermore, we observed that glutamate-induced p38-dependent neuronal death is caspase-independent. It may be a useful model for further investigation as glutamate has been considered to contribute to ischemia, traumatic brain injury and chronic neurodegenerative disorders.

Inhibition of the stress-activated protein kinases may prevent neuronal apoptosis, but these kinases are also implicated in physiological functions (Coffey et al., 2000; Chang et al., 2003), which suggests that the SAPKs may also be unsuitable drug targets. Therefore our neuroprotective construct nNOS-PBD, which can target the PSD95-nNOS interface selectively and specifically, may have therapeutic importance. It would increase the number of possible drug targets that could be considered for neurological diseases involving excitotoxicity. Furthermore, compared with small molecule catalytic site inhibitors, agents that disrupt the interaction between PSD95-nNOS may increase the specificity. Moreover, as the present study demonstrated an essential requirement for Rho GTPase in the pathway linking calcium influx with stimulation of neuronal p38α and apoptosis, inhibition of Rho may also have the beneficial effect of increasing survival subsequent to excitotoxic challenge. However, other possible upstream and downstream regulators of the glutamate-induced p38 MAPK pathway and subsequent neuronal death still remain to be elucidated.

7. SUMMARY

The experiment carried out in this thesis sought to distinguish the role of p38α and JNK stress-activated protein kinases in cell death induced by two different stress stimuli in cerebellar granule neuron cultures, thus making it possible to investigate the regulators in response to glutamate-induced neurotoxicity. On the basis of the present study, the following results were obtained:

1. Stress-activated protein kinases p38α and JNK are required in different forms of apoptotic neuronal death in response to glutamate and withdrawal of trophic support stimulation. JNK2/3 isoforms mediate the activation of c-Jun in response to trophic support withdrawal.

2. The interaction between nNOS and PSD95 and subsequent NO productions are essencial for glutamate-induced p38 activation and the ensuing cell death. The PSD95-nNOS interface may provide a potential target for design of neuroprotective drugs.

3. The Rho GTPase RhoA is required, but not sufficient, in the pathway which links between calcium influx and the activation of stress-activated protein kinase p38α and subsequent neuronal death.

In conclusion, glutamate-induced neuronal death requiring stress-activated protein kinase p38α pathway is distinct from withdrawal of trophic support induced neuronal death requiring JNK. The importance of interaction between nNOS and PSD95, and the essential role of Rho in glutamate induced activation of p38α and subsequent neuronal death makes them possible target candidates for neuroprotection.

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