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Stress Activated Protein Kinase Regulation of Gene Expression in Apoptotic Neurons: A Dissertation

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Stress Activated Protein Kinase Regulation of Gene Expression in Apoptotic Neurons

A Dissertation Presented

By

Gerard S. De Zutter

Submitted to the Faculty of the

University of Massachusetts

Graduate School of Biomedical Sciences

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Interdisciplinary Graduate Program

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Summary

Basic biological processes require gene expression. Tightly regulated molecules known as transcription factors mediate the expression of genes in development and disease. Signal transduction pathways, which respond to environmental cues or stressors are major regulators of the transcription factors. Use of macromolecular synthesis inhibitors in models of normal neurodevelopment and neurodegenerative cell death has led to the discovery that gene expression is required for these processes to occur (Martin et. al., (1988), *J Cell Biol* **106** p829). To date, however, the identities of very few of the genes required in these events have been revealed. Hence, the activation or requirement of specific signaling pathways leading to the expression of known apoptotic genes is not well established. Utilizing the neurothrophic factor deprivation and neurotoxin models of programmed cell death we address these gaps in our understanding of the molecular mechanism of apoptosis as it occurs in neuronal cell death.

Nerve growth factor (NGF) withdrawal from PC12 cells leads to the activation of p38 and apoptosis. The functional significance of 38 activation in this paradigm of cell death is not known. To increase our understanding of apoptosis I examined the requirement for p38 activity in pro-apoptotic gene expression in PC12 cells. I performed a subtractive hybridization that led to the identification of the monoamine oxidase (MAO) gene as induced in response to NGF withdrawal. Using the p38 inhibitor PD169316 I showed that the NGF

withdrawal stimulated induction of the MAO gene and apoptosis is blocked by inhibition of the p38 MAP kinase pathway. I also determined that the MAO inhibitor clorgyline blocked cell death indicating that MAO activity contributes to the cell death caused by NGF withdrawal. Together, these data indicate that the p38 MAP kinase pathway targets the MAO gene in response to apoptotic stimuli.

To study the requirement for the JNK signaling pathway in neurodegeneration I stimulated primary cortical neurons with the neurotoxin arsenite. Arsenite treatment of primary neurons leads to both JNK and p38 activation and subsequently apoptosis. Utilizing transgenic mice lacking the JNK3 gene I demonstrated that JNK3 specifically contributes to the effects of arsenite in these cells. Ribonuclease protection assays were used to identify Fas ligand as a molecule whose arsenite-induced expression is dependent on the JNK3 signal transduction pathway. Furthermore, I have shown that neurons deficient in signaling mediated by the receptor for Fas ligand are resistant to cell death due to arsenite treatment. These results in total have established that the JNK3 mediated expression of Fas ligand contributes to the arsenite induced death of cortical neurons.

In summary, the work presented in these studies identifies the JNK and p38 MAP kinase signal transduction pathways as mediators of apoptosis in neuronal cells. Importantly, I have provided evidence that these stress activated pathways are responsible for the expression of specific genes in apoptotic neuronal cells.

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List of Abbreviations

Act D	actinomycin D
AP-1	activator Protein-1
ATF2	activating transcription factor 2
Bcl-2	B-cell lymphoma-2
CHX	cycloheximide
DA	dopamine
Clor	clorgyline
ERK	Extracellular signal-regulated kinase
FasL	Fas ligand
FADD	Fas associated death domain protein
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
H_2O_2	hydrogen peroxide
KCl	potassium chloride
KA	kainic acid
lpr	lymphoproliferation ; mutation
MAO	monoamine oxidase
MAPK	mitogen activated protein kinase
MEKK	MAPK or ERK kinase kinase

MKK	MAPK kinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	thiazolyl blue
NGF	nerve growth factor
PC12	pheochromocytoma cell –12
PI-3K	phosphoinositol 3′ kinase
ROS	reactive oxygen species
SAPK	stress activated protein kinase
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor associated factor

CHAPTER I

GENERAL INTRODUCTION TO APOPTOSIS

Evolution and Characteristics of Apoptosis. 3.5 billion years ago, during the Archean era, cyanobacteria first appeared on the earth (6). These photosynthetic organisms produce oxygen as a byproduct of their respiration. It wasn't until 1 billion years later that enough cyanobacteria existed to raise the earth's atmospheric oxygen from 1% to approximately 15%. It is likely that at that time, the paleoproterozoic era (2.5 billion years ago), the first reactive oxygen species were generated in significant quantities. Sometime in the neoproterozoic era (540-900 million years ago) cyanobacteria took up residence within lower eukaryotic cells, becoming what we now call mitochondria. Also during this late proterozoic era multicellular organisms began to develop. Conceivably, the deleterious, reactive oxygen species generated by mitochondrion became important in causing cell death during the processes of development and cellular degeneration. Many millions of years later the morphological hallmarks of this cell death, termed apoptosis, were observed and documented (51). Apoptosis was eventually described as programmed cell death when it was discovered that a genetic program consisting of the activity of several genes is required for the cell death which occurs in proper *Caernorhabditis elegans* development (21). Homologs of the nematode cell death genes were soon thereafter identified in higher eukaryotic species and the field of apoptosis research experienced

exponential growth as researchers realized the importance of apoptosis in development and disease. Subsequently apoptosis was demonstrated to be an essential component of normal processes such as nervous system development, immune response and cell turnover. During neurodevelopment, neurons that fail to gain sustenance from survival factor producing cells are eliminated by apoptosis (79). Negative selection in the immune system during development occurs through the process of apoptosis. An immune response is terminated when cells that are generating the response are cleared by an apoptotic mechanism. The consequences of aberrant apoptosis include neurodegenerative and autoimmune diseases as well as tumorigenesis.

Morphologically, apoptosis can be defined in cells that display nuclear chromatin condensation, cell shrinkage, and plasma membrane blebbing (15). Eventually cells undergoing apoptosis re-distribute themselves into smaller membrane bound particles which are removed by a non-inflammatory, phagocytic process. In contrast, another form of cell death termed necrosis does not appear to be an ordered or programmed process but rather a catastrophic death. Necrosis is characterized by cell and mitochondrial swelling followed by membrane rupture and spillage of the cell contents into the external milieu which can trigger inflammatory responses in surrounding cells.

Chromatin cleavage by a specific nuclease is a biochemical hallmark of apoptosis. The caspase activated Dnase (CAD) enters the nucleus of

apoptotic cells and cleaves chromatin preferentially between nucleosomal particles resulting in their release from the DNA polymer (22). These particles are classically detected as a ladder pattern on agarose gels and also photometrically by more modern ELISA techniques. The effects of CAD can also be quantitated by the TUNEL method which detects broken ends of DNA. Apoptotic insults also activate specific, intracellular protease cascades which cause the ordered disintegration a cell's ultrastructure. Colorimetric and fluorimetric assays detecting substrate cleavage by these proteases, termed caspases, are now reliably used as markers for apoptosis. The caspases exist as inactive pro-enzymes and their cleavage to active enzymes can be detected by specific antibodies against their active isomer(s). Loss of membrane depolarization and an opening of the mitochondrial permeability transition pore also occur during apoptosis and can be measured with electrophysiological techniques. Observation of the release of mitochondrial constituents such as cytochrome c has been shown to be a reliable indicator of apoptosis. In fact it is now becoming clear that mitochondria are central regulators of some apoptotic pathways.

Defining mechanisms of apoptosis. The machineries of apoptosis are activated by a wide variety of stimuli such as U.V. light, redox stress, cytokines and other receptor ligands. Several death mechanisms may exist in the same cell and their activation is dependent on the stimulus and cell cycle status. For example, trophic factor withdrawal induced apoptosis of

differentiated, post-mitotic, rat pheochromocytoma cells (and primary

neurons) can be blocked by transcriptional or translational inhibitors (69, 83).

This observation indicates that new gene expression is required for apoptosis

to occur in these cells. However, undifferentiated, mitotic PC12 cells cannot

be rescued from trophic factor withdrawal induced apoptosis by these

inhibitors. In general, it appears that differentiated or post-mitotic cells

exposed to mild or more physiologically relevant death stimuli depend on

new gene expression to carry out a death program while proliferating cells or

massively insulted cells do not seem to require gene expression to die.

Pro- and anti-apoptotic members of the Bcl-2 family of proteins which

reside in or are closely associated with mitochondria regulate cell death

pathways which travel through mitochondria (Fig. 1-1) (29). Many cell death

stimuli cause the release of cytochrome c from mitochondria during a major

commitment step of apoptosis. Overexpression of anti-apoptotic molecules

Bcl-2 or Bcl-XL prevent the release of cytochrome c (52) while overexpression

of the pro-apoptotic Bcl family members Bax and Bak promote the

translocation of cytochrome c out of the mitochondria (91). Once cytosolic,

cytochrome c binds to the apoptosis protease-activating factor-1 (Apaf-1)

causing it's oligomerization (35). Apaf-1 contains a caspase recruitment

domain (CARD) which it utilizes to recruit an activator caspase, procaspase 9.

Procaspase 9 is cleaved and activated in an ATP dependent manner and then

goes on to cleave procaspase 3 resulting in an activated caspase 3. An

additional level of regulation is conferred upon death
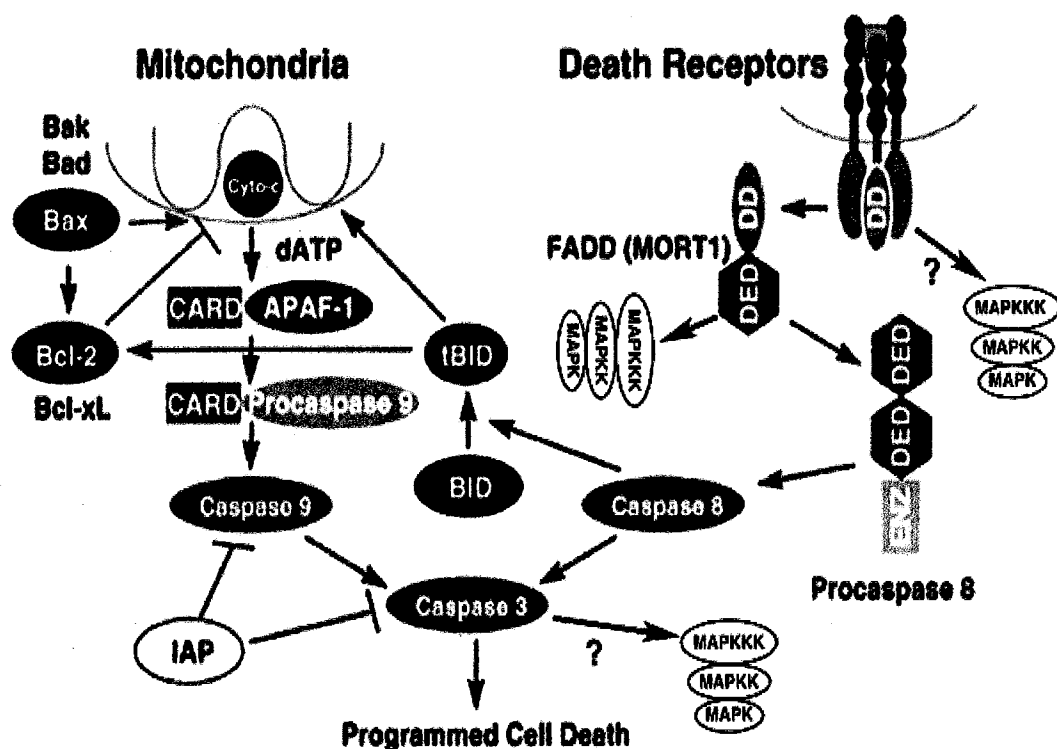
Fig. 1-1. Molecular Machinery of Apoptosis

Caspase activating pathways, Bcl-2 regulators and potential involvement of MAP kinase signaling cascades are shown. (Modified from Fesik, S. W., (2000) Cell vol. 103, p273-282

pathways by the inhibitor of apoptosis proteins (IAPs). These proteins, which exist in organisms from viruses to mammals, have been shown to modulate caspases by binding, inhibiting and possibly marking the enzymes for disposal by the ubiquitin degradation pathway (124). Recently a seemingly double negative was discovered with the identification of a protein which represses IAP function called Smac/DIABLO (106, 118). This repressor is apparently released concurrently with cytochrome c and acts to prevent the

inhibition, by IAPs, of caspase activation. Caspases contain an obligatory cysteine residue at their active site and prefer to cleave substrates next to aspartate residues (29). Many caspase substrates have been identified and they include other caspases, cytoskeletal proteins and a number of other molecules which regulate cellular homeostasis by maintaining membrane and DNA integrity and mitochondrial function (29). Conversely, inflammatory responses such as that caused by necrosis result in the release, from storage vesicles, of many different types of proteolytic enzymes which wantonly and catastrophically cleave many substrates they contact.

Another distinction between apoptotic mechanisms can be made based on whether or not they involve mitochondria. In contrast to the cytochrome C-Apaf-1-caspase mechanism described above, some pathways resulting in cell death do not require mitochondrial constituents or functions. TNF superfamily members such as FASL and TRAIL bind cognate receptors which have cytoplasmic portions termed death domains. Through their death domains activated receptors oligomerize with other death domain containing signaling molecules such as FADD/MORT1, which in turn activate an initiator caspase, caspase 8 (Fig. 1-1) (65). Caspase 8 directly cleaves procaspase 3 to release and activate the major death effector, caspase 3 (29).

Mitochondrion dependent and independent pathways leading to cell death are not, however, without their promiscuity. Recently it was demonstrated that Bcl-2 inhibits cell death triggered by Fas(95). Subsequently it was shown that Bcl-2 works to prevent a Fas induced release of cytochrome

c from the mitochondria (66). The direct link was established when it was found that caspase 8 cleaves and activates a proapoptotic molecule, Bid, which then localizes to the mitochondria and facilitates Bax oligomerization and cytochrome c release (Fig. 1-1) (61, 66).

Alternatively, mitochondria may also contribute to cell death by generating reactive oxygen species (ROS) such as that produced by the electron transport chain in the inner mitochondrial membrane. During normal respiration, superoxide production leads to formation of hydrogen peroxide which can then be converted to a highly reactive hydroxyl radical by an iron catalyzed reaction. Under normal physiological conditions antioxidant defense molecules such as reduced glutathione and glutathione peroxidase inactivate the ROS produced by such endogenous mechanisms and also that which may be produced by ambient radiation. It is likely that when the production of ROS, induced by an insult or metabolic imbalance, overcomes the capacity of the antioxidants, cellular damage occurs. Conversely, antioxidant downregulation, such as that which occurs in Parkinson's disease (78) would achieve the same effect. Reactive oxygen species such as hydroxyl radicals can directly cause damage by oxidizing protein thiols, peroxidating lipids or altering DNA structure (3). However, ROS also have been demonstrated to function as signaling molecules. Redox imbalance caused by oxidative stress activates stress response signaling pathways and has effects on stress response transcription factor binding to

DNA (67, 99). Indeed, both signaling and cell damage together may be the conduits through which ROS mediate their deleterious effects.

The molecular mechanisms and machinery downstream from the initiator caspase 8 and the mitochondria are rapidly being identified and seemingly work to funnel signals into a common final death effector mechanism involving caspase 3. If the entire process of apoptosis were likened to a screen play, the machinery or effectors of cell death are the inescapable, dramatic finale.

Signaling pathways upstream of the death machinery could be considered the second act of this tragedy. Several different signaling pathways can be activated by life or death stimuli. Generally, noxious stimuli initiate stress activated protein kinase (SAPK) mediated pathways while mitogenic or trophic factors stimulate pathways involved in survival, proliferation and differentiation.

Mitogen activated protein kinase (MAPK) mediated signaling pathways can regulate apoptosis, probably upstream of the caspases and mitochondria. Two groups of MAPKs, the c-jun N-terminal kinases (JNKs) and the p38 MAP kinases are activated by and propagate cell death signals. These kinases can be grouped and sub-categorized as SAPKs. The extracellular-signal regulated kinases (ERKs) form a third group of MAPKs, are activated by mitotic or trophic stimuli and oppose the death pathways. The MAP kinases phosphorylate various downstream substrates which carry out the desired effect of the initial stimulus. The most well known substrates

of these kinases are transcription factors which regulate gene expression through their binding to specific promoter sequences. The genes expressed are important in diverse processes such as mitosis, differentiation and apoptosis. It is important to note that a cell death signal, such as that propagated by the SAPKs, may promote death or alternatively, supress survival. Likewise, it is possible that ERK functions as an anti-apoptotic or a pro-survival molecule.

Crosstalk, redundancy and opposition between pathways prevent straightforward interpretations or predictions of stimulus induced signal transduction. Furthermore, signaling or second messenger induced events are also likely to be important downstream of the death machinery and may participate in positive or negative feedback loops. The activation of protein kinase cascades has received much attention with regards to regulating signaling pathway activation. However, specific phosphatases which de-phosphorylate and inactivate kinases are also very important in modulating signaling activity.

The opening act of the cell death screen play could be considered one in which the ultimate fate of the cell is determined. How cells respond to environmental stress as well as other extracellular stimuli is central to this potentially fateful decision. For example, in the case of TNF or FASL treatment, the ligand binds to the receptor but the immediate downstream events are complex to say the least. The ligated receptor recruits adaptor molecules (e.g. FADD) to it's cytoplasmic domain which then activate life or

death signaling pathways (65). How the cell decides which adaptor (s) to recruit and which pathway (s) to activate is unknown but of utmost importance. Molecules such as phoshoinositide 3-kinase, AKT and NFκB have been proposed to be central regulators of this decision making process (4, 48) but it is likely that fate is determined by a combination of pathway activation, feedback mechanisms that positively or negatively reinforce survival signals and threshold barriers.

Gene expression in the neurotrophic theory of programmed cell death. Many Americans who spent their formative years during the 1960's will contend that you can't possibly have too many neurons. They may also agree that too much of a good thing can be bad. This is apparently the case for neurons during normal vertebrate development. On average, half of the neuronal cells generated during embryogenesis die by apoptosis before they reach a fully differentiated, functional state (79). For example, neuronal precursors migrate to form the cortical layers and then fully differentiate into functional neurons. However, in a survival of the fittest scenario, only those neurons whose probing growth cones contact a neurotrophic source survive. The neurons which fail to connect with the limited trophic sources die a programmed death (79). In most cases the trophic support comes from glial cells which synthesize and secrete neurotrophins such as nerve growth factor (NGF). Indeed, nerve growth factor is the best characterized member of the family of neurotrophins. Exogenously supplied NGF supports the survival of

sympathetic and sensory neurons even in cases where death stimuli are present. Clearly, defects in apoptosis during development can lead to abnormalities and improper brain function.

Descriptive studies of cell death during nervous system development appeared as early as the middle 1900's (32). Biochemical and cell biological evidence of neuronal programmed cell death in vivo wasn't presented until 1990. Using a dye which stains DNA, Oppenheim et al. detected condensed nuclear chromatin in motoneurons and dorsal root ganglion cells of an embryonic day 8 chick (80). These results demonstrate that a large portion of developing neurons in the chick embryo are dying by programmed cell death. These investigators also found that when these embryos were treated with the protein synthesis inhibitor cycloheximide, the naturally occurring cell death was significantly reduced. In summary their data established that apoptosis occurs during normal neurodevelopment and the inhibition of cell death by cycloheximide suggested that the cell death was dependent on new protein synthesis. At that time the straightforward conclusion was drawn that programmed cell death requires new protein synthesis and the newly synthesized proteins actively participate in killing the neuronal cell(s) in which they are expressed. More recently it has been proposed that apoptotic protein synthesis or gene expression could be required to suppress survival signals instead of being a pro-apoptotic mechanism. Around the same time that the chick embryo studies were carried out several investigators recapitulated those results in cell culture using primary cells from rat and

mouse brain as well as the rat PC12 neuronal cell line. In all cases the results showed that neurons deprived of trophic support, akin to a developmental scenario, died by apoptosis which could be prevented by the addition of macromolecular synthesis inhibitors or NGF to the cultures (69, 83). Interestingly, these experiments also demonstrated that up to ~13 hrs after the removal of trophic support, adding back NGF or the transcriptional inhibitor actinomycin D to the trophin starved neurons rescued them from apoptosis (69, 83). This result suggests that a period of time prior to commitment to death is necessary for the accumulation of enough death stimulus for the majority of the cells to proceed irreversibly down the death pathway. The precipitating event for a cell to commit to death is not known. One possibility is that a threshold barrier must be overcome by a build up of pro-apoptotic signaling events. Another is that the initial lag period is necessary so that a critical mass of newly synthesized pro-apoptotic (or survival suppressing) molecules is reached which commits a cell to die. These two possibilities do not have to be mutually exclusive and there is likely to be several barriers which must be overcome along the commitment path to cell death. In fact it may be that in the initial lag phase of developmental cell death, signaling events and gene expression, or a lack thereof, is how a cell ponders or decides it's own fate.

The death of specific subsets of neurons causes the symptoms of neurodegenerative diseases such as Parkinson's disease. Histochemical and morphological studies of neurons which are degenerating reveal that many of

these cells display characteristics of apoptotic death (104). While it is understood that the death of the neurons underlies the disease there is little understanding of why the cells die. Mice administered the Parkinson's syndrome inducing neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), display significant apoptotic neuronal cell death in brain tissue slices (129). Primary neurons and PC12 cells also die programmatically when treated with MPTP (44, 111). Furthermore, cycloheximide reduces the cell death caused by the neurotoxin in cell cultures (13, 44, 111). Therefore, neurodegenerative and neurodevelopmental apoptosis differ in context but are the same morphologically and, to some degree, mechanistically.

If apoptotic neuronal cell death depends on protein synthesis or gene expression, then logical questions arise; what are the identities of the proteins or genes being expressed? In addition, how are these newly synthesized molecules involved in the program of cell death, i.e. in which act do these molecules appear? To completely consider the mechanism of apoptosis one must also acknowledge that some genes may be expressed in defense of the cell responding to a death stimulus. Previous, large scale screens designed to characterize genes expressed in apoptotic PC12 cells have led to the identification of rat orthologs of human *TAFII70*, *Ring 3* and the *Drosophila fsh* gene (46, 108). While the expression of these genes during cell death may be useful as markers, a role for these genes in apoptosis has not been established. Traveling backwards up the pathway leading to expression of an apoptotic gene other questions arise. What are the transcription factors which regulate

apoptotic gene expression? What signaling pathways regulate the transcription factors involved? More directed studies utilizing RT-PCR and northern blotting demonstrated that the cell cycle gene cyclin D1 and the immediate-early response transcription factors c-Jun and c-Fos are induced in apoptotic neuronal cells (25, 30). Dominant negative, microinjection experiments and analyses of mice with germline mutations of the c-Jun gene have established that c-Jun is an important mediator of apoptosis in neurons (5, 39, 110). The activation of JNK in neurons in response to apoptotic stimuli is also well established (121). To date, however, very few studies have addressed the functional significance of JNK activation or c-Jun induction. Furthermore, the involvement of the other family of stress activated protein kinases, p38 MAP kinases, in apoptosis is not well established. The goal of the present study is to identify genes expressed during apoptosis. In addition the work also examines upstream signaling pathways mediating the expression of these genes.

CHAPTER II

APOPTOTIC GENE EXPRESSION MEDIATED BY THE p38 MAP KINASE SIGNAL TRANSDUCTION PATHWAY

Background

<u>NGF withdrawal induced apoptosis.</u> Apoptosis contributes to the normal development of the nervous system and also to the progression of some neurodegenerative diseases. For example, neurons that fail to make a functional connection with survival factor producing target cells during development are eliminated by apoptosis (79). Furthermore, subpopulations of neurons associated with pathological neurodegeneration, including Parkinson's, Huntington's, and Alzheimer's diseases, may die by apoptosis (24, 72, 84, 94, 103). The death of these neurons is thought to contribute to the symptoms of these neurodegenerative disorders. Thus, the degeneration of nigrostriatal dopaminergic neurons causes Parkinson's syndrome (104, 132). While the degeneration of these cells has been attributed to apoptosis (104, 132), the mechanisms that contribute to the apoptotic process remain to be established.

PC12 pheochromocytoma cells can be considered to be a model for studying both developmental and disease-associated apoptosis. In normal

serum containing media these cells grow and divide as a relatively non-descript cell type. When treated with nerve growth factor (NGF), PC12 cells differentiate into a post-mitotic neuronal cell type (38). Because neuronal PC12 cells synthesize, store and secrete large amounts of dopamine they most closely resemble dopaminergic neurons of the sympathetic nervous system (37, 38). Expression of mutated amyloid precursor protein, exposure to iron, or exposure to reactive oxygen species (e.g. H_2O_2 or peroxynitrites) causes neuronal PC12 cells to undergo apoptosis, mimicking the etiology of neurodegenerative disease (47, 76, 130). Depriving differentiated PC12 cells of NGF reproduces a developmental scenario whereby the failure of a neuron to connect to a source of trophic support causes that neuron to undergo programmed cell death (79). The wide variety of stimuli that can cause apoptosis suggests that several molecular pathways leading to death exist within these cells (81, 82). NGF withdrawal-induced apoptosis of PC12 cells is blocked by the addition of macromolecular synthesis inhibitors up to 14 hours after the removal of NGF (83). These data indicate that gene expression is required, either to enhance death signals or to suppress survival signals, to carry out the death program.

At the cell surface, the Trk tyrosine kinase and the p75NTR receptors bind the neurotrophins NT-3, NT4/5, CNTF, GDNF and NGF and mediate differentiation, survival and apoptosis of neurons (43, 48). The TrkA and p75 receptors bind NGF and initiate signaling events through their cytoplasmic domains. Interestingly, NGF binding to p75 causes apoptosis in the absence

of TrkA. When p75 and TrkA are present, NGF treatment does not cause cell death leading to the idea that the TrkA pathway is dominant over p75 signaling and suppresses the death signal generated by the activated p75 receptor. In fact TrkA and p75 heterodimerize, possibly modulating each other's downstream activities. The temporal expression of these receptors during neurodevelopment may be a mechanism by which developmental apoptosis is regulated. The cytoplasmic domains of these receptors can be considered the upper most activators of a variety of signaling pathways that operate in parallel or in opposition to each other. Positive and/or negative signals emanating from these receptors depend on the presence of the receptors on the cell surface and the neurotrophins that they bind. In general, positive signals, enhancing survival and growth are transmitted by the Trk receptors while p75 initiates both positive and negative signals. p75, in addition to being the first identified neurotrophin receptor was also the first known member of the TNF receptor super family (48). However, the Trk receptors are much better understood in terms of their neurotrophic specificties and the molecular mechanisms of their signaling pathways. The Trk receptors bind neurotrophins and oligomerize, stimulating transphosphorylation of their cytoplasmic domains. This tyrosine phosphorylation event attracts several adaptor proteins which in turn recruit other molecules to initiate signaling pathways. Recruited molecules include the GTP binding proteins, Ras and Cdc42/Rac which are upstream activators

of the MAPKKKs. The most well studied pathway leading from the Trk

receptor is one which activates phosphoinositide 3-kinase (PI-3K) in response

NGF

↓

TrkA

↓

PI-3K

↓

PDK

↓

AKT

⊥

Apoptosis
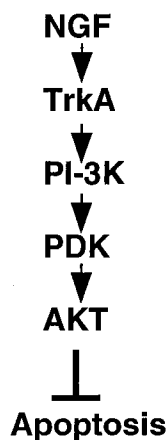
Fig. 2-1. NGF mediated pro-survival pathway

The anti-apoptotic effect of AKT is activated by a PI-3K mediated pathway.

to neurotrophin binding (Fig. 2-1). In fact, in many neuronal cell types PI-3K

is the primary mediator of the survival effects imparted by the neurotrophins.

Once activated, PI-3K can stimulate the serine/threonine kinase Akt which

inhibits pro-apoptotic molecules such as BAD, Bax and the transcription

factors p53 and Forkhead. In addition, the anti-apoptotic effects of Bcl-2 are

regulated by the inhibitor of apoptosis proteins (IAPs) which in turn are

activated by PI-3K. Trk and p75 can directly interact and through

bidirectional crosstalk, they modulate the various signaling pathways

initiated by them. For instance, in the absence of Trk signaling, NGF

stimulation of p75 leads to the death of sympathetic neruons (2). If TrkA is

activated in addition to p75, the apoptotic effect of p75 is blocked coincident

with the activation of the pro-survival players, Ras, PI-3K and the

serine/threonince kinase Akt (70). In normal growing neurons, Schwann cells, and oligodendrocytes, however, p75 causes the activation of NFkB, a pro-survival transcription factor (9, 40, 58). The presence of TrkA receptor does not affect this activation leading to the hypothesis that Trk and p75 together can mediate survival signaling pathways.

The p38s form one family of stress activated protein kinases, JNKs form the other. Because many noxious stimuli and environmental stresses activate p38 MAP kinases and JNKs they may act synergistically to mediate apoptosis. Identity of p38 protein sequences across the higher eukaryotes is very well conserved while it is somewhat less conserved for the lower eukaryotes. At least four known homologous kinases, α, β, γ, δ, are synthesized from separate genes and exhibit wide tissue distribution (23). Phosphorylation on the Threonine 180 and Tyrosine 182 residues within a Thr-Gly-Tyr stretch of subdomain VIII renders the kinase active (89). Environmental stress(s) and stimulated receptors initiate signaling events which lead to the activation of cascades of kinases (Fig.2-2). At the top of the core p38 kinase cascade, a relatively large group of MAP kinase kinase kinases which include ASK1, TAK1 and members of the MLK group phosphorylate and activate the downstream MAP kinase kinases, MKK3 and MKK6 (57). In vitro, MKK3 prefers to phosphorylate the p38α and p38γ while MKK6 activates the p38α, p38β2 and p38γ MAP kinases (23). It remains to be determined if these specificities hold true in vivo.

Cytoplasmic substrates of p38. NGF withdrawal from neuronal PC12 cells

activates p38 and causes these cells to die by apoptosis. Expression of a

dominant interfering mutant

Stimulus

Cdc42/Rac ?

GTPases

MEKKs ASK1
TAO
TAK1 ?

MKK3/MKK6 MKK4

?

p38
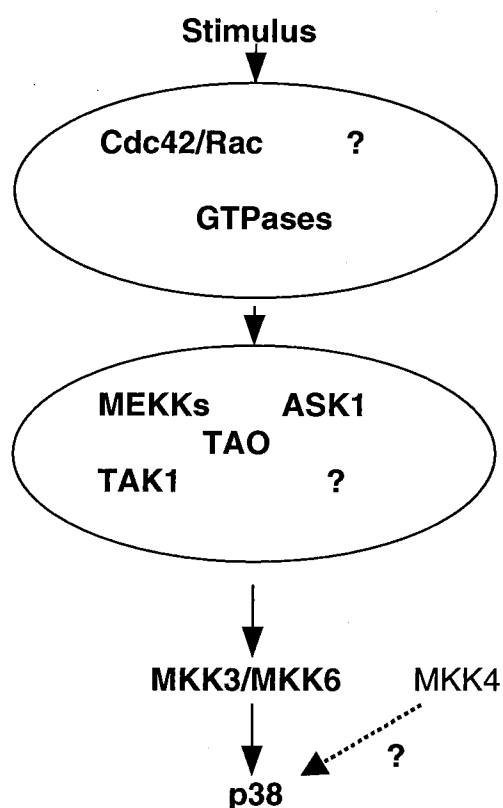
Fig. 2-2. Core p38 signaling pathways

Components circled are the least well defined groups and represent the
MAPKKK's (e.g. MEKKs, ASK1) and their upstream activators (e.g. GTPases)

of MKK3 reduces the apoptotic effect of NGF deprivation (121). Small

molecule inhibitors of p38 also prevent this apoptosis (56). Activated p38

phosphorylates a variety of substrates that are not transcription factors but

could be involved in apoptosis nonetheless (Fig. 2-3).

Tau protein is a cytoplasmic substrate of p38. In addition to other residues, p38 phosphorylates tau at Ser422, a site which is phosphorylated on tau proteins that form neurofibrillary tangles in degenerating neurons of Alzheimers disease patients (90). In normal brain tissue, which does not contain neurofibrillary tangles, Ser422 of tau proteins are not phosphorylated. Neurofibrillary tangles are markers for degenerating neurons in Alzheimers patients but the functional significance of the tangles and the p38 dependent phosophorylation of Ser422 has not been established.

Interestingly, p38 has been shown to target an AU-rich element (ARE) in cytokine mRNA that promotes it's stability (117). In addition, MAP kinase-interacting kinases 1/2 are phosphorylated by p38 and go on to phosphorylate eukaryotic initiation factor-4E (eIF-4E) (109). Phosphorylated eIF-4E has enhanced affinity for the 7-methyl-guanosine cap of all eukaryotic mRNAs. By binding, in a complex, with the 5'-cap eIF-4E regulates translation in mammalian cells.

p38 phosphorylates another substrate which may be part of a cellular defense or repair mechanism. MAP kinase activated protein kinase (MAPKAP) 2 is activated by p38 and goes on to phosphorylate the heat shock protein, Hsp27 (98). Hsp27 is known to facilitate the polymerization of actin, thereby enhancing the repair of the filament network which is dismantled, to some degree, during cell stress (59).
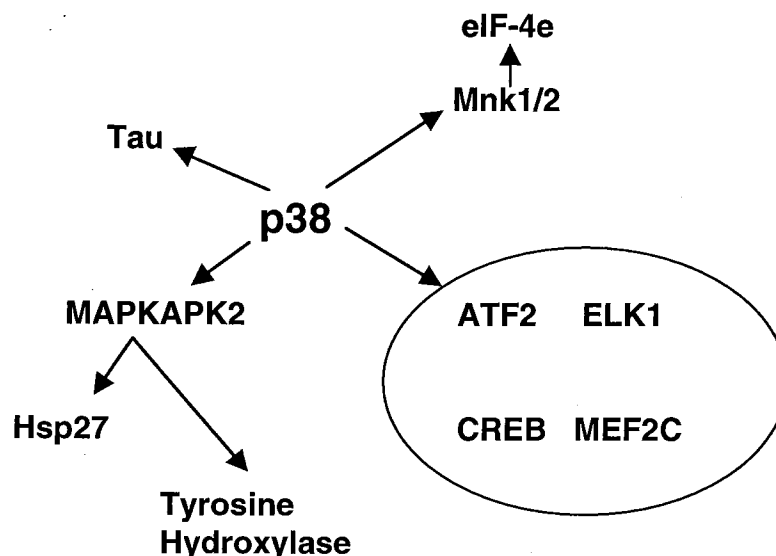
Fig. 2-3. p38 Substrates

The substrates for p38 are illustrated with the targeted transcription factors encircled.

Transcription Factor Substrates of p38. In response to U.V. light, the

transcription factor Elk-1 is phosphorylated by p38 (Fig. 2-3), binds with the

serum response factor (SRF) to the serum response element (SRE) of the c-Fos

gene and induces it's expression (87). Similarly, ATF-2, a member of the AP-1

family of transcription factors, is phosphorylated by p38 in neuronal cells. c-

Jun gene expression can be driven through the binding of ATF-2 to AP-1-like

sites in the c-Jun promoter. Moreover, c-Jun gene expression is also induced

by the transcription factor MEF-2C after it is transactivated by p38 dependent

phosphorylation (41). c-Jun forms homodimers and heterodimers with other

AP-1 family members such as c-Fos to activate transcription from AP-1

binding promoter sites. An amino terminal truncation which removes the

transactivation domain of c-Jun does not prevent it from dimerizing or

binding to AP-1 promoters. This dominant-negative c-Jun is incapable of stimulating AP-1 driven gene expression in neurons (39). Interestingly, microinjection of this mutant c-Jun into sympathetic neurons protects these cells from NGF withdrawal induced death (39). Phosphorylation at Ser63 and Ser73 regulates the functions of c-Jun (5, 25, 110). Mutation of the Serine residues to Alanine prevents phosphorylation at those sites. Mice which have had the mutant c-Jun allele "knocked-in", replacing the wild type c-Jun allele, are resistant to seizures and neuronal apoptosis caused by excitotoxins (5). In addition, primary neurons derived from the mutant mice are resistant to apoptotic insults. These data establish an important role for c-Jun in neuronal apoptosis. The role of p38 in neuronal apoptosis is not well defined and appears to depend on the cell type. In PC12 cells it is well documented that p38 is activated and phosphorylates ATF-2 in response to NGF withdrawal (121). In addition, pretreatment of PC12 cells with small molecule inhibitors of p38 also blocks the apoptotic response to trophic factor withdrawal. Conversely, NGF withdrawal from rat primary sympathetic neurons does cause apoptosis but does not activate p38 over it's low basal activity (20, 110). Also, KCl deprivation induced apoptosis of rat primary cerebellar granule neurons does not cause an increase in the low p38 activity. However, by way of preventing generalizations about the importance of p38 in apoptosis of primary vs tumor cells, several groups have demonstrated that p38 is important in mediating the survival of primary neurons. Developing fetal chick neurons and PC12 cells contain a high basal level of p38 activity that is

abolished by treating the neurons with the trophic factor, insulin (42, 121). Other data suggested that insulin may regulate p38 activity by activating a phosphatase. p38 inhibition also reduces the trophic factor withdrawal induced apoptosis of sympathetic ganglia, dorsal root ganglia, ciliary ganglia and motoneurons derived from chick embryos (44). Serum withdrawal causes the apoptosis of dopaminergic neurons in rat primary ventral mesencephalic cultures. Treatment of these cultures with small molecule p38 inhibitors restores the viability of the serum starved dopaminergic neurons back to serum treated levels (129). Additionally, pre-incubation of mesencephalic tissue with p38 inhibitors dramatically improved the survival of dopaminergic neurons after transplantation into Parkinson's syndrome affected rat brains.

To summarize, the phosphorylation of transcription factors by p38 mediates or induces the expression of other, pro-apoptotic, transcription factors whose gene targets are unknown. While somewhat controversial, p38 activity is an important regulator of cell survival in some primary cell types and brain tissues. It is likely that, as yet unknown, genes other than transcription factors are induced by and downstream of p38 which play a role in cell survival or cell death. Importantly, both the JNK and p38 MAP kinases are activated during neurotrophic factor withdrawal (56, 121). It is thought that these MAP kinase signal transduction pathways may mediate pro-apoptotic gene expression (16). Targets of the JNK pathway in neurons

include increased expression of FasL (60), but little is known about potential targets of the p38 MAP kinase pathway.

NGF deprivation of neuronal PC12 cells initiates a continuum of events collectively known as apoptosis. Early events in this process include; flipping of phosphatidyl serine lipids from the inner to the outer leaflet of the plasma membrane and immediate-early response gene (e.g. c-Jun) expression. In the mid to later stages of this initial, reversible, stage of apoptosis other genes are also likely to be expressed, enabling the apoptotic process to continue to an execution phase. Cytochrome c release and caspase activation ocurr at the beginning of the execution phase and committ a cell to die. These processes are irreversible and manifest themselves by inducing the condensation and cleavage of chromatin, leading ultimately to nuclear breakdown and plasma membrane blebbing. We examined gene expression in NGF-withdrawal induced apoptosis by using subtractive hybridization and northern blotting. We utilized endpoint assays which detect nuclear morphology, chromatin condensation and cleavage to quantitate the apoptotic PC12 cells.

The purpose of this study was to identify p38 MAP kinase-dependent gene expression in apoptotic neuronal PC12 cells. We report the identification of MAO as a gene that is regulated by the p38 MAP kinase pathway in apoptotic cells. Interestingly, MAO activity strongly potentiates neurotrophic factor withdrawal-induced apoptosis.

Methods and Materials

Cell Culture. PC12 cells were maintained at 37°C and 10% CO_2 in Dulbecco's

Modified Eagles Medium (DMEM) containing 10% heat inactivated horse

serum(Life Technologies, Rockville, MD), 5% Fetal bovine serum (Omega

Scientific), 0.05U/ml Penicillin, 0.05U/ml Streptomycin (Life Technologies,

Rockville, MD) and 2mM Glutamine (Life Technologies, Rockville, MD).

PC12 cells were differentiated on plates coated with 0.2 mg/ml rat tail

collagen (Biomedical Technologies, Stoughton, MA) in DMEM supplemented

with 1% heat inactivated horse serum, the same amounts of Penicillin,

Streptomycin and Glutamine as listed above, and 75ng/ml nerve growth

factor, NGF (Austral Biologicals) for 10-12 days. The media was changed

every other day. NGF withdrawal was initiated by washing differentiated

PC12 cells once with NGF-free medium and then incubating the cells with the

NGF-free medium including a neutralizing antibody (Sigma Chemical Co.) to

the 2.5S unit of NGF (13/mg ml; 1:1000 dilution). Control cells were

incubated in the NGF containing differentiation media after the wash in

NGF-free media. Clorgyline (0 – 10 µM; Research Biochemicals Intl.),

deprenyl (0 – 10µM; Research Biochemicals Intl.), PD169316 (10 µM;

Calbiochem), SP600125 (10µM; Celgene Corp.) and PD98059 (25 µM;

Calbiochem) were added to the culture medium. To treat cells with

dopamine, the media was replaced with differentiation media including

300µM dopamine plus 75ng/ml of NGF.

Poly A⁺ RNA isolation. mRNA was extracted from PC12 cells with the

Invitrogen Micro-Fast Track 2.0 mRNA isolation system using the

manufacturer's protocol. Briefly, cells were lysed for 20 minutes at 45°C in

2% SDS buffer and the lysate incubated at room temperature with oligo dT

cellulose powder for 20 minutes. This solution, containing oligo dT-mRNA

complexes, was added to spin columns and serially washed with low salt

buffer followed by elution of the purified mRNA with 10mM Tris-Cl. The

eluted mRNA was quantified, ethanol precipitated and stored at –80°C until

use. Prior to use the precipitated mRNA was centrifuged at 15,000xg for 15

minutes at 4°C, the supernatant was discarded and the pellet resuspended in

6-10 µl of the 10mM Tris-Cl elution buffer.

Subtractive Hybridization. Control, differentiated PC12 cell mRNAs were

subtractively hybridized to NGF withdrawn, apoptotic PC12 mRNAs with

the CLONTECH PCR-Select cDNA Subtraction kit using the manufacturer's

protocol. Briefly, 2 µg poly A⁺ RNA was reversed transcribed using 200U/µl

of MMLV reverse transcriptase to achieve first strand cDNA synthesis. The

single stranded cDNAs were used as templates by T4 DNA polymerase to

yield double stranded cDNAs. The double stranded cDNAs were phenol-

chloroform extracted, ethanol precipitated and digested with RSA 1 to give

blunt ended cDNA fragments. Two different adaptor oligonucleotides

containing T7 promoter sequences and Not 1 restriction sites were ligated,

separately, by adding 400U/µl T4 DNA ligase to NGF withdrawn double stranded cDNAs only. Control PC12 cell cDNAs were added to the two separate, adaptor ligated, double stranded cDNA populations and the samples were heat denatured at 98°C for 1.5 minutes followed by an annealing step at 68°C for 8 hours. The two separately hybridized cDNA populations were mixed together in the presence of control cDNA and allowed to further anneal at 68°C overnight. After an adaptor extension step, PCR primers for the adaptors were used to drive the exponential amplification of the apoptosis associated cDNAs. Nested PCR primers also for an adaptor sequence were then added and another PCR amplification step performed to further enrich the apoptosis associated cDNAs. The resulting PCR products were then run on a 2.0% TAE/ agarose gel. The cDNA bands observed to be amplified in the subtracted, apoptotic cDNA lane were excised from the gel, purified away from agarose and ethidium by the maufacturer's protocol for the Geneclean II kit. These cDNAs were PCR amplified, purified, cloned into Invitrogen's pCR™II vector, transformed into DH5α competent bacterial cells and 15 clones per amplified band were picked for DNA sequence analysis.

Northern Blotting. 2-4 µg of poly(A)+ RNA from NGF treated and NGF withdrawn PC12 cells (0, 12, 24 hrs) was prepared as described previously. The mRNA was fractionated on 1.2% agarose/formaldehyde gels, transferred in DEPC treated 20XSSC to Stratagene's duralose membrane and hybridized

with 5-10x10^6 cpm of ^{32}P labeled oligonucleotides synthesized by random primer labeling from the pCR™II vector cDNA inserts and a 1.8-kb human β-actin cDNA fragment (Genbank acc. # M10278) from Clontech. Pre-hybridization and hybridizations were done in 50% deionized formamide, 10% dextran sulfate, 1% SDS, 1M NaCl and 100 μg/ml of denatured, sonicated salmon sperm DNA overnight (12-16hrs) at 42°C. After serial washing the hybridized blots in 2XSSC/0.1% SDS and 0.2XSSC/0.1% SDS at RT and 42°C, the membranes were exposed to film to obtain autoradiographic images and to a Molecular Dynamics Storm phosphorimager for quantitation.

Cell Death Determination. Differentiated PC12 cells (6-7 days) were harvested, counted by trypan blue staining and equal numbers were re-plated (5X10^4 cells/ml) and allowed to continue differentiating for 5-7 more days before beginning NGF withdrawal. Nucleosomal fragmentation in differentiated PC12 cells, differentiated cells which had been NGF deprived for 24 hours, and dopamine-treated cells was examined using the Cell Death Detection ELISA assay (Roche). Cells were harvested by gentle pipetting, combined with the culture supernatant, centrifuged for 5 minutes at 200xg (4°C), resuspended in 1 ml 4°C phosphate buffered saline and transferred to a pre-chilled microcentrifuge tube. The samples were centrifuged for 5 minutes, 200xg at 4°C, the supernatant aspirated and the pellet resuspended for lysis in 500 μl of lysis buffer for 30min at 4°C. Following centrifugation of 15,000xg at

4°C for 15 minutes, the supernatants were diluted 1:10 with the lysis buffer and 100 µl used in a multiwell plate for a sandwich ELISA assay with anti-histone and anti-DNA peroxidase conjugated antibodies to detect free nucleosomes in the lysates. A 2,2'-azino-di-[ethylbenzthiazoline sulfonate] peroxidase substrate was added and incubated at room temperature with orbital shaking for 10-20 minutes before reading the multiwell plate at 405nm.

Monoamine Oxidase Activity Assay. Monoamine oxidase activity was assayed by a method adapted from Wurtman and Axelrod (120). NGF-deprived and NGF-treated PC12 cells were harvested by pipetting and centrifuged at 200xg (4°C) for 5 minutes. The supernatant was discarded, the pellet was resuspended in 1 ml 0.01M potassium phosphate buffer (pH 7.2), and was transferred to a microcentrifuge tube. The cell suspension was sheared by passage through a 20 gauge needle 10 times. The protein concentration was determined by the Bradford method and equal amounts (20µg) of protein were added to 15 µl of 100µM^{14}C-5-Hydroxytryptamine (5-HT) (0.1mCi/ml) in a 0.1M potassium phosphate buffer pH 7.4. The reactions were incubated for 20 minutes at 37°C and then terminated by the addition of 2M citric acid. The reaction cocktail was extracted by addition of toluene : ethyl acetate (1:1) and 2 mls of the organic phase containing the ^{14}C byproduct of the MAO catalyzed deamination of the labeled 5-HT was added to an equal volume of ScintiSafe Econo 1 scintillation fluid (Fisher Scientific). The radioactivity was quantitated on a Beckman Coulter LS6500 scintillation counter.

Immunocytochemistry of PC12 cells. Glass coverslips (Corning co.) were ethanol flamed and coated with 66.7 µg/ml poly-D-lysine, 6.7 µg/ml laminin in sterile water overnite, the solution was aspirated and the coverslips washed 3X with sterile water before differentiating PC12 cells were plated at $5x10^4$ cells/ml. After NGF deprivation, NGF treatment or dopamine treatment of the cells the media was aspirated and replaced with a fixative solution containing 4% para-formaldehyde, and 1mM $MgCl_2$ in 1X phosphate buffered saline, ph 7.3 for 45 minutes at room temperature. The fixative was aspirated and the cells washed 3 x 5 minutes in 1X phosphate buffered saline containing 10mM glycine and 0.1% Triton X-100 at room temperature followed by permeabilization for 30 minutes in 1X phosphate buffered saline containing 0.5% NP-40 at room temperature. Following permeabilization cells were washed once with 1X phosphate buffered saline containing 0.1% Triton X-100, 5mM sodium flouride, and 1mM sodium molybdate. Blocking solution consisting of 0.1% Triton X-100, 2.5% BSA, 2.5% horse serum in 1X phosphate buffered saline was added to the cells for overnight incubation at 4°C. The coverslips were then washed 3 x 5 minutes in 1X phosphate buffered saline containing 10mM glycine and 0.1% Triton X-100 at room temperature with the exception that the second wash also contained 0.1 µg/ml of the DNA stain 4,6-diamidino-2-phenylindole. Coverslips were then mounted with 4 µl of vectashield mounting media (Vector Laboratories Inc.)

onto slides, sealed with nail polish (Revlon) and then viewed at 10,000x

magnification on a Zeiss Axioplan 2 fluorescence microscope.

Results

Gene expression is required for NGF withdrawal-induced apoptosis.

Neuronal PC12 cells undergo apoptosis when deprived of NGF (121).

Previous studies have demonstrated that NGF withdrawal-induced death of

PC12 cells (and primary neurons) can be blocked by inhibitors of

transcription or translation (69, 83). We confirmed this observation by

measuring nucleosome fragmentation of DNA using the PC12 cells employed

in this study.

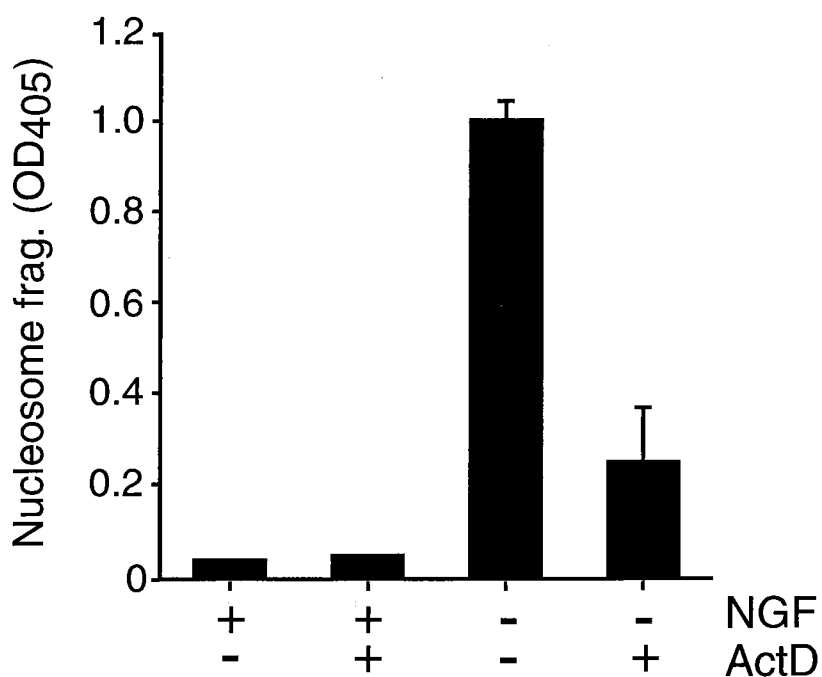**Fig. 2-4.** Macromolecular synthesis dependence of NGF withdrawal-induced apoptosis of neuronal PC12 cells. Differentiated PC12 cells were incubated without and with NGF (75ng/ml) in the presence or absence of 1 μg/ml of the transcriptional inhibitor actinomycin D (ActD). Cell lysates were prepared after 24 hrs. and assayed for nucleosomal DNA fragmentation (mean ± SD; n=3). Similar data were obtained in three independent experiments.

Treatment with actinomycin D did not significantly affect NGF-treated PC12 cells, but this treatment dramatically reduced apoptosis caused by NGF deprivation (Fig. 2-4). These data suggest that gene expression is required to execute the apoptotic program induced by NGF-withdrawal.

To identify genes induced during NGF withdrawal-induced apoptosis, we used subtractive hybridization of cDNA isolated from control neuronal PC12 cells and from PC12 cells 12hr. post NGF withdrawal. cDNA clones identified in this assay were tested in a secondary screen by Northern blot analysis of polyA$^+$ mRNA isolated from control and apoptotic PC12 cells. This strategy led to the identification of the A isoform of MAO (Fig. 2-5A). The MAO mRNA was induced 2.8 and 4.0 fold at 12 and 24 hrs respectively, post NGF withdrawal (Fig 2-5B). In contrast, the expression of SNAP-25 mRNA, which is selectively expressed in differentiated neuronal PC12 cells, was not induced by NGF withdrawal (Fig. 2-5B).

MAPK requirement for MAO gene expression. The JNK and p38 MAPK (but not the extracellular signal-regulated kinase (ERK) MAPK) are activated in response to trophic factor deprivation (121). To test the role of these MAP kinase signal transduction pathways in MAO gene expression following NGF withdrawal, we examined the effect of small molecules that are known to inhibit MAP kinase signal transduction. The ERK inhibitor PD98059 did not rescue PC12 cells from apoptosis (Fig. 2-6A) and did not prevent the expression of MAO mRNA in response to NGF withdrawal (*data not shown*).

A

	Undiff	12	12	24	24	Time(hr)
		+	-	+	-	NGF

MAO

SNAP-25

β-Actin

B
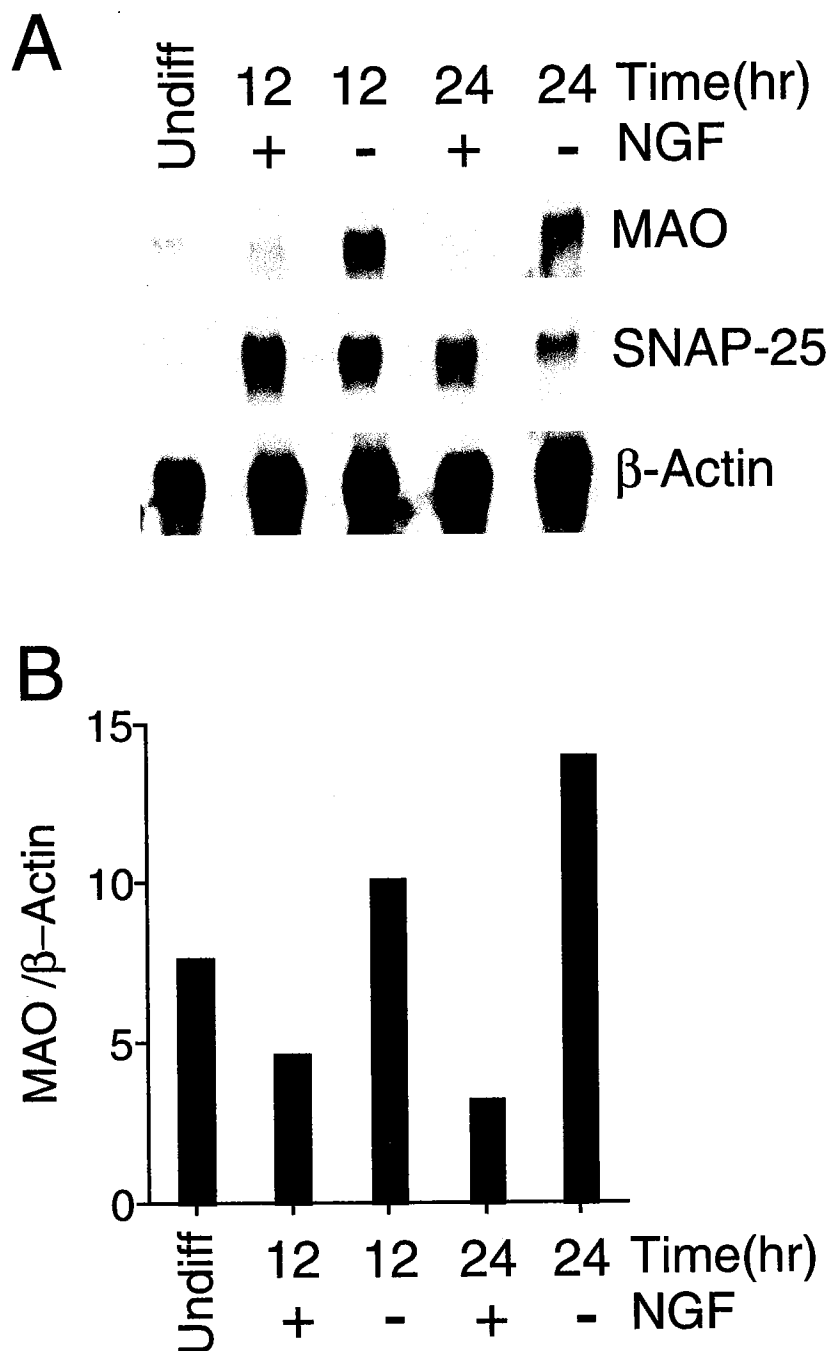
Fig. 2-5. MAO expression is induced following NGF withdrawal. Differentiated PC12 cells were incubated without or with NGF (12 or 24 hrs.). Naive (undiff) PC12 cells were also examined. The expression of MAO, SNAP-25, and β-actin was examined by Northern blot analysis. The hybridization was detected by autoradiography (A) and was quantitated by Phosphorimager analysis (B). The ratio of MAO / β-actin is presented (*arbitrary units*) as the mean ± SE (n= 4).

The JNK inhibitor SP600125 (7) reduced PC12 cell apoptosis (Fig. 2-6A), but did not affect the apoptosis-induced expression of MAO (*data not shown*). In contrast, the p38 MAPK inhibitor PD169316 reduced PC12 cell apoptosis (65% inhibition) and markedly reduced the basal expression of MAO (Figs. 2-6A & B). Strikingly, the induction of MAO mRNA upon NGF deprivation was also abolished by the p38 MAPK inhibitor PD169316 (Fig. 2-6B). These data suggest that p38 MAPK activity contributes to the expression of MAO in differentiated PC12 cells. Furthermore, these data indicate that p38 MAPK is required for the increased expression of MAO caused by NGF withdrawal.

Effect of NGF withdrawal on MAO activity. Two isoforms of MAO (A and B) are expressed in the brain (112, 113). MAO A is found predominantly in the substantia nigra (a region rich in dopaminergic neurons), the locus coeruleus, the striatum and other areas containing catecholaminergic neurons (18, 113). MAO B is expressed at a lower level in the same brain regions as MAO A and is most abundant in the raphe nuclei and glial cells (53, 112). MAO A and B differ in their substrate preferences. MAO A has high activity towards the biogenic amines 5-HT, norepinephrine, and dopamine and is specifically inhibited by clorgyline (96). MAO B has high activity towards phenylethylamine and benzylamine as substrates and is inhibited by deprenyl, a drug with reported effects on Parkinson's disease (96).

PC12 cells resemble catecholaminergic neurons by synthesizing, storing and releasing dopamine (37, 62). PC12 cells also contain predominantly the
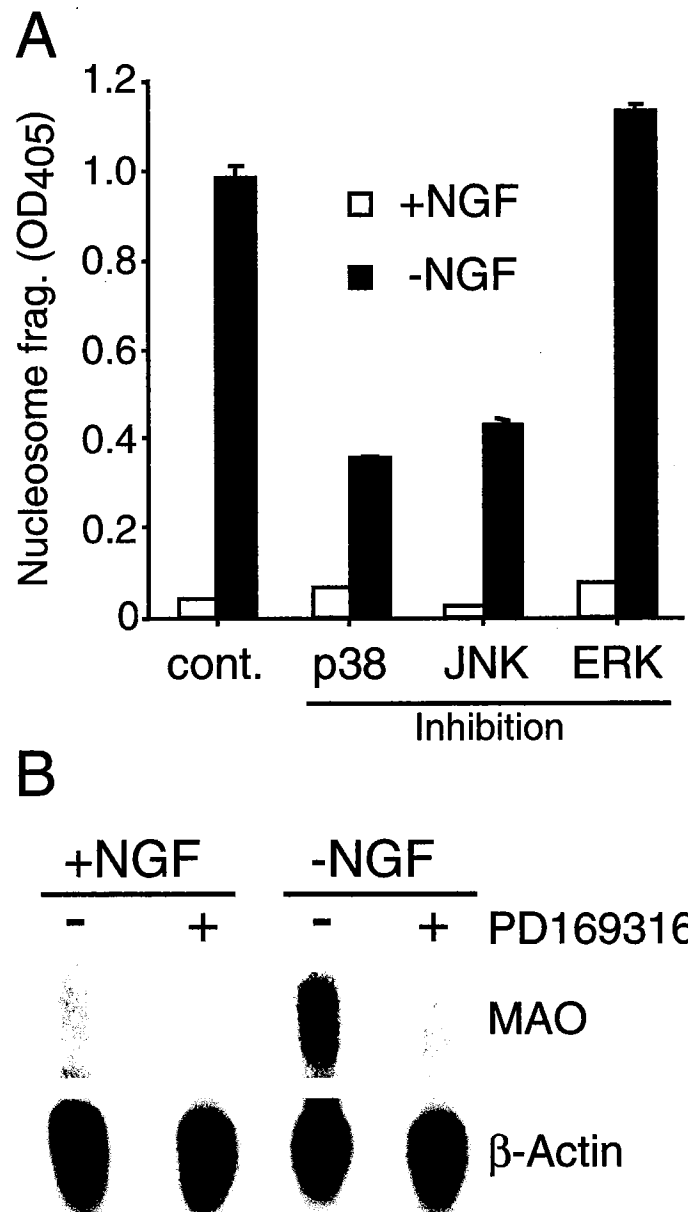
Fig. 2-6. Role of MAPK in apoptosis and expression of MAO. Neuronal PC12 cells were incubated in the presence and absence of NGF (24hrs.). (A) Apoptosis was measured using the nucleosomal DNA fragmentation assay (mean ± SD; n=3). The effect of treatment of the cells with inhibitors of p38 MAPK (PD169316), JNK (SP600125)and the ERK pathway (PD98059) was examined. Similar data were obtained in three independent experiments. (B) MAO A and β-actin mRNA expression was examined by Northern blot analysis. The effect of treatment with the p38 inhibitor PD169316 is presented. Similar data were obtained in three independent experiments.

A isoform of MAO and undetectable levels of MAO B (127). To determine if MAO activity is affected by the withdrawal of NGF an enzyme assay was performed using 5-HT as the substrate. A time course of NGF deprivation indicated that MAO substrate deamination (assayed in cell lysates) was increased following NGF withdrawal (Fig. 2-7A). The maximum 5-HT deamination was observed at 24hrs. post NGF withdrawal and was inhibited by the irreversible MAO A inhibitor clorgyline (Fig. 2-7A). Nanomolar concentrations of the MAO A inhibitor clorgyline effectively eliminated the metabolism of the 5-HT substrate while the MAO B inhibitor deprenyl had no effect at the same concentration (Fig. 2-7B). As reported previously, micromolar and millimolar concentrations of deprenyl and clorgyline inhibit both MAO A and B isoforms (Fig. 2-7B). Collectively, these data demonstrate that withdrawal of NGF from differentiated PC12 cells causes an increase in MAO activity and addition of the irreversible MAO inhibitor clorgyline strongly inhibits MAO activity in PC12 cells.

Effect of MAO inhibition on PC12 cell apoptosis. MAO expression and activity are increased following NGF withdrawal. To test whether MAO activity was relevant to NGF withdrawal induced apoptosis, we examined the effect of the MAO inhibitor clorgyline. Neuronal PC12 cells were deprived of NGF and treated with or without clorgyline for 24 hours and then stained with 4,6-diamidino-2-phenylindole. Cells exhibiting chromatin condensation, visualized by fluorescence microscopy, were scored as
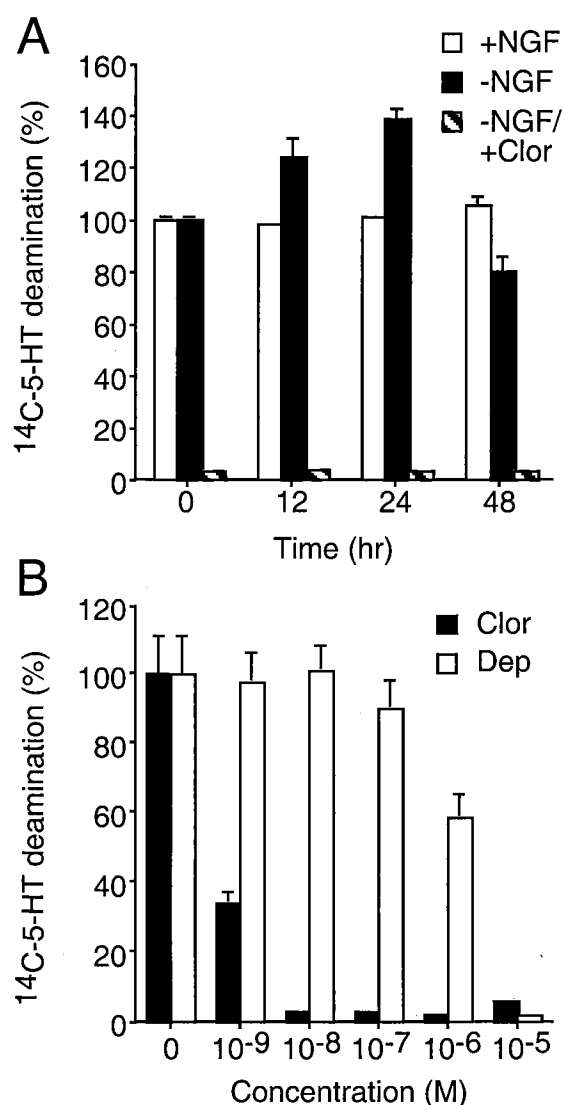
Fig. 2-7. MAO activity is increased in apoptotic PC12 cells. (A) Neuronal PC12 cells were incubated in the presence and absence of NGF (12, 24 or 48 hrs.). The effect of addition of the irreversible MAO inhibitor clorgyline (10^{-7}M) was examined. Extracts were prepared from the cells and MAO activity was measured. The data presented are the rates of 5-HT deamination (mean ± SD; n=3) and are normalized to the rate of deamination measured in control neuronal PC12 cells. Similar data were obtained in three independent experiments. (B) Comparison of clorgyline (Clor) and deprenyl (Dep) on MAO activity in neuronal PC12 cell lysates. Clorgyline and deprenyl are selective inhibitors of the MAO isoforms A and B, respectively. The effect of different concentrations of the drugs on MAO activity was examined. The data presented are the rates of 5-HT deamination (mean ± SD; n=3) and are normalized to the rate of deamination measured in control cell lysates. Similar data were obtained in three independent experiments.

apoptotic (Fig. 2-8). At least 1,000 cells were examined in each treatment group. NGF-deprivation caused a 10-fold increase in apoptotic cells after 24 hrs (Fig. 2-9A). Addition of clorgyline to NGF-treated cells had no effect on apoptosis (Fig. 2-9A). In contrast, clorgyline treatment resulted in a 70% reduction in NGF withdrawal-induced apoptosis (Fig. 2-9A). These observations were confirmed by measurement of nucleosome fragmentation of DNA, a different parameter of cell death (Fig. 2-9B). Collectively, these data indicate that MAO activity potentiates the NGF-withdrawal induced death of PC12 cells. Furthermore, MAO inhibition causes reduced apoptosis.

MAO substrate-induced apoptosis of PC12 cells. Substrates of MAO include the neurotransmitter dopamine. MAO deaminates dopamine producing the product 3,4-dihydroxyphenyl-acetic acid (DOPAC) and the byproduct H_2O_2. While the effects of DOPAC on cell survival have not been established, the potent and deleterious effects of H_2O_2 and related reactive oxygen species are established (77). Low concentrations (< 1μM) of exogenous dopamine have been shown to promote PC12 proliferation and survival, but higher concentrations (>100μM) kill PC12 cells, sympathetic ganglia neurons, and cerebellar granule neurons (131). In order to investigate the effect of MAO inhibition on dopamine-induced apoptosis, 300μM dopamine was added to NGF maintained PC12 cells. The effect of addition of the MAO inhibitor clorgyline was examined. Exogenous dopamine caused a robust increase in PC12 cell apoptosis measured by chromosome condensation (staining with

Fig. 2-8. Effect of dopamine and NGF withdrawal on nuclear morphology. Neuronal PC12 cells were incubated (24 hrs.) in the presence and absence of NGF, or in the presence of NGF plus 300 μM dopamine (DA). The effect of addition of 0.1 μM clorgyline (Clor) was examined. The cells were fixed, stained with 4,6-diamidino-2-phenylindole and inspected by fluorescence microscopy. Representative images of PC12 cells are illustrated. Similar data were obtained in three independent experiments.
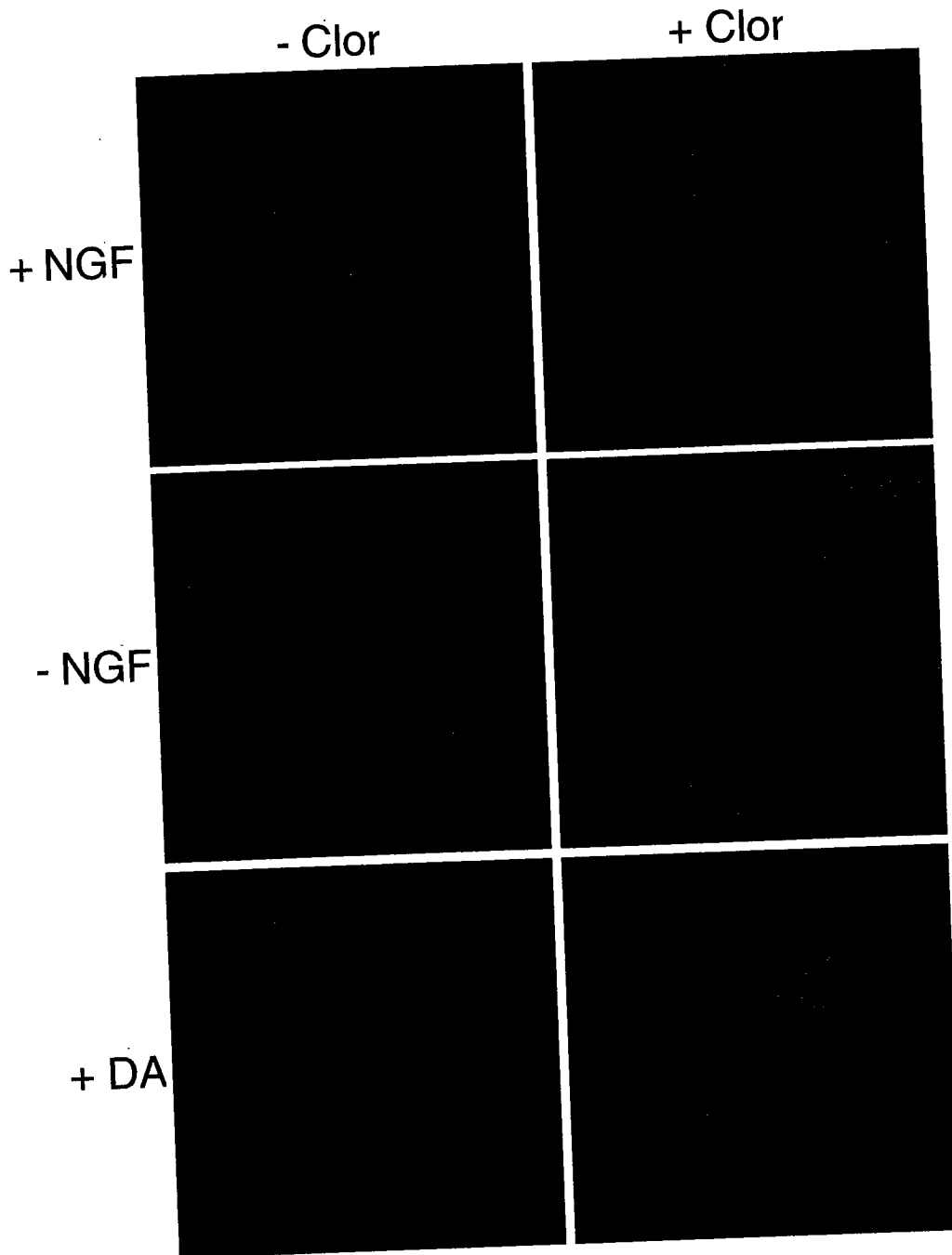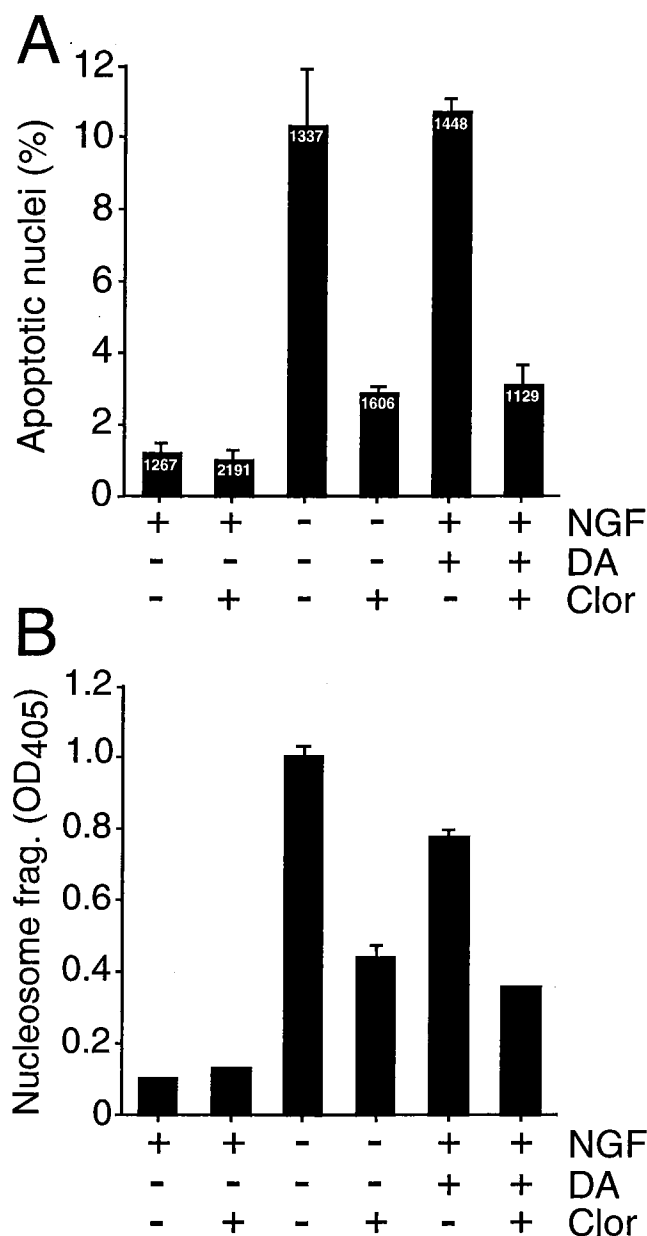
Fig. 2-9. Effect of MAO inhibition on dopamine and NGF withdrawal-induced apoptosis. Neuronal PC12 cells were incubated (24 hrs.) in the presence and absence of NGF, or in the presence of NGF plus 300 μM dopamine (DA). The effect of addition of 0.1 μM clorgyline (Clor) was examined. (A) The cells were fixed, stained with 4,6-diamidino-2-phenylindole and inspected by fluorescence microscopy. Cells with condensed chromatin and/or fragmented nuclei were scored as apoptotic. The number of cells examined for each treatment group is shown (inset). The percentage of apoptotic cells (mean ± SEM; n=3) is presented. (B) Apoptosis was examined by measurement of nucleosomal DNA fragmentation (mean ± SD; n=3). Similar data were obtained in three independent experiments.

4,6-diamidino-2-phenylindole) and the nucleosomal DNA fragmentation assay (Figs. 2-8, 2-9A & B). Furthermore, the dopamine-induced apoptosis was inhibited by the addition of the MAO inhibitor clorgyline (Fig. 2-9A & B). Together, these data indicate that dopamine-induced death of neuronal PC12 cells is reduced by the MAO inhibitor clorgyline and suggest that MAO catalyzed deamination of dopamine contributes to PC12 cell apoptosis.

Discussion

The p38 MAPK pathway is required for pro-apoptotic expression of MAO.

The JNK and p38 MAPK are activated by many stimuli that cause apoptosis (16). JNK phosphorylation and activation of the transcription factor c-Jun during cell death is well established (16). However, the roles of other substrates of JNK are not defined (16). Similarly, the targets of the p38 MAP kinase pathway in apoptosis are poorly understood. It is likely that these MAP kinase signal transduction pathways contribute to the control of gene expression in apoptotic cells. Gene expression is known to be required for neurotrophic factor withdrawal-induced apoptosis of PC12 cells (69). Candidate genes that may be induced by pro-apoptotic signal transduction by the JNK pathway include FasL (60). However, genes that are expressed by a p38 MAPK-dependent mechanism in apoptotic cells have not been described. Here we report that MAO expression is increased in neuronal PC12 cells following NGF withdrawal and that this expression of MAO requires the p38 MAP kinase signal transduction pathway.

The p38 MAPK inhibitor PD169316 prevents increased MAO expression by PC12 cells following NGF withdrawal and also suppresses apoptosis. These data, together with the observation that NGF withdrawal causes p38 MAPK activation (56, 121) suggests that the p38 MAPK signaling pathway functions as a regulator of MAO gene expression in apoptotic cells. Further studies are required to identify the mechanism by which the p38 MAPK

pathway increases the expression of MAO mRNA. It is possible that p38

MAPK may regulate MAO mRNA stability. Alternatively, p38 MAPK may

regulate transcription of the MAO gene. The observation that p38 MAPK

phosphorylates and activates a number of transcription factors (e.g. ATF2,

Elk-1, Ets, and MEF2) and that p38-activated protein kinases can

phosphorylate and stimulate the transcription factor CREB, suggests that the

effects of p38 MAPK on MAO gene expression may be complex.

Nevertheless, MAO expression is dependent on the p38 MAPK signal

transduction pathway (Fig. 2-6B).

MAO activity contributes to PC12 cell apoptosis. The observation that MAO

expression and activity are increased in response to NGF withdrawal

indicates that this oxidase may play a role in trophic factor deprivation-

induced apoptosis of PC12 cells. Indeed, the byproducts of MAO catalyzed

deamination of dopamine cause apoptosis of neuronal cells (49, 77). While

both autooxidation and MAO-catalyzed deamination of dopamine have been

reported to cause apoptotic death (34, 49, 77), the results of the present study

indicate that MAO activity does contribute to PC12 cell death (Figs. 2-7 & 2-

8). Dopaminergic neurons and PC12 cells sequester millimolar

concentrations of the neurotransmitter dopamine in releasable pools of

synaptic vesicles (62). Apoptosis induced defects in exocytosis, transporter

molecules or synaptic vesicle integrity may lead to increased cytosolic

dopamine, which can then be metabolized by MAO. The dopamine may also

be derived from exogenous sources (e.g. release from neighboring cells). Together, these considerations indicate that MAO provides a potential source of reactive oxygen species during the apoptotic process. Other sources of reactive oxygen species include NADH oxidases (101). Inhibition of MAO may therefore protect cells from apoptosis by decreasing the formation of reactive oxygen species. This proposed mechanism implies that the role of MAO is to potentiate apoptosis rather than acting directly in the apoptotic death mechanism (e.g. caspase activation). The possibility that MAO may contribute to the death of dopaminergic neurons following the death of neighboring neurons (and subsequent release of dopamine) is intriguing because it suggests a potential mechanism for neurodegeneration.

MAO is a resident protein of the outer mitochondrial membrane (36). This localization of MAO is interesting because an important role for mitochondria in apoptosis has been established (17). This sub-cellular localization suggests that MAO may alter mitochondrial function. Indeed, it has been demonstrated that MAO activity can cause mitochondrial dysfunction (14). Further studies are required to demonstrate that MAO does cause mitochondrial dysfunction in apoptotic cells.

MAO A deficiency in mice and humans is associated with increased aggressive behavior (8, 10). Studies of MAO A knockout mice indicate elevated brain levels of dopamine and this neurotransmitter mediates, in part, a stress response (10). A link between aggression and neuronal apoptosis has not been established. However, the results of our study suggest that

apoptotic defects may contribute to the phenotype of MAO A-deficient mice.

Nevertheless, the results of the present study establish that MAO is a target of

pro-apoptotic signal transduction by the p38 MAPK signal transduction
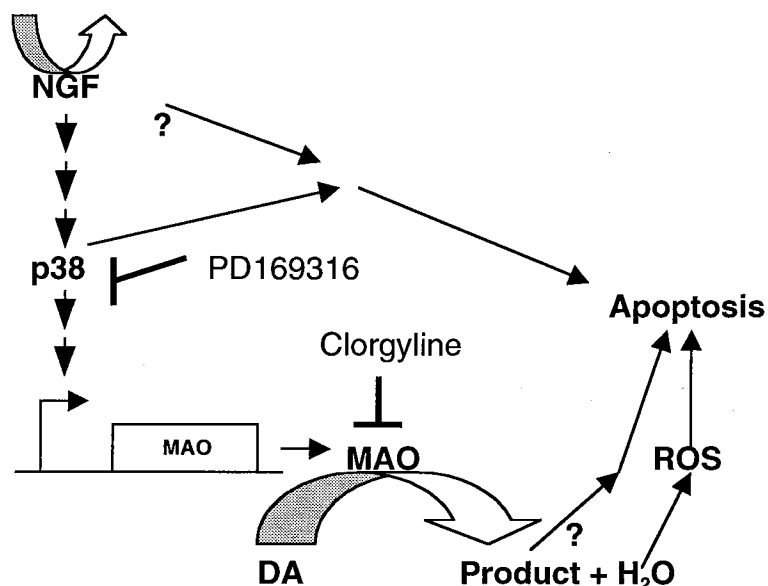
pathway.

Fig. 2-10. Proposed mechanism for p38 contribution to PC12 cell death.

Illustrated is the involvement of p38 and MAO as supported by the data
presented in this study.

CHAPTER III

PRO-APOPTOTIC GENE EXPRESSION MEDIATED BY JNK DURING ARSENITE INDUCED NEURODEGENERATION

Background

<u>JNK Characteristics.</u> Degeneration of neurons *in vitro* and *in vivo* caused by apoptotic stimuli has been shown to involve the activity of JNK. The kinase's activity increases in response to death stimuli and blocking the functions of JNK causes perturbations in apoptotic processes.

Three separate genes make up the JNK family of stress activated protein kinases (SAPK) (16). These 3 genes are alternatively spliced, giving rise to 10 known JNK isoforms. Expression of the transcripts leads to the formation of 46 kDa and 55 kDa isoforms of each of the JNK molecules. It is not known what the functional differences, if any, exist between the JNK variants. Some clues are given by examining the tissue distribution of the JNKs (16). Universal expression of JNK1 and JNK2 occurs in mammalian tissues. JNK3 has a more restricted expression pattern and is predominantly expressed in the brain and to a smaller extent in the heart and testis. JNKs are

Serine/Threonine kinases, preferentially phosphorylating Ser/Thr-Pro motifs on substrates. JNK targets are also required to have specific binding domains to allow JNK to dock and perform a phosphate transfer reaction (102). The high resolution structure of inactive JNK3 has been determined and reveals that this kinase is typical of other MAPK (122). In simple terms the kinase consists of two globular domains sandwiched around an active site cleft. ATP binds in the active site which appears to be relatively well ordered. Activation of the kinase by phosphorylation on the Thr and Tyr residues is likely to change the structure of the active site to allow it to accommodate substrates. Determination of the structure of an active kinase will allow a comparison to the inactive structure and reveal the changes that occur to enable the kinase to phosphorylate substrates. Dual specificity Ser/Thr-Tyr MAP kinase phosphatases (MKPs) regulate the MAP kinases by de-phosphorylating and inactivating them. In vitro experimentation reveals that MKP isoforms display different specificities towards JNK, p38 and ERK MAP kinases (71). It remains to be established that these preferences are also relevant in vivo. Knockout of the MKP-1 gene in mice results in no phenotype suggesting that redundant functions are mediated by the MKP isoforms (19).

Cascades of JNK activation. As with the p38 signal transduction pathway, JNK activation is mediated by protein kinase cascades (Fig. 3-1) (16). The

Stimulus

Rho GTPases

?

TRAF2

MEKKs ASK1 TAK

TPL2 MLK ?

MKK4/MKK7

JNK
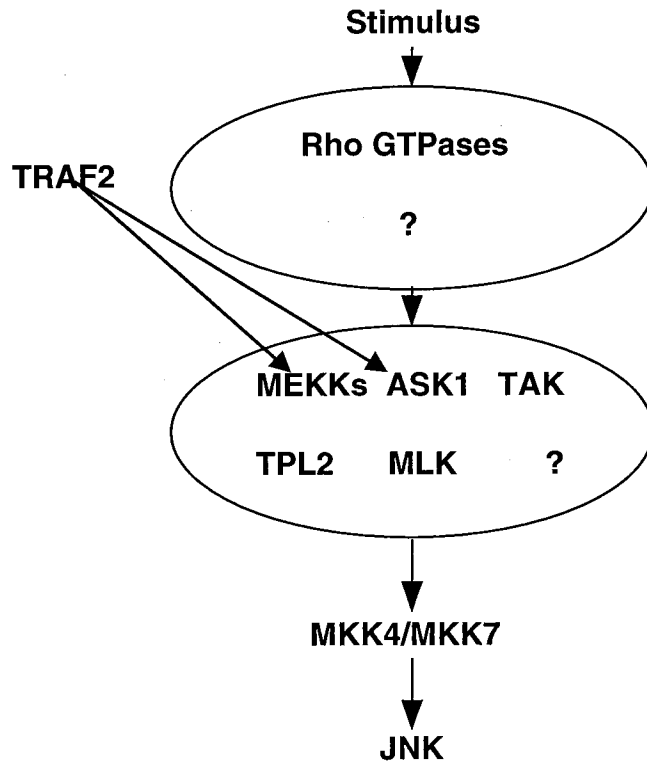
Fig. 3-1. Model of core JNK signaling pathways

Known components of the pathways are shown. Question marks represent unkown components that are likely to exist.

molecular mechanism (s) leading to JNK activation have been best described in fibroblasts, tumour cells and immune cells. JNK pathway activation is achieved in neurons by treating them with many of the same stimuli used on non-neuronal cells. For example, ligation of FAS and TNF receptors with agonistic molecules results in adaptor recruitment to the receptor, activation of protease cascades and activation of stress kinase cascades within neuronal cells (33).

In order to perform an ATP dependent phosphoryl transfer, JNK must first be activated by phosphorylation of Threonine and Tyrosine residues in it's subdomain VIII. Upstream activating MAP kinase kinases, MKK4 and MKK7 phosphorylate and activate JNK (16). At present three isoforms of MKK4, differing in their amino terminal domains, have been identified. Likewise, alternative splicing and utilization of unique inititation sites generates six MKK7 isoforms which differ in their carboxy-terminal and amino-terminal sequences. Unlike the upstream activators of the p38 pathway, numerous studies have established some specificities of the JNK activation pathways. In general, cytokines such as IL-1 and TNF activate JNK through MKK7 while activation by environmental stress is mediated by MKK4. Both MKKs can phosphorylate JNK on Thr and Tyr but MKK7 and MKK4 prefer to phosphorylate Thr and Tyr respectively. MEKKs, MLKs, ASK1 and numerous other MAP kinase kinase kinases (MAPKKK) dual phosphorylate the MKKs on their T-loop, activating them (16). The large number of MAPKKKs complicate interpretations of specific signaling responses to physiologically relevant stimuli. Functional redundancies are likely to exist among these kinases. The upstream activators of MAPKKKs are not well characterized but are known to include members of the Rho family of GTPases (16, 48). These GTPases have been shown to target members of the MEKK and MLK groups of the MAPKKKs. In addition, the adaptor protein TRAF2 binds MEKK1, ASK1 and other unidentified molecules to activate JNK and p38 in response to TNF treatment (45, 128).

More specifically, activated TNF receptors recruit TRAF2 to its cytoplasmic domain which can then bind the MAPKKKs such as ASK1 (126). Prior to it's recruitment by TRAF2, ASK1 dissociates from it's inhibitor, thioredoxin in a reacitve oxygen species dependent manner (93). This allows ASK1 to homodimerize, an event thought to be necessary for TRAF2 to function through that particular MAPKKK. The mechanism of interaction between TRAF2 and MEKK1 is unknown.

JNK activation and neuronal cell death. JNK is activated in response to a variety of apoptosis inducing agents. Presently, the only known kinase to phosphorylate and activate the pro-apoptotic transcription factor c-Jun, is JNK. Therefore, JNK has been strongly implicated in apoptotic responses. Initial experiments demonstrated that NGF withdrawal from neuronal PC12 cells causes JNK activation and subsequent cell death (121). Catalytically inactive and constitutively active components of the JNK pathway induce an apoptotic response in PC12 cells. Specifically, differentiated PC12 cells over expressing the JNK activator, MEKK1 undergo increased apoptosis as compared to cells expressing an inactive, mutant MEKK1 (121). Co-expression of dominant-interfering c-Jun mutants prevent the MEKK1 induced apoptosis. Transgenic mice, lacking the JNK gene (s) have provided the best evidence for JNK mediation of apoptosis. Mice which have been treated with the glutamate receptor agonist kainic acid (KA) undergo marked seizures and tremor. Kainic acid injection also causes an initial necrotic insult to the CA3 region of the hippocampus in mouse brain followed by an

increase in phosphorylated c-Jun staining immediately surrounding the necrotic area (123). Coincident with the phospho-c-jun staining pattern, a large amount of hippocampal cell apoptosis was observed up to 5 days after KA injection. AP-1 driven gene expression in the hippocampus of these mice was also significantly increased. Conversely, mice which have had the neuron specific JNK3 isozyme ablated, undergo a striking decrease in seizures and tremors due to KA injection. Hippocampal tissue slices from KA treated, *jnk 3 -/-* mice display initial necrotic damage but the latent cell death observed in the wild type mice is absent (123). The increase in AP-1 driven gene expression which occurs in the wild type mice is abolished in the *jnk3 -/-* mice. These data demonstrate the importance of the JNK pathway in neuronal apoptosis. More specifically, the decrease in AP-1 driven gene expression concomitant with the decrease in hippocampal apoptosis in the *jnk 3 -/-* mice indicates that JNK may activate gene expression to cause neurodegeneration. The JNK1 and JNK2 kinases have been implicated as mediators of apoptosis during early embryonic development of the mouse brain (55). Mice harboring a compound mutation of both *Jnk1* and *Jnk2* genes die at E11.5. At this stage an anomaly of hindbrain exencephaly is obvious on the *Jnk1/Jnk2* null embryos (55) and presumably this defect stimulates the lethal absorption of the unborn fetuses. Histological examination of hindbrain tissues from normal and double mutant E9.0 embryos reveals a lack of apoptotic cells in the lateral edges of the neural folds in the double mutant as compared to the wild type. This lack of apoptosis in a Jnk1/Jnk2

mutant embryo prevents the neural tube from folding properly, causing the observed exencephaly and subsequent embryonic lethality.

Intriguingly, an anti-apoptotic role for JNK is suggested by data obtained from the same Jnk1/2 double mutant embryos. The forebrains of these mice display a substantial increase in the number of neuroepithelial cells undergoing apoptosis, relative to wild type mice (55). In addition, this particular area of the brain also displayed an increase in caspase-3 activity. Furthermore, TNF treatment of some non-neuronal cell types leads to JNK activity which does not contribute to the resulting apoptosis of those cells (64). Similarly, lymphocytes from mice which don't express the JNK pathway activator, TRAF2, are more sensitive to TNF-induced apoptosis (126). These findings contrast with the pro-apoptotic role of JNK and display the potential complexities of JNK activity in different cell types and tissues regions.

JNK regulation of transcription events in cell death. Downstream of JNK activation, much focus has been placed upon the phosphorylation of c-Jun in response to apoptotic stimuli (Fig. 3-2). Activated c-Jun can dimerize with another transcription factor, ATF-2 (which is also phosphorylated by JNK and/or p38) and then bind to TRE elements in it's own promoter to induce c-Jun and other gene's synthesis (41, 116). Phosophorylated c-Jun can also heterodimerize with c-Fos to form AP-1 complexes which have been suggested to be important for driving apoptotic gene expression in neurons

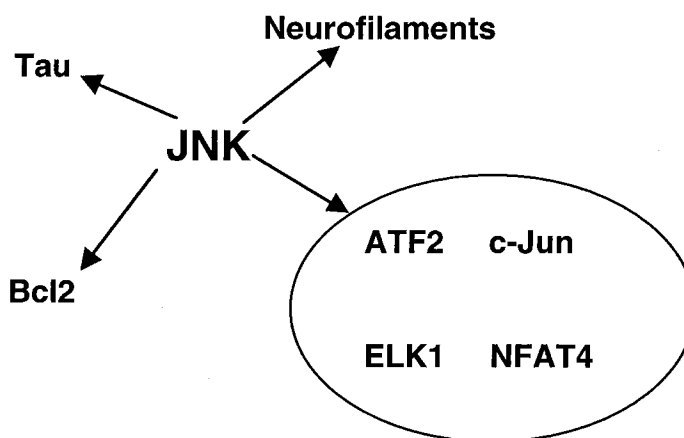(123). Developmental (e.g. trophic factor withdrawal) and neurotoxic (e.g. kainic acid treatment) models

Neurofilaments

Tau

JNK

Bcl2

ATF2   c-Jun

ELK1   NFAT4

Figure. 3-2. Substrates of JNK

Known substrates of JNK are pictured. Molecules encircled represent transcription factors that are activated by JNK

of cell death have been shown to depend on new gene expression to occur. JNK is activated by apoptosis stimuli and a role for it in macromolecular synthesis dependent cell death is implied through it's regulation of the pro-apoptotic transcription factor c-Jun. But, as is the case with p38 MAP kinase, little is known about the transcriptional consequences downstream of JNK and c-Jun activation in neuronal apoptosis. However, one area of investigation, mostly in non-neuronal cells, has identified Fas ligand as a gene induced by JNK in response to apoptotic stimulus (16). In a Jurkat T-cell line, activation of the JNK specific MAPKKK, MEKK1 results in increased JNK activity, increased Fas ligand promoter activity and apoptosis of those cells

(26). Transfection of dominant interfering mutants of c-Jun and ATF-2 into the same cells decreases Fas ligand message and apoptosis. Cerebellar granule neurons, undergoing apoptosis by trophic factor withdrawal display increased JNK levels, increased phospho-c-Jun staining and increased Fas ligand message as measured by RT-PCR (60). Also, addition of a Fas:Fc decoy receptor into the media of cells undergoing trophic factor withdrawal reduces cell death caused by this treatment. This study suggests that Fas ligand is an important mediator of neurodevelopmental death in these cells. However, it remains to be determined if a Fas ligand mediated mechanism is activated in other neuronal types or in neurotoxic models of neurodegeneration. Furthermore, no studies have examined which JNK isozyme is responsible for inducing Fas ligand expression during apoptosis. Given the number of JNK splice variants, the tissue distribution of the JNKs and the evidence for both pro and anti-apoptotic activities of JNK, the answers to these questions may be complex but important nonetheless in defining specific roles for JNK in regulation apoptotic mechanisms.

Arsenite model of neurodegeneration. Gestational day 9 mouse embryos can be explanted from the uterus and grown in culture for at least 24 hours during which embryonic growth and development mimics that which occurs in vivo (92). One event which takes place during this time is cranial and spinal neural tube closure (100). Introduction of micromolar concentrations of sodium arsenite into the culture media of the E9 mice results in non-closure of the cranial neural tube or exencephaly (100). Jnk1/Jnk2 null

embryos display an exencephaly anomaly and die by E11.5. In both examples of neural tube non-closure, evidence was presented demonstrating that defective cell death processes lead to the lethal brain defects. Therefore it may be that JNK also regulates apoptosis in the arsenite or neurotoxic model of brain malformation. Thus, arsenite treatment of rat cortical neurons increases JNK and p38 MAP kinase activities and causes apoptosis of those cells (73). Although the same phenotype is reached in models involving JNK activation and JNK deletion, it is likely that the mis-regulation of JNK activity contributes to the exencepahly. However, as is the case in many studies of JNK in neurodegeneration, no functional significance is described for JNK (or p38) activation in these neurons.

The purpose of this study is to identify genes that are regulated by JNK activity in response to apoptotic stimulus. We report the identification of Fas ligand as a gene dependent on JNK for it's arsenite induced expression. Furthermore, our results indicate that the JNK3 isoform, specifically, upregulates Fas ligand in response to arsenite treatment.

Methods and Materials

Targeted disruption of the Jnk3 gene. Disruption of the Jnk3 gene was performed as described previously (123). A λ Fix II phage library from a 129/SV mouse strain was utilized to clone a 10 Kb *Jnk3* fragment (NotI-EcoRI). A 4.0 Kb, 5′ fragment from the genomic fragment, a 1.6 Kb PGK-neo cassette and a 1.8 Kb 3′ fragment from the Jnk3 genomic fragment were inserted into pBluescript KS vector (Stratagene) to construct the targeting vector. The vector also contained a PGK-tk cassette for negative selection of the mutant ES cells. After linearizing, the vector was injected into W9.5 ES cells and the resultant transfectants resistant to G418 and gancyclovir were screened by Southern blotting. B6.MRL-Tnfrsf6^{lpr} mice (Jackson Laboratory) which harbor a mutation in the Fas antigen were generously supplied by the laboratory of Dr. Ralph Budd (University of Vermont).

Primary Neuronal Cell Isolation and Culture. Wild type, *Jnk3 -/-* and *lpr* mice were mated and pregnant females were sacrificed by cervical dislocation at day E17.5. The E17.5 embryos were removed from the uterus, decapitated and the cortices dissected away from the rest of the brain. The cortical tissue was dissociated by trituration until a homogenous suspension was obtained. After counting viable cells by trypan blue exclusion, 1.25×10^5 cells/ml were plated on poly-L-ornithine coated plates in Neurobasal medium (Life

Technologies Inc.) containing 1X B27 supplement (Life Technologies Inc.),

0.5mM L-Glutamine (Life Technologies Inc.) and 1% Fetal Bovine Serum

(Omega Scientific). The plated cells were incubated in a 5% CO_2 incubator at

37° C. After 2 days more of the same media was added without serum but

including 5-Fluoro-2'- Deoxyuridine (10µM final, Sigma) and Uridine 5'-

Triphosphate (10µM final, Sigma) to reduce glial cell contamination. 5 days

after the intitial plating all media was aspirated and replaced with

Neurobasal media containing B27 and 0.5mM Glutamine. Neuronal cells

were utilized for experiments 7-9 days after initial plating.

Cell Survival Assay. Cells were pre-treated with plain media or media

containing indicated inhibitors for 1-2 hours prior to the addition of media

containing none or 2µM Sodium m-Arsenite (Sigma). At various time points

indicated after arsenite treatment, a tenth volume (of the culture medium) of

3- [4, 5–Dimethylthiazol–2–yl]– 2, 5–diphenyltetrazolium bromide (MTT; 5

mg/ml in sterile PBS) was added to the cells and allowed to incubate for 2

hours. All supernatant was then aspirated from the cells and .04 N

HCl/Isopropanol was added to solubilize the cells. 200 µl of the solvate was

then transferred to a 96 well plate and the plate was read at 570nm

(background of 650nm subtracted) on a microtiter plate reader.

Total RNA preparation. Total RNA was extracted from neurons in culture

utilizing the RNeasy Mini kit by Qiagen and following the manufacturer's

protocol. After aspiration of the supernatant cells were lysed in the wells by

addition of a guanidinium isothyocyanate buffer. Total RNA in the lysates binds to a silica based column through which the lysates are passed. Following serial washes with ethanol based solutions to remove contaminants and impurities, the total RNA was eluted with 30 µl of diethyl pyrocarbonate- treated water. The RNA was quantified and the purity determined by measuring the absorbance of the samples at 260 and 280 nm. Samples were then aliquotted and stored at –80°C until use.

Ribonuclease protection assay. Multi-probe ribonuclease protection assays were performed with the Riboquant RPA kit by Becton Dickinson-Pharmingen and following the manufacturer's protocol. Multi-probe templates were radiolabeled with 10µl of α- ^{32}P- Uridine Triphosphate (10mCi/ml; New England Nuclear) by T7 RNA polymerase for 1 hour at 37° C. After terminating the reaction with Dnase, the cocktail was phenol:chloroform extracted (1:1 vol/vol) and the resulting aqueous phase was transferred to a new tube. After another chloroform extraction the total RNA was precipitated from the aqueous phase in a new tube by adding ammonium acetate (0.57M final concentration) and 100% ethanol incubating for 30 minutes at –80° C. The samples were spun 15,000 x g for 15 minutes at 4° C. The supernatant was removed and the pellet washed once with 4° C 90% ethanol. After removing the supernatant and air drying, the pellets were re-suspended in 8µl of a hybridization buffer and 2µl of 4 x 10^5 cpm/µl labeled probe was added. These samples were placed on a heating block at 90° C which was immediately turned to 56° C and maintained at that temperature

for 16 hours. Prior to removing the samples the heating block was turned to 37° C and when the temp reached 37° C the samples were incubated for 15 minutes. Single stranded RNA was digested by adding Rnase (250U/µl) to the samples and incubating for 45 minutes at 37° C. Tris-saturated phenol and chloroform (1:1 vol/vol) were added to the digests and vortexed into an emulsion followed by a 15,000 x g centrifugation at room temperature. The upper aqueous phase was transferred to a tube containing ammonium acetate (0.57M final concentration) and 100% ethanol and incubated at –80° C for 30 minutes to precipitate RNAs. Samples were microfuged at 15,000 x g for 15 minutes at 4° C. Supernatants were removed and the pellets were washed once with 90% ethanol. After removing the supernatant the pellets were air dried and then re-suspended in 5µl of a bromophenol blue containing loading buffer. 1.5 x 105 cpms of the unhybridized template set (s) and the total volumes of the hybridized samples were resolved by running them on a 6% acrylamide gel. The gel was run at 50 watts constant power for 2.5-3 hours (until the bromophenol blue reached the bottom edge of the gel). After disassembly of the gel running apparatus, the gel was adsorbed onto filter paper, wrapped in saran wrap and dried on a vacuum gel dryer. The dried gel was exposed to a phosphorimager for quantification and to film for autoradiography.

Immunocytochemistry of primary cells. Glass coverslips (Corning co.) were ethanol flamed and coated with a 0.1% solution of poly-L-ornithine (Sigma). The solution was aspirated and the coverslips washed 3X with sterile water

before freshly isolated cortical neurons were plated at 1×10^5 cells/ml. After inhibitor and/or arsenite treatment the media was aspirated and immediately replaced with a fixative solution containing 4% para-formaldehyde, and 1mM $MgCl_2$ in 1x phosphate buffered saline, ph 7.3 for 45 min at room temperature. The fixative was aspirated and the cells washed 3 x 5 min in 1Xphosphate buffered saline containing 10mM glycine and 0.1% Triton X-100 at room temperature. The cells were permeabilized for 30 min in 1X phosphate buffered saline containing 0.5% NP-40 at room temperature. Following permeabilization cells were washed 1X with 1X phosphate buffered saline containing 0.1% Triton X-100, 5mM sodium flouride, and 1mM sodium molybdate. After aspirating off the previous wash A blocking solution consisting of 0.1% Triton X-100, 2.5% BSA, 2.5% horse serum in 1x phosphate buffered saline was added to the cells for overnight incubation at 4°C. Primary antibodies were then added to blocking solution at various dilutions (1:500 for α-MAP2; 1:500 for α-JIP 3 (50)1:300 for α–JIP1 (125) and 1:500 for α–phospho-JNK (Promega) and incubated with the cells for 3-4 hours at room temperature with gentle rocking. The coverslips were then washed 3X 5 min in 1X phosphate buffered saline containing 10mM glycine and 0.1% Triton X-100 at room temperature. Goat anti-mouse Cy3 (1:2,000) or Donkey anti-rabbit FITC (1:1000) conjugated secondary antibodies (Molecular Probes) in blocking solution were then added to the cells for 1.5 hours at room temperature with gentle rocking. FITC conjugated α-tubulin antibodies (1:750; Sigma) was also added to some coverslips during the secondary

incubation. The coverslips were then washed 3X 5 min in 1X phosphate buffered saline containing 10mM glycine and 0.1% Triton X-100 at room temperature except that the second wash also contained $0.1\mu g/ml$ of the DNA stain 4,6-diamidino-2-phenylindole. Coverslips were then mounted with $4\mu l$ of vectashield mounting media (Vector Laboratories Inc.) onto slides, sealed with nail polish (Revlon) and then viewed at 10,000x magnification on a Zeiss Axioplan 2 fluorescence microscope.

Results

<u>Establishment of cortical neuron cultures.</u> Neuronal degeneration occurs in response to excitotoxic and neurotoxic agents as well as in neurodegenerative disease. These insults or diseases target specific populations of neurons in brain tissues. Cortical neurons are often damaged by noxious stimuli and in disease conditions. Therefore we prepared primary cortical cell cultures for the purpose of studying neurodegeneration in response to the neurotoxin, arsenite. Embryonic cortices fron wild type, *Jnk3 -/-* and *lpr* mice were dissected and dissociated cultures were established by triturating the tissue. Primary antibodies to JNK, the scaffolding molecules JIP1, JIP3 and PSD95, and the microtubule associated proteins MAP2 and β tubulin were utilized to examine the location of these proteins in the primary cells. The MAP2 staining (Fig. 3-6B) and the FITC conjugated tubulin (Fig. 3-3) antibody revealed that approximately 90% of the cells were neurons. In addition, western blotting for the glia specific protein GFAP, produced a very low signal from the cells that were in culture for 7 days (*data not shown*). Immunofluorescence images reveal that JNK, JIP1 and PSD95 are localized at the tips of neuritic processes with a faint signal along the neurites and in the cell body (Fig. 3-3 through 3-5). In contrast, JIP3 staining exhibited a strong signal in the cell soma or peri-nuclear with little signal detected elsewhere in the

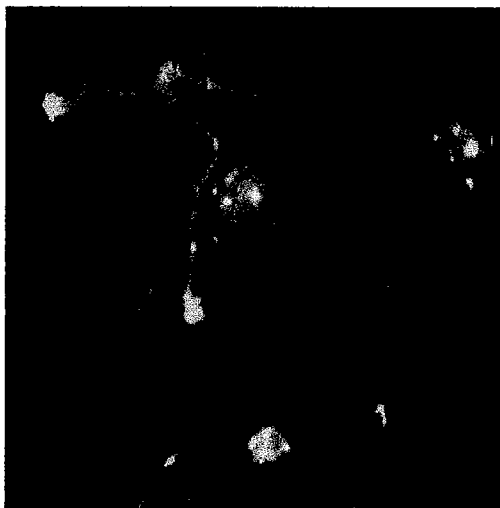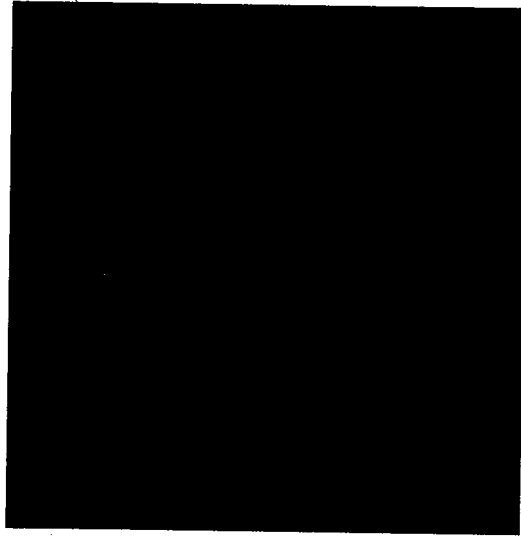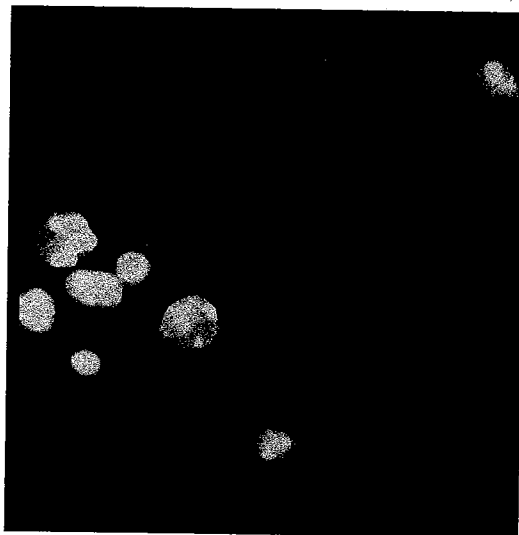α Phospho-
JNK

Fig. 3-3

α Tubulin
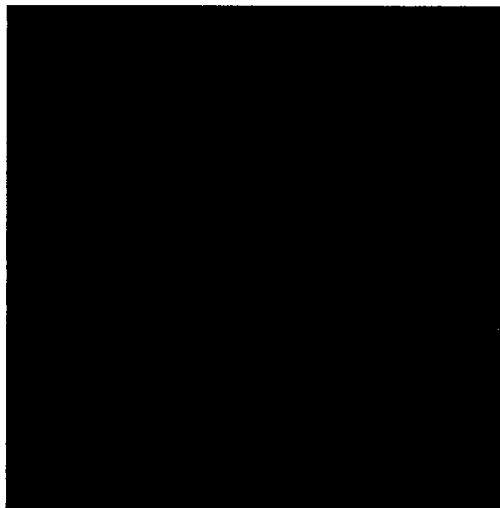
Overlay

α Phospho-JNK
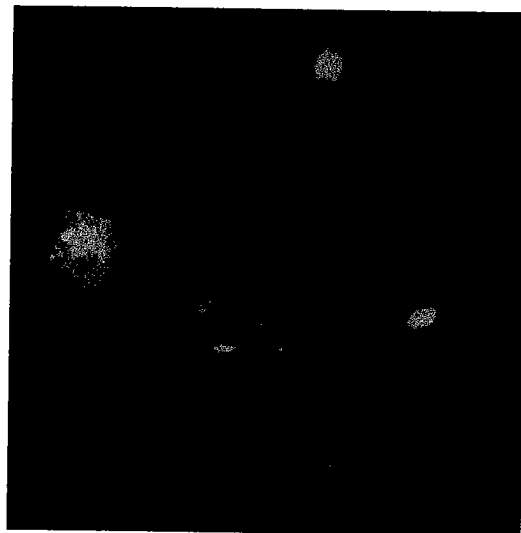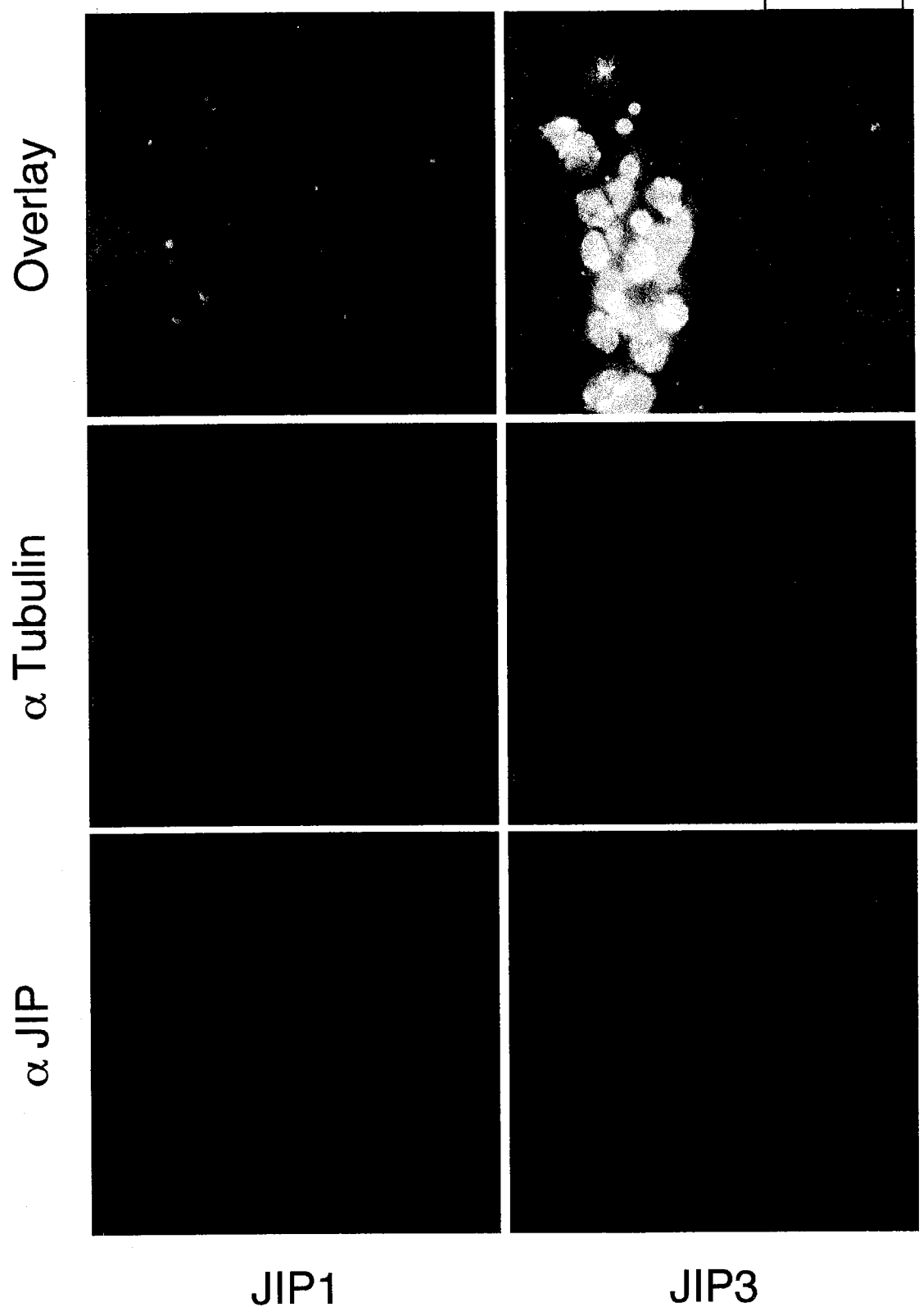
α PSD95

Overlay

Fig. 3-4

Fig. 3-5

Figure 3-3. Immunolocalization of JNK in cortical neurons. Primary cells that had been cultured for 7 days were fixed in p-formaldehyde and subjected to immunocytochemistry with antibodies to JNK and tubulin as indicated. Cy3 (red) and FITC (green) fluorophors were detected by immunofluoresence microscopy, separately and in combination (overlays) as shown. The pictures were taken at 630 X magnification. These images are representative of cells observed in at least 5 fields of view.

Figure 3-4. Co-localization of JNK and the putative growth cone scaffold PSD95. Primary cells that had been cultured for 7 days were fixed in p-formaldehyde and subjected to immunocytochemistry with antibodies to JNK and PSD95 as indicated. Cy3 (red) and FITC (green) fluorophors were detected by immunofluoresence microscopy, separately and in combination (overlays) as shown. The pictures were taken at 630 X magnification. These images are representative of cells observed in at least 5 fields of view.

Figure 3-5. Immunolocalization of JNK pathway scaffolding molecules in cortical neurons. Primary cells that had been cultured for 7 days were fixed in p-formaldehyde and subjected to immunocytochemistry with antibodies to JIP1, JIP3, and tubulin as indicated. Cy3 (red) and FITC (green) fluorophors were detected by immunofluoresence microscopy, separately and in combination (overlays) as shown. The pictures were taken at 630 X magnification. These images are representative of cells observed in at least 5 fields of view.

resting neurons (Fig. 3-5). Theses staining patterns were the same in *Jnk 3 -/-* cortical neurons also (*data not shown*).

Arsenite induces apoptosis of mouse cortical neurons. Varying concentrations of the environmental toxin, sodium arsenite (0-2μM) were used to treat mouse cortical neurons in culture. The effects of these treatments were then quantitated by measuring cell viability with the mitochondrial dehydrogenase substrate, 3-[4, 5–Dimethylthiazol–2–yl]–2, 5–diphenyltetrazolium bromide (MTT). This dose-response treatment of the cortical neurons resulted in a 70% decrease in cell viability after a 48 hour

exposure to 2μM arsenite (Fig. 3-6A). Visual examination of arsenite treated
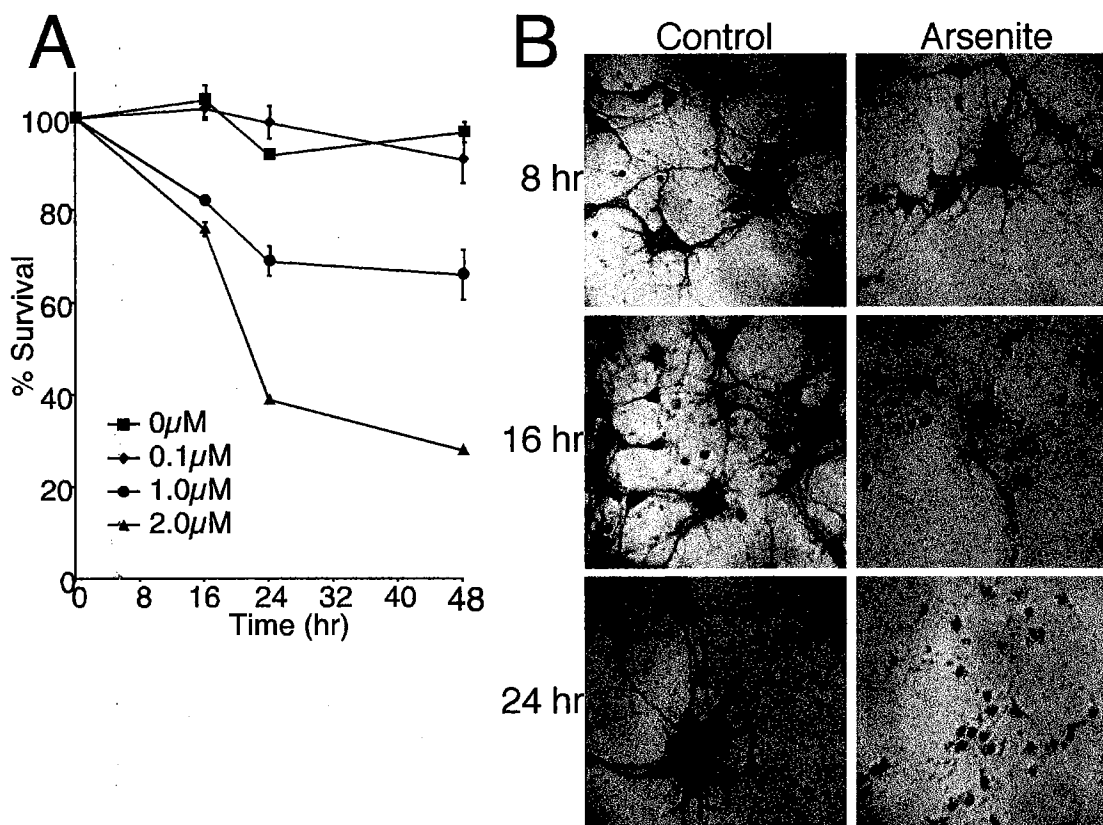
Figure 3-6. Effect of sodium arsenite on cortical neuron viability. (A) Cortical neurons in culture were treated with 0μM (squares), 0.1μM (diamonds), 1.0μM (circles) or 2.0μM (triangles) of sodium arsenite. At the indicated time points cell survival was determined by measuring mitochondrial dehydrogenase metabolism of 3-[4, 5–Dimethylthiazol–2–yl]–2, 5–diphenyltetrazolium bromide (MTT). Data is normalized to the untreated control cells (mean ± S.D., n=3). Similar data were obtained in at least 3 independent experiments. (B) Neurons were treated with or without 2.0μM arsenite for 0-24 hours, fixed with p-formaldehyde and stained with α-MAP2 antibody to examine neuronal morphology at the time points indicated. Light microscopic images were taken at 200 X magnification and are representative of 5 fields viewed.

cells revealed increasing neurite degeneration and cell body shrinkage,

typical characteristics of neuronal apoptosis, from 8 to 24 hours exposure (Fig.

3-6B).

Arsenite induced neuronal death is mediated by JNK. JNK activity has been reported to be activated by arsenite in rat neurons (73). If JNK mediates arsenite induced death then ablation of the JNK gene(s) or inhibition of JNK activity should block the effect of arsenite treatment.
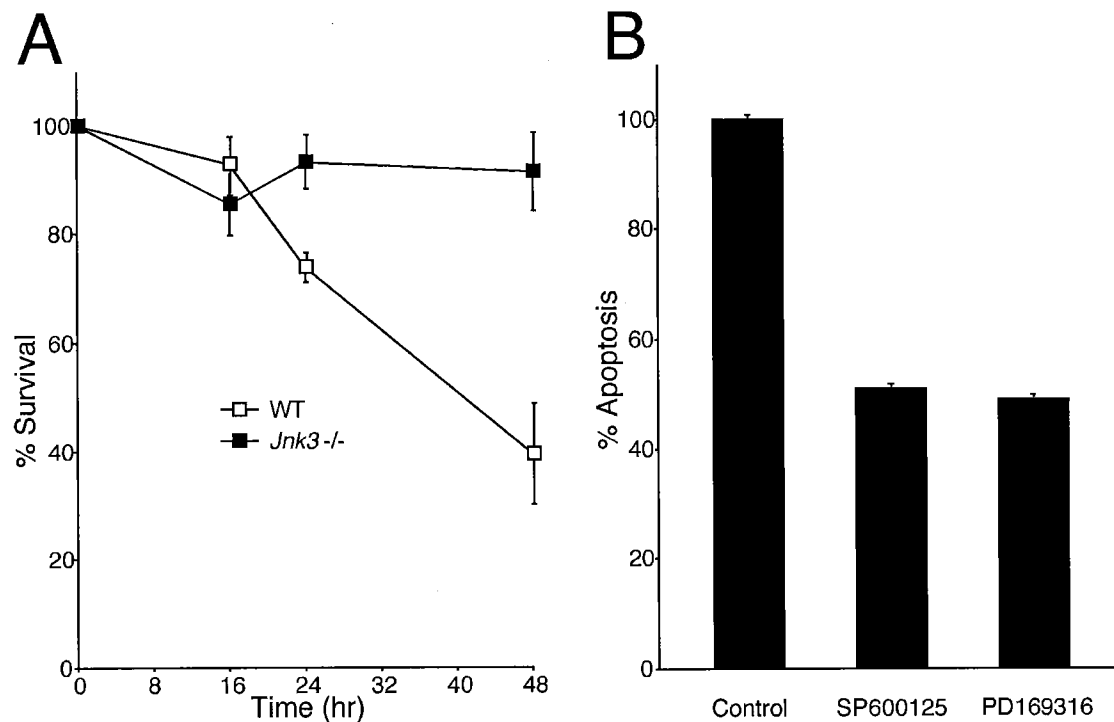
Figure 3-7. JNK3 gene ablation and JNK inhibition effects on arsenite-induced cell death. Cortical neurons were incubated in the presence or absence of 2μM arsenite. (A) Cells cultured from wild type (open squares) or *Jnk3 -/-* (closed squares) were treated with arsenite for 0-48 hours and then assayed for cell survival using MTT. The data presented are normalized to the untreated control cells (100% survival) for each genotype (mean ± S. D., n=4). (B) Wild type neurons were treated with inhibitors of JNK (SP600125; 10μM), p38 MAPK (PD169316; 10μM) or vehicle (DMSO) for 1.5 hours prior to incubation with 2μM arsenite for 24 hours. The MTT assay was used to assess cell death and the data are presented as relative to the amount of death (assigned as 100% cell death) in the vehicle (and arsenite) treated cells (mean ± S. D., n=4).

In order to examine the requirement of JNK in arsenite induced cell death cortical neurons were isolated and cultured from E17.5 mouse embryos which

lack the neuron specific *Jnk3* gene. These neurons in addition to primary cortical neurons from wild type mice were then incubated with 2μM arsenite for 0-48 hours. At the indicated time points the cells were assessed for cell death by the MTT viability assay. Compared to wild type neurons, the *Jnk3-/-* cells displayed a robust resistance to arsenite induced death at 24 and 48 hours after beginning treatment (Fig. 3-7A). Additionally, treatment of wild type neurons with SP600125, a small molecule inhibitor that directly and specifically blocks JNK activity (7), resulted in a substantial decrease in the amount of death caused by arsenite (Fig. 3-7B). These data demonstrate that arsenite induced death is mediated, at least in part by JNK3. The results also indicate that JNK activity is an important component of this model of neurodegeneration.

Macromolecular synthesis is necessary for arsenite induced

neurodegeneration. Previous studies have suggested that kainic acid induced death of hippocampal neurons may require a gene expression component. In order to examine the requirement for gene expression in arsenite induced death, neuronal cells were treated with either the transcriptional inhibitor actinomycin D (ActD, 1.0 μg/ml) or the translational inhibitor cycloheximide (CHX, 0.5 μg/ml) prior to and during arsenite incubation. In both cases the arsenite induced death of the wild type neurons was completely abolished relative to the control, inhibitor treated cells (Fig. 3-8A & B). These results demonstrate that gene expression is necessary for arsenite induced cortical cell death.
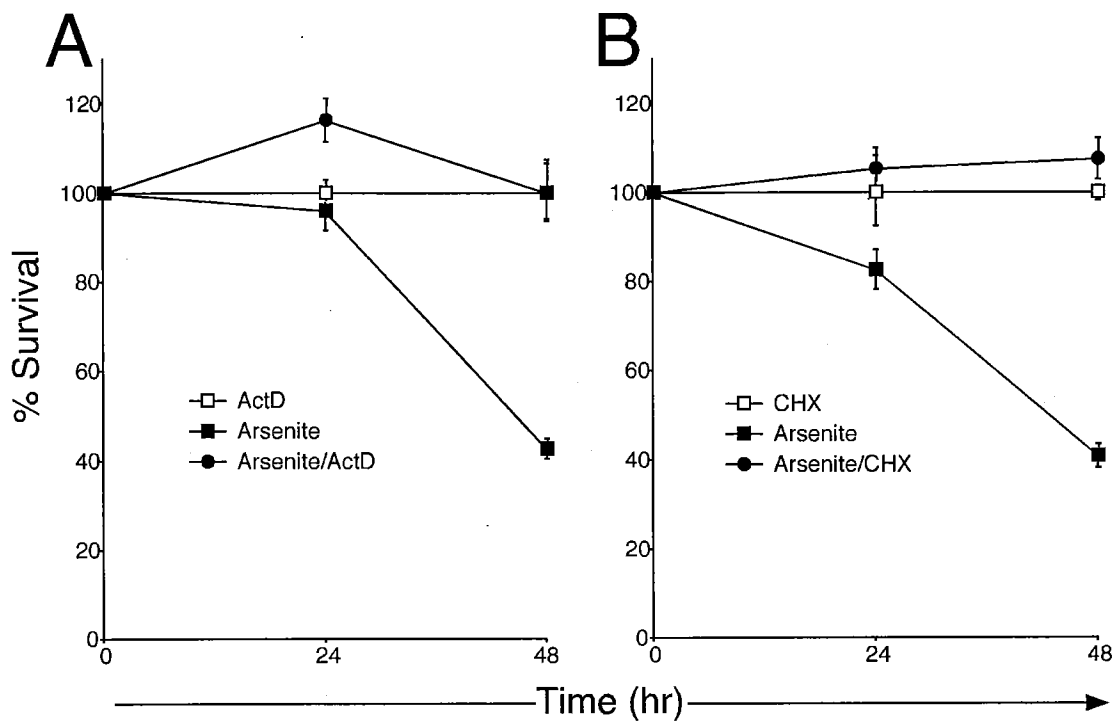
Figure 3-8. Gene expression dependence of arsenite-induced death of cortical neurons. Primary neurons were treated with or without (closed squares) macromolecular synthesis inhibitors for 2 hours prior to incubation with 2μM sodium arsenite for 0-48 hours. (A) The transcriptional inhibitor actinomycin D (ActD; 1μg/ml) was added to untreated (open squares) or arsenite treated (closed circles) cells. (B) The protein synthesis inhibitor cycloheximide (CHX; 0.5μg/ml) was added to untreated (open squares) or arsenite treated (closed circles) cells. Cell survival was determined by MTT assay at the time points indicated. Data for arsenite treated neurons in the presence or absence of macromolecular synthesis inhibitors (closed symbols) were normalized to the values of inhibitor treated cells (open symbols) at each time point (mean + S. D., n=4). Similar data were obtained in 2 independent experiments.

Arsenite induced expression of Fas ligand depends on JNK3. The functional

consequences of arsenite induced JNK activation are unkown. The resistance

of *Jnk3* -/- neurons to arsenite induced cell death (Fig. 3-7A) combined with

the ability of macromolecular synthesis inhibitors to block the effects of

arsenite (Fig. 3-8A & B) suggests that JNK3 contribute to cell death by

inducing pro-apoptotic gene expression. To examine this possibility we performed ribonuclease protection assays on the wild type and *Jnk3 -/-* neurons which had been treated with 2µM arsenite for 0-24 hours. We found that over a 24 hour time course of arsenite treatment, the pro-apoptotic molecule Fas ligand was dramatically induced in wild type neurons (Fig. 3-9A & B). Strikingly, *Jnk3 -/-* cells did not display increased Fas ligand expression over the same time course (Fig 3-9A & B). Expression of the Fas ligand receptor Fas, the apoptosis inducing ligand TRAIL, or the housekeeping gene L32 did not change in response to arsenite incubation of wild type or *Jnk3 -/-* neurons (Fig. 3-9A, C & D). Bcl-2 family members play an important role in regulating apoptosis. Therefore the expression of these molecules in arsenite treated, wild type and *Jnk3 -/-* neurons was examined by ribonuclease protection assay. The expression of these molecules did not change in either cell population relative to the untreated cells (Fig. 3-10A-E and *data not shown*). Collectively these data indicate that arsenite stimulation of cortical neurons triggers a JNK3 dependent expression of the pro-apoptotic, Fas ligand gene. Furthermore the anti-apoptotic molecules, Bcl-2 and Bcl-XL are not down regulated in response to arsenite suggesting that JNK3 does not act to decrease pro-survival gene expression in this model of neurodegeneration.
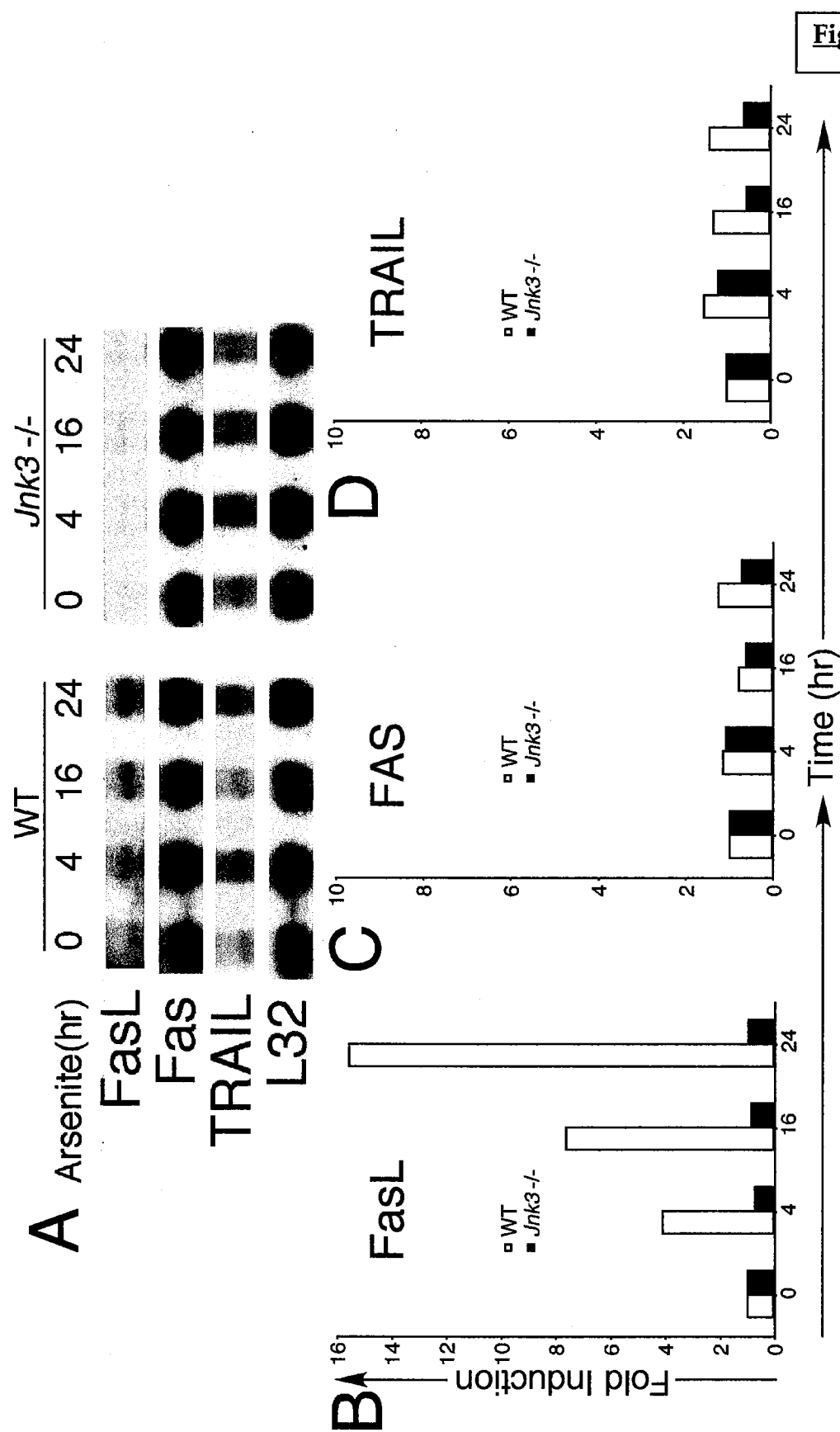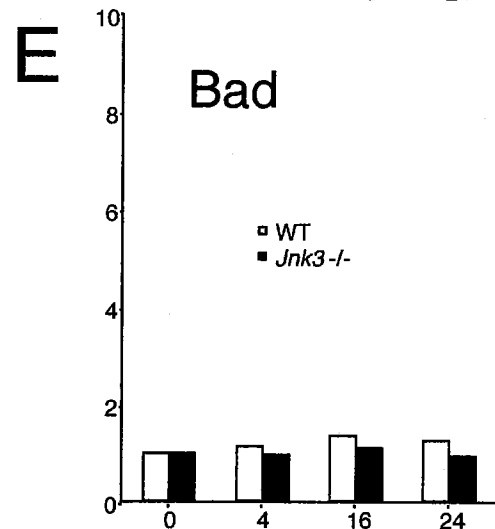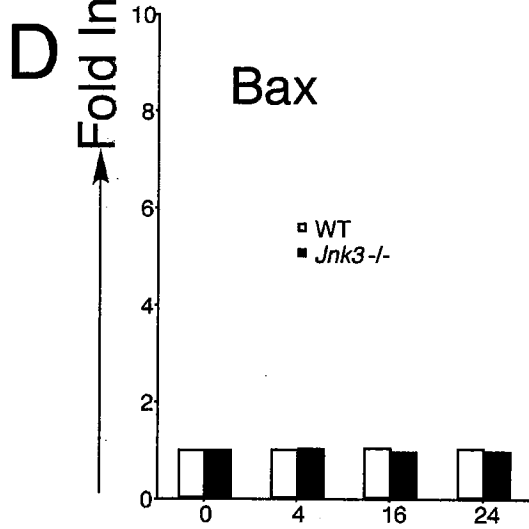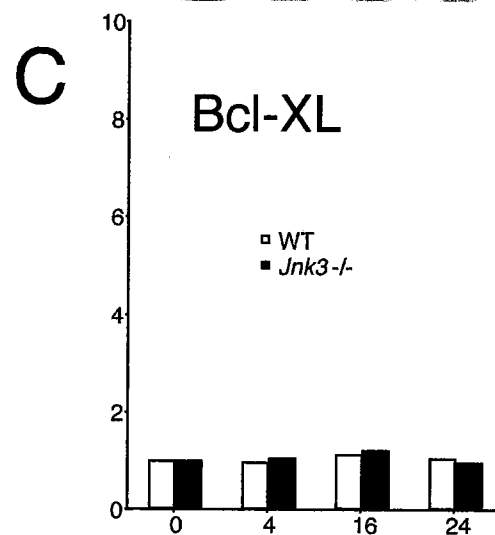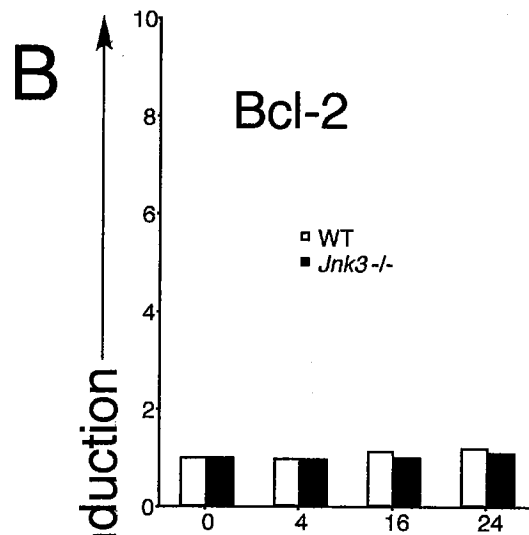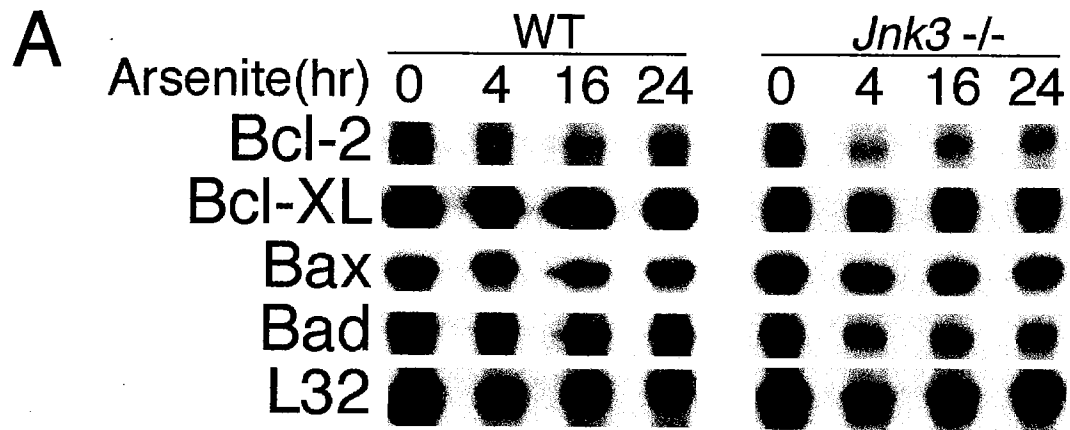
Fig. 3-9

Figure 3-9. Effect of JNK3 gene ablation on pro-apoptotic gene expression induced by arsenite. Primary cortical neurons from wild type and Jnk3 -/- mice were incubated with 2µM arsenite for 0-24 hours. At the time points indicated total RNA was extracted from the cells and 5µg was utilized in ribonuclease protection assays. (A) Autoradiograph of genes examined by ribonuclease protection assay. Expression of the apoptosis inducing ligands; Fas ligand and TRAIL, the Fas ligand receptor Fas and the the L32 housekeeping gene are shown. (B) Quantitation (by phosphorimager) of Fas ligand expression in wild type (open bars) and *Jnk3* -/- (closed bars) cells. (C) Quantitation of TRAIL expression done as described in (B). (D) Quantitation of Fas done as described in (B). Each gene's expression is represented as fold induction over (or under) that which occurs at 0 hours. The data is further normalized for loading differences to the housekeeping gene L32. Data for wild type expression was obtained in two independent experiments.

Fas/Fas ligand mediated pathways contribute to arsenite induced

neurodegeneration. The observed induction of Fas ligand caused by arsenite

prompted an examination of the Fas/Fas ligand pathway in this model of

neurodegeneration. *lpr* mice contain a transposon insertion in intron 2 of the

Fas antigen gene (*lpr* mutation) (1). This mutation prevents Fas mediated

apoptosis and causes lymphoproliferative disorders and autoimmune disease

(119). *lpr* mice were obtained and used to establish cultures of primary

cortical neurons. Initially, an agonistic α Fas antibody was used to

demonstrate that cortical neurons were indeed sensitive to Fas/Fas ligand

induced death. Wild type neurons were treated for 48 hours with or without

the α Fas antibody and cell survival was assayed by MTT. This treatment

caused a 45% decrease in neuronal viability, relative to control, untreated

cells (Fig. 3-11) α-Fas treatment of *lpr* neurons did not reduce the viability of

these cells (*data not shown*). *lpr* and wild type cortical cells were subjected to

treatment with 2µM sodium arsenite. Remarkably, the *lpr* neurons displayed

a substantial,

Figure 3-10. Effect of Jnk3 gene ablation on expression of Bcl-2 family members in arsenite treated neurons. Cortical neurons cultured from wild type and Jnk3 -/- mice were incubated with 2 μM arsenite for 0-24 hours. At time points indicated cells were harvested and total RNA was extracted. 5μg of RNA was utilized in ribonuclease protection assays. (A) Autoradiography of genes examined by ribonuclease protection assay. Expression of selected members of the Bcl-2 family is shown. (B-E) Quantitation of selected Bcl-2 family members was performed as described in Fig. 4B (B) Quantitation of the anti-apoptotic Bcl-2 gene. (C) Quantitation on anti-apoptotic gene Bcl-XL. (D) Quantitation of the pro-apoptotic gene Bax. (E) Quantitation of the pro-apoptotic gene Bad. Fold induction was calculated for these genes and is presented as described in Fig. 4.

78% decrease in cell death compared to the wild type neurons (Fig. 3-12).

Additionally, a soluble Fas:Fc decoy receptor, which will bind secreted Fas ligand, was added to the media of wild type neurons prior to arsenite treatment to try and block the resultant cell death. The Fas:Fc treated cells were resistant to arsenite induced cell death to a similar extent (68% decrease in cell death) as the Fas deficient, *lpr* neurons (Fig. 3-13). These data strongly suggest that arsenite induced Fas ligand expression results in the synthesis and secretion of Fas ligand which then activates a Fas mediated cell death mechanism.
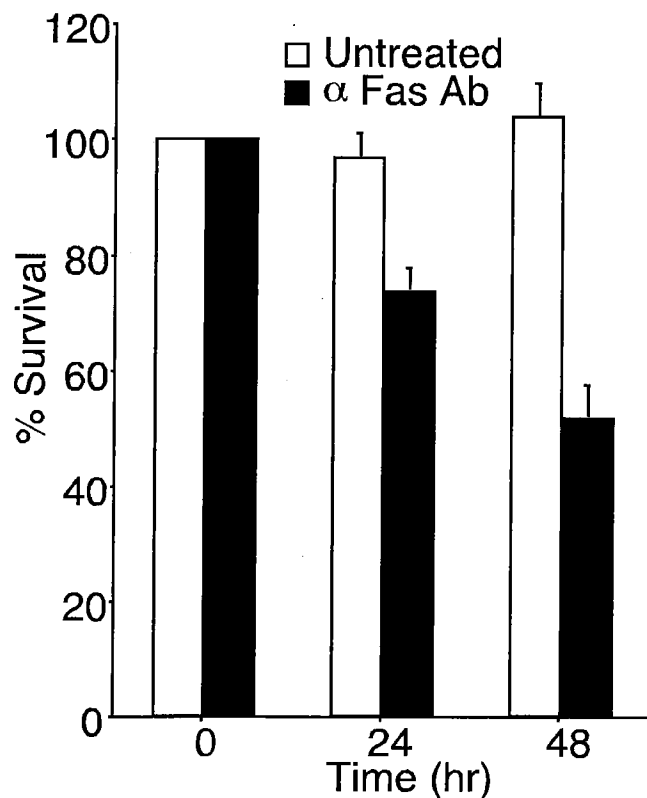
Figure 3-11. Effect of Fas ligation on wild type primary neurons. Cortical neurons in culture were treated with (dark bars) or without (open bars) 1μg/ml of an α Fas agonistic Ab (Jo2; Pharmingen). Cells were harvested and assessed for viability by MTT at 0, 24 and 48 hours after beginning treatment. Data is relative to the control untreated cells (100% survival) and presented as the mean ± S. D., n=4.
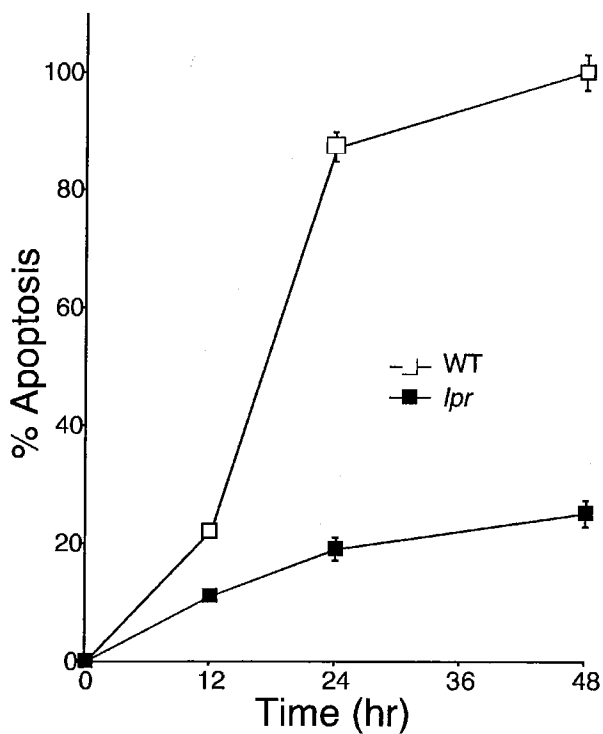
Figure 3-12. Effect of arsenite treatment on cortical neurons from Fas defective and wild type mice. Wild type (open squares) and *lpr* neurons (closed squares) were treated with 2μM arsenite for 0-48 hours and viability of the cells was determined by MTT assay at the time points indicated. Data are expressed as % cell death relative to the death of WT neurons after 48 hours of arsenite treatment (set to 100% cell death).
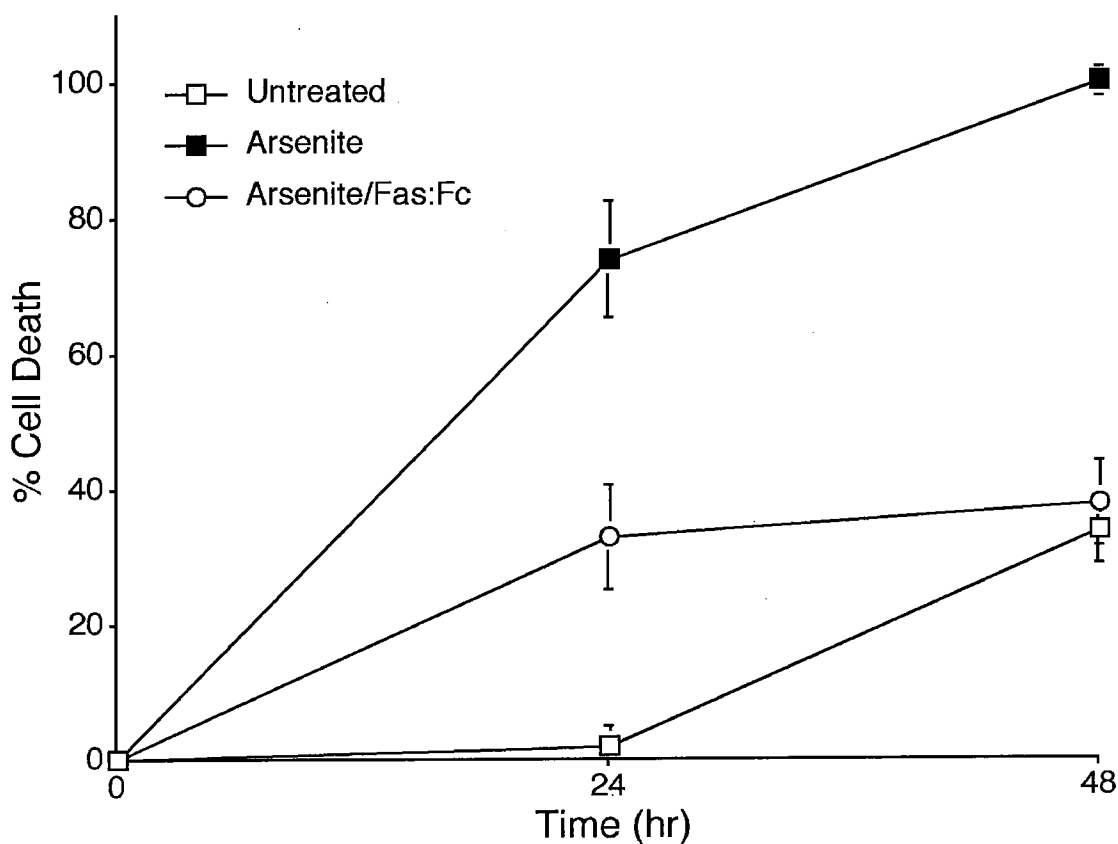
Figure 3-13. Effect of Fas ligand decoy receptor on arsenite induced cell death. Wild type cortical neurons were pre-incubated with (open circles) or without (closed squares) a soluble, chimeric Fas:Fc decoy receptor (5µg/ml; Alexis) for 2 hours and then treated for 0-48 hours with arsenite. Cell viability was assessed by MTT assay at the time points indicated. Data is represented as % cell death relative to the amount of cell death in the population of neurons treated with arsenite for 48 hours (set to 100% cell death) compared to the untreated cells (open squares). Values are expressed as the mean ± S. D., n=4.

Discussion

Previous studies have demonstrated the activation of JNK and p38 in response to arsenite treatment. Dominant-interfering and constitutive-active mutants of c-Jun and upstream activators of JNK and p38 were used to show the importance of these pathways in neurodegeneration induced by arsenite (73). An immunodepletion strategy led to the identification of JNK3 as the specific JNK isozyme activated by arsenite. Additionally, hippocampal neurons which do not express JNK3 are resistant to kainic acid induced apoptosis and display a decrease in AP-1 driven luciferase expression and activity (123). In the NGF withdrawal induced death of PC12 cells SAPK activation is correlated with Fas ligand induction (60). In support of this model of cell death, trophic factor (KCl) withdrawal induced death of cerebellar granule neurons was shown to be blocked by a Fas:Fc decoy receptor suggesting that Fas ligand mediates this developmental model of neuronal cell death (60).

These studies, however, still do not provide a mechanism for how JNK mediates neruonal apoptosis. In one case (arsenite model) an apoptosis mechanism downstream of JNK activation is lacking. In the other case (KCl withdrawal), the dependence of primary cell death on JNK activation is not established.

Utilizing gene knockout technology, the results presented in this study demonstrate that arsenite induced apoptosis and expression of the pro-apoptotic Fas ligand gene is dependent on the neruon specific JNK3 isozyme. The work also defines a role for Fas ligand in a neurotoxic (as opposed to developmental) model of neurodegeneration.

Fas ligand is an important mediator of immune cell death. In immune cells, T cell receptor ligation leads to JNK activation, Fas ligand expression and apoptosis (26). Electrophoretic mobility shift assays demonstrated c-Jun and ATF-2 bind to a unique MEKK1 responsive element in the Fas ligand promoter in response to MEKK1 activity (27). Activation of tumor necrosis factor alpha (TNF-α) type 1 receptor also activates the JNK signaling pathway and causes immune cell death (64). These results suggest a pro-apoptotic role for JNK by inducing Fas ligand gene expression. However, other data generated in the same cell types demonstrated that JNK activation may not be directly linked to apoptosis per se. Inactivating mutation of TRAF2, an adaptor protein which binds TNFR1 cytoplasmic domain, blocks TNF induced JNK activation but does not prevent apoptosis (64). In addition, *Mkk4 -/-* thymocytes are more susceptible to apoptosis (74). These results do little to determine the location or importance of JNK signaling cascades in the apoptosis pathway but do provide precedence for the transcriptional induction of Fas ligand by JNK. Our results, in neuronal cells, demonstrate a JNK dependent induction of Fas ligand in neuronal apoptosis (Fig. 3-9).

To date the limited studies of a JNK-Fas ligand mediated death mechanism in neurons have not revealed the intricacies and conflicting results as described in other cell types. In fact, recent studies performed on primary cerebellar granule neurons and sympathetic neurons have helped to demonstrate the importance and location of c-Jun and the JNK pathway in neuronal apoptosis (88, 114). NGF withdrawal from sympathetic neurons or KCl withdrawal from cerebellar granule neurons induced the expression of a pro-apoptotic Bcl-2 family member, Bim (88). Expression of a dominant negative c-Jun molecule abolishes the induced expression of Bim (114). In addition, the apoptosis caused by these treatments is prevented by ablation of the Bim gene. The inability of a general caspase inhibitor to block the Bim induction in the apoptotic neurons demonstrates that the c-Jun (and presumably JNK) driven expression of a pro-apoptotic molecule occurs upstream of the caspases. Interestingly, the induction of Bim was not observed in some other non-neuronal cell types exposed to apoptosis stimuli (75). It will be important to find out if Bim induction is a mechanism for neurotoxic or excitotoxic models of neurodegeneration.

JNK3 regulates the expression of Fas ligand in response to arsenite. Use of *Jnk3 -/-* cortical cells enabled us to determine that JNK3 specifically induces Fas ligand in response to arsenite (Fig. 3-9). More experimentation needs to be done to examine JNK activation and consequently Fas ligand expression in response to different apoptotic stimulus and in different neuronal cell types.

Thus, kainic acid treatment of hippocampal neurons from *lpr* mice causes abundant apoptosis of those cells (54). This result contrasts with the resistance of *Jnk3 -/-* hippocampal neurons to kainic acid and indicates that the mechanism of cell death in this cell type is not dependent on Fas or JNK3. The role of JNK in neuronal apoptosis may be tissue or cell type specific. While the neural tube closure defect in *Jnk1/Jnk2* null embryos is attributed to a decrease in apoptosis, another area of the brain (forebrain) exhibits increased caspase activity and apoptosis suggesting an anti-apoptotic role for JNK (55). In total the JNK knockout results demonstrate that, despite JNK3's brain specific expression, the other JNK isoforms can regulate neuronal survival in development and potentially in neurotoxicity. Our studies examining cortical neurons demonstrate that JNK plays an important role in the neurotoxin induced apoptosis of these cells. The resistance of *Jnk3 -/-* cortical neurons to arsenite (Fig. 3-7) and the JNK3 dependent induction of Fas ligand (Fig. 3-9A & B) indicate that JNK3 specifically, regulates a pro-apoptotic mechanism in response to apoptotic stimulus. More work needs to be done to address the involvement of specific JNK isoforms in different neuronal cell types in response to various apoptotic stimuli.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Pro-apoptotic role of MAO and the p38 pathway

In post-mitotic or differentiated cell types the crux of an apoptotic process may be gene expression. While much attention has been focussed on the activation of MAP kinases in cell death, little is known about the events directly downstream of the activated terminal kinase (e.g JNK, p38). In many studies a transcription factor is the final, known molecule in signaling pathways demonstrated to be activated by apoptosis stimuli. Presumably, the transcription factors can then contribute to the expression of genes which are involved in the apoptotic process. However, to date few genes, besides those for the transcription factors themselves, have been identified as being regulated in response to death stimuli. The present work identifies MAO as a gene whose expression is dependent on and up-regulated by the p38 MAP kinase signaling pathway in response to an apoptotic stimulus. This study, in conjunction with others, establishes the framework of a death inducing pathway from NGF withdrawal to the cell death contributing expression of MAO. A possible mechanism of how MAO may function to cause cell death is also presented. Of course, some "black box" areas still exist in the

pathway. Relative to the work presented here, the mechanism or transcription factor between activated p38 and MAO gene expression has not been identified. Half life measurements of MAO transcripts may reveal that the mechanism leading to increased MAO message is related to p38 mediated stability of the message. Alternatively, mutational analysis of the MAO promoter may reveal specific promoter sites and transcription factors that are responsive to p38 activity. Very recently the transcription factor Sp1 was demonstrated to be activated by p38 MAP kinase in a monocytic cell line (68). This finding elucidates a potential link between p38 and MAO expression because the MAO promoter is known to contain several Sp1 binding sites. In fact, characterization of the MAO promoter revealed that the Sp1 sites are necessary for promoter activity and that Sp1 is the major transcription factor binding to the promoter (97). To address this issue in the PC12 cell model system used for the present study a supershift or far western assay can be used incorporating the p38 specific inhibitor PD169316. These experiments would probe the importance of p38 and the Sp1 sites in apoptosis induced MAO expression.

Under normal conditions dopamine is sequestered in vesicles inside the cell and does not exist in large quantities in the cytosol. However, the cytoplasm is where MAO functions to oxidatively deaminate biogenic amines such as dopamine. To date, a mechanism for the apoptosis induced increase in cytoplasmic dopamine has not been described. Some areas of investigation can be pursued to examine ways in which dopamine levels become elevated

in the cytosol. Neurotransmitter containing synaptic vesicles have approximately 30 resident proteins which mediate transmitter uptake, vesicle targeting, docking and membrane fusion processes (28). Vesicular monoamine transporters (VMATs) are responsible for the energy dependent uptake of dopamine into vesicles. Reserpine, a specific VMAT inhibitor, could be applied to differentiated PC12 cells and the cells then harvested periodically over a 24 hour time course of NGF withdrawal and assayed for apoptosis. These results could be compared to those obtained from the same experiment with the exception that the PC12 cells are not subjected to NGF withdrawal. By preventing the vesicular uptake of dopamine (as well as other monoamines) into vesicles, more neurotransmitter would be available for oxidation by MAO. If a reserpine induced increase in apoptosis were observed and it's effect was mediated by MAO, clorgyline treatment of the cells would be expected to decrease this cell death.

Radiolabeled dopamine could be used to observe (by density centrifugation and liquid scintillation counting) the presence or absence of dopamine in a vesicular fraction during NGF withdrawal. Concurrently, reactive oxygen species could be measured by commercially available ELISA kits to determine if changes in oxygen radical levels correlate with the reserpine induced distribution of dopamine.

Immunostaining of NGF deprived neuronal PC12 cells with antibodies that recognize synaptic vesicle components may indicate defects in synaptic

vesicle docking in these apoptosing cells. SNAP-25 is part of a plasma membrane associated core complex which regulates proper targeting and docking of synaptic vesicles to the plasma membrane. Likewise, the GTP-binding, synaptic vesicle associated protein Rab3a regulates the fusion and exocytosis of vesicles at the plasma membrane. Immunolocalization of Rab3a in conjunction with that of SNAP-25 and/or the VMATs may reveal apoptosis induced changes in these protein's distribution. Results of these experiments could suggest roles for these proteins in the aberrant release of dopamine into the cytosol.

MAO deficient mice display abnormal aggressive behavior (8, 10). These mice also exhibit increased dopamine content in specific areas of the brain, presumably due to the lack of metabolism by MAO (10). In fact aggression is directly linked to dopamine activity in the brain. It would be interesting to determine if the death of specific subpopulations of neurons is the underlying mechanism through which dopamine acts. A panel of brain slices from the MAO deficient mice could be subjected to dopamine treaments and histological examination to reveal the effects of that treament on cell viability. Furthermore, whole animal treatments with dopamine followed by histological examination of various brain tissues could provide definitive *in vivo* evidence for the effect of dopamine on neuronal viability. These type of experiments address the involvement or importance of MAO activity in cell death. The activity of the MAP kinases could also be examined in different areas of the brains of dopamine treated MAO deficient mice as compared to

wild type. A positive activation feedback loop mediated by MAO activity

may be revealed in the wild type mice that could be absent in the MAO

knockout mice.

JNK pathway in neuronal apoptosis

An intriguing conceptual issue concerns the location of the SAPK cascades in the overall apoptotic pathway. One possibility, that also addresses the importance of JNK signaling in immune cell apoptosis, is that the JNK induced expression of Fas ligand occurs in parallel or in addition to a caspase mediated apoptosis mechanism. Dominant interfering mutants of MEKK1, TRAF2 and JNK do not block TNFR1 or Fas mediated death of immune cells (64). However, prolonged activation of the JNK cascade by constitutive active MEKK1, UV radiation and Fas agonist antibody does lead to Fas ligand expression and immune cell apoptosis (12, 26). A straightforward interpretation of the current study is that the JNK cascade induces Fas ligand synthesis (Fig. 3-9A & B) which can then bind Fas, leading to activation of the downstream death machinery (i.e. caspases). Thus, *Jnk1-/- /Jnk2 -/-* fibroblasts do not translocate their cytochrome c and are resistant to apoptosis caused by UV radiation (105) suggesting that JNK operates upstream of mitochondria and the caspases in this model of cell death. The work describing the placement of the JNK cascade in apoptosis pathways has been performed mostly in non-neuronal cells. Nevertheless, in one case JNK has been demonstrated to be important for death signaling through mitochondria and in another case it is important in mediating the pro-apoptotic expression of Fas ligand.

Recently identified and partially characterized scaffolding molelcules may play an important role in cell death by organizing signaling molecules into discreet modules that are responsive to specific death stimuli (16). JNK interacting protein (JIP) 1 has been shown to bind MLK, MKK7 and JNK as well as potentiate JNK activation, defining itself as a scaffold for the JNK signaling pathway (115). JIP2 and JIP3, other molecules with homology to JIP1 have been identified but are incompletely characterized. Interestingly, hippocampal neurons that lack JIP1 are resistant to kainic acid induced cell death. An attractive hypothesis would be that JIPs define JNK signaling pathways by modularizing pathway components in response to specific stimulus and depending on cell type.

The emerging technology of gene arrays will enable researchers to perform large scale screens that can identify individual or groups of genes regulated by diverse biological stimuli. Because the activities of JNK may change depending on the cell type and stimulus used it will be important to examine JNK activation in various biological models. One such set of models are mice which are lacking the JNK genes, separately and in combinations. Array experiments examining gene expression or lack thereof in these genetically altered mice (or primary cells derived from) may reveal the importance of the JNK signaling pathways in various tissues. It is likely that secondary screens such as ribonuclease protections assays will be required to confirm and more closely examine initial leads developed by the gene array.

Pertaining to the current work, interesting observations have been made in cells treated with arsenite. As reported here and in other studies (121) JNK activation by arsenite leads to neuronal degeneration. However, other work has demonstrated that arsenite induced JNK activation is correlated with tumor progression in non-neuronal cells (11). In fact the activation of different MAP kinase family members in response to arsenite has been reported in different cell types. In cells where arsenite treatment leads to degeneration, JNK is activated (11, 63). In these cells the JNK activation is linked to pro-apoptotic gene expression (11).

Mice that are deficient in the inducible enzyme, heme oxygenase-1 display pathological iron loading defects and increased susceptibility to stress induced oxidative damage (85, 86). This data indicates that the oxygenase plays a protective role in response to stress stimulus. Interestingly, arsenite treatment of a hepatoma cell line led to the induction of the heme oxygenase-1 gene (31). Furthermore, arsenite did not activate JNK in these cells but did activate p38 which was required for heme oxygenase expression (31). In addition, arsenite treatment of *Jnk 1-/- / Jnk2 -/-* fibroblasts (that do not express JNK3) leads to increased apoptosis as compared to the wild type fibroblasts (De Zutter, G. S., unpublished results). These data, in total, suggest that JNK can act as a pro-survival or pro-apoptotic molecule in response to the same stimulus but in different cell types.

By holding one variable constant (e.g. arsenite treatment) comparison of gene arrays or ribonuclease protection assays performed on different

tissues or cells isolated from various *Jnk -/-* mice could demonstrate the importance of specific JNK isoforms in those tissues or cell types. More work, examining JNK activity, gene expression and viability between cell or tissue types may reveal cell type specific functions for JNK.

Alternatively, primary cell cultures established from different tissues of the JNK deficient mice could provide excellent model systems for the examination of JNK activity in various models of cell survival. The major advantage of the cell cultures is that they are easily manipulated. For example, a comparison of different primary cell types which have been transfected or infected with various JNK molecules could be done. The cells would be treated with arsenite and then examined for apoptosis and specific JNK isozyme activity.

In addition, it would be interesting to observe the location of JNK scaffolding molecules in response to apoptotic stimuli. Given the striking differences in immunostaining patterns between JIP1 and JIP3 it is likely that they differentially modulate kinase activities in response to stimuli or depending on cell type. Recently it was discovered that JIPs are cargo for the molecular motor kinesin (107). While the importance of the association has not been established, it appears that the localization of JIP to the nerve terminals is dependent on the function of kinesin. Different stimuli may effect the localization of the JIPs and could be correlated with altered kinesin function. Also, the importance for JNK activity in the association of JIPs with

kinesin could be probed by using primary cells from the JNK knockout mice or by the use of the JNK inhibitor SP600125.

Besides arsenite, there are a multitude of stimuli that can activate the MAP kinase signal transduction pathways. As the activities of these kinases in different cell types are established the future in this field will involve the molecular dissection of these anastamosing signaling pathways in response to different environmental cues. The strategies or approaches that have been discussed above may be applied in examining the effects of almost any stimulus. However, it is apparent that many avenues of investigation will be opened due to the availability of MAPK knockout mice and gene array technology.

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