FACTORS CONTRIBUTING TO ADULT DRG NEUROTROPHIN-INDEPENDENT NEURONAL SURVIVAL

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Factors contributing to adult DRG neurotrophin-independent neuronal survival

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A thesis submitted to the School of Graduate Studies in partial

fulfilment of the requirements for the degree of Doctor of Philosophy

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St. John's, Newfoundland and Labrador

June 2006

St. John's Newfoundland and Labrador

ABSTRACT

Understanding mechanisms of cellular survival in the adult dorsal root ganglion (DRG) neuron is important in identifying targets for disease intervention and treatment in the peripheral nervous system (PNS). I have used DRG neurons to study signal transduction mechanisms involved in adult DRG neurotrophin-independent survival and response to stressful stimuli to understand mechanisms of neuroprotection and to enhance cellular survival after insult so that other processes, such as regeneration, can occur.

Dorsal root ganglion (DRG) sensory neurons become less dependent upon neurotrophins for their survival as they mature. In Chapter 2 we show that adult DRG sensory neuronal cultures are able to survive for at least two weeks in culture in the absence of nerve growth factor (NGF). The use of pharmacological inhibitors of cellular signalling pathways confirmed the importance of the phosphoinositide kinase-3 (PI 3-K) and protein kinase C (PKC) pathways in this neurotrophin-independent neuronal survival.

Chapter 3 shows concurrent studies investigating the role of stress-activated signalling pathways and the small heat shock protein, Hsp27, in protecting PC12 cells from heat shock (HS) and NGF withdrawal-induced apoptosis. PC12 cells and a stable cell line overexpressing Hsp27 (HSPC cells) were subjected to a physiological heat stress. Hsp27 associated with Akt and p38MAPK after stress and HS resulted in rapid activation of Akt followed by p38 MAP kinase signalling, with phosphorylation and intracellular translocation of Hsp27 also detectable. Hsp27 protected NGF-differentiated PC12 cells

against NGF-withdrawal treatment and phosphorylation of Akt was maintained in both the heat-shocked and NGF-withdrawal treated HSPC cells compared to the parental cells.

Our studies of Hsp27 in PC12 cells formed the basis for the experimental work outlined in Chapters 4 and 5. We investigated the possibility that Hsp27 may contribute to adult DRG neurotrophin-independent survival. Unlike adult DRG neurons *in vitro*, neonatal DRG neurons require NGF for survival; withdrawal of NGF results in apoptosis of a majority of neonatal neurons. Constitutive Hsp27 expression was higher in adult DRG neurons compared to neonates. After HS, Akt activation and upregulation of Hsp27 expression occurs in both adult and neonatal neurons. Increasing endogenous Hsp27 by HS in neonatal neurons was able to inhibit NGF withdrawal-induced apoptosis. Hsp27 siRNA treatment of adult neurons effected a decreased expression of Hsp27, which correlated with increased apoptosis. Hsp27 siRNA also blocked the HS-induced rescue of neonatal neurons after NGF withdrawal. These results indicate that physiologically induced upregulation of Hsp27 is sufficient to provide some degree of neuronal protection. This induction appears to be regulated by transcriptional activation of HSF-1 as shown by HSF-1 nuclear translocation and EMSA analyses of HSF-1 binding to nuclear protein.

These results provide further evidence that adult DRG neuronal survival depends on multiple factors; one of significant importance is Hsp27. My studies show that this protein and its interactions with other signalling intermediates play important roles in neuronal survival. Further assessment of Hsp27's protein interactions and regulation will hopefully contribute to elucidating factors important for adult DRG neurotrophin-independent neuronal survival.

Acknowledgments

There are many people who have contributed to this work and to whom I owe many, many thanks. My supervisor and mentor, Dr. Karen Mearow, has been an endless source of guidance, support and advice. Thank you for giving me the opportunity to work on a project that I thoroughly enjoyed. Thanks are also extended to Masuma, who provided not only immense technical support but was also a good friend. The two of you made my experience memorable.

I must also thank my lab mates (past and present) for their support. Thanks Dave, for being there at the beginning to help me get started. Budd, thanks for the technical support and the many chats that helped me keep my sanity through everything, especially comps. Kristy and Sherri, thanks for your friendship and help with aspects of my project. Thank you, Jinguo for your help and guidance as well.

Of course, I probably would never have gotten as far as I did without the support of my parents. You have been amazingly supportive and confident in my abilities. I feel very lucky to have you as such a huge part of my life.

Lastly, thank you Lloyd, for helping me through the daily ups and downs of the past five years. Day to day you were supportive and always believed in me. You gave me encouragement, inspiration and the ability to believe that I can accomplish anything.

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LIST OF ABBREVIATIONS

1H NMR	Proton Nuclear Magnetic Resonance
Akt/PKB	Protein Kinase B
ATP	Adenosine Triphosphate
Bcl-2	B-Cell Lymphoma 2
BDNF	Brain-Derived Neurotrophic Factor
CNS	Central Nervous System
DIC	Days in Culture
DRG	Dorsal Root Ganglion
ER	Estrogen Receptor
ERE	Estrogen Response Element
GAB	Grb2 Associated Binder 2
GDNF	Glial Cell Line-Derived Neurotrophic Factor
Grb2	Growth Factor Related Bound Protein 2
GSK	Glycogen Synthase Kinase
HSE	Heat Shock Element
HSF1	Heat Shock Factor 1
HSF2	Heat Shock Factor 2
HSF3	Heat Shock Factor 3
HSF4	Heat Shock Factor 4
Hsp22	Heat Shock Protein 22
Hsp27	Heat Shock Protein 27
Hsp 40	Heat Shock Protein 40
Hsp70	Heat Shock Protein 70
Hsp90	Heat Shock Protein 90
Hsps	Heat Shock Proteins
HSV	Herpes Simplex Virus
IAP	Inhibitor of Apoptosis Protein
IEF	Isoelectric Focusing
IGF	Insulin-like Growth Factor
IL-1β	Interleukin-1β
ILK	Integrin Linked Kinase
JNK	C-Jun N-Terminal Kinase
MAPK	Mitogen Activated Protein Kinase
MAPKAP-K2	Mitogen Activated Protein Kinase Activated Protein-Kinase 2
MEK	Mitogen Activated Protein Kinase Kinase
MMP	Mitochondria Membrane Permeabilization
MTS	3-(4,5-dimethylhiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NF-κB	Nuclear Factor-κB
NGF	Nerve Growth Factor
NT	Neurotrophin
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
OD	Optical Density

P38 MAPK	p38-Mediated Mitogen Activated Protein Kinase
P75	p75 Neurotrophin Receptor
PC12	pheochromocytoma-12 cells
PDK1	3-Phosphoinositide-Dependent Protein Kinase-1
PDK2	3-Phosphoinositide-Dependent Protein Kinase-2
PH	Pleckstrin Homology domain
PI3-K	Phosphoinositol-3 kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PMS	phenazine methosulfate
ROS	Reactive Oxygen Species
SCG	Superior Cervical Ganglion
SMAC/DIABLO	Second Mitochondrial Activator of Caspases/Direct IAP Binding Protein With Low pI
TNF	Tumor Necrosis Factor
TrkA	Tyrosine Kinase Receptor A
TrkB	Tyrosine Kinase Receptor B
TrkC	Tyrosine Kinase Receptor C

CHAPTER 1

GENERAL INTRODUCTION

1.1 The Dorsal Root Ganglia

Somatosensory information from the periphery must be conveyed long distances in order to be perceived and processed by the brain. Dorsal root ganglion (DRG) neurons mediate the sensory modalities of touch, nociception, proprioception, and temperature, as they transduce stimuli and transmit information from the periphery to the central nervous system (CNS). Positioned at all spinal levels (cervical, thoracic, lumbar and sacral), DRGs contain heterogeneous populations of sensory neurons that have a pseudo-unipolar morphology. These neurons extend a central axon that bifurcates into two branches: one that projects to the dorsal horn of the spinal cord, and the second to the periphery (Gardner *et al.*, 2000). In cell culture, extensions protruding from the cell body are referred to as 'neurites'. The sensory neurons contained within a DRG express receptors for various neurotransmitters and substances, such as neurotrophins, and these play a fundamental role in their development and function.

1.1.1 Development of the rodent DRG and the role of neurotrophins

Throughout embryonic and postnatal development, DRG neurons are dependent on trophic support from various neurotrophins (NTs) not only for their survival, but also for axonal growth and proper target innervation (Snider and Silos-Santiago, 1996). NTs are target-derived soluble factors that serve many functions during neuronal development

– they promote survival of neurons at various stages of development, regulate proliferation, axonal growth and target innervation (Snider and Silos-Santiago, 1996; Lentz *et al.*, 1999; Ernfors, 2001) . The first neurotrophin to be discovered and characterized, nerve growth factor (NGF) (Levi-Montalcini and Booker, 1960; Mobley *et al.*, 1976), paved the way for discovery of the other family members, such as brain-derived nerve factor (BDNF) (Leibrock *et al.*, 1989; Hohn *et al.*, 1990), neurotrophin-3 (NT-3) (Maisonpierre *et al.*, 1990), and neurotrophin 4/5 (NT-4/5) (Berkemeier *et al.*, 1991; Ip *et al.*, 1991), and their importance has been intensely studied over the years. In DRG neurons, the neurotrophins NGF, NT-3, NT-4/5, and BDNF, which are all structurally related, regulate growth and survival of neurons as well as influence the production of different neuronal phenotypes (Thoenen, 1991; Lewin, 1996; Lentz *et al.*, 1999).

The type of neurotrophin that promotes survival and growth of sensory neurons is specific to the particular cell type and age (Phillips and Armanini, 1996). For example, many cells in the embryonic DRG are dependent on NGF for survival (Ernfors *et al.*, 1993; Mu *et al.*, 1993; Phillips and Armanini, 1996), as evidenced in studies where inhibition of transduction initiated by NGF binding to its receptor resulted in neuronal cell death (Vogelbaum *et al.*, 1998). Similarly, studies have shown that a proportion of rodent DRG neurons at age E12.5 depend on BDNF for survival (Acheson *et al.*, 1995). Remaining embryonic DRG neurons past age E14.5 and until birth depend on NGF, NT-3, and NT-4/5 for survival, and post birth are primarily dependent on NGF in the early postnatal period (Memberg and Hall, 1995). During early postnatal life, there is a change in neurotrophin receptor expression such that there is a decrease in the percentage

of cells expressing TrkA (40%) and a concomitant increase in the proportion of neurons expressing the receptors for glial cell line-derived neurotrophic factor (GDNF) (40%) (Bennett *et al.*, 1996; Molliver and Snider, 1997; Molliver *et al.*, 1997). Thus, compared with the embryonic period, the adult DRG has significantly fewer neurons expressing TrkA and, although these cells remain NGF responsive, they no longer require NGF for their survival (Lindsay, 1988; Verge *et al.*, 1992; McMahon *et al.*, 1994; Snider, 1994) (see Figure 1.1).

1.1.1.1 The role of neurotrophins in DRG neurons *in vitro*

To date, the mechanism responsible for the switch in NGF-dependence to NGF-independence following the maturation of DRG neurons is unclear. However, it is clear that trophic factors play an important role in proper neuronal development. Based on *in vitro* experiments, late embryonic (E15) and early postnatal (P2) rat DRG neurons will undergo significant cell death following NGF withdrawal (Eichler and Rich, 1989; Wagstaff *et al.*, 1999). Additionally, embryonic DRG neurons that have been cultured to age postnatal day 7 or less undergo apoptosis when NGF is withdrawn from the culture medium (Lindsay, 1988; Memberg and Hall, 1995; Lewin, 1996). However, experiments have also shown that when embryonic neurons (E15) are cultured for three weeks in the presence of NGF, they are subsequently able to survive NGF withdrawal (Eichler and Rich, 1989; Tong *et al.*, 1996). It is evident that sensory neurons are dependent on NGF for survival if cultured during late embryonic or early postnatal development, but will lose this dependence once adulthood is reached.

1.1.1.2 The role of neurotrophins in DRG neurons *in vivo*

Studies employing *in vivo* methodologies have demonstrated the dependence of embryonic and early postnatal neurons on neurotrophins for survival. Treatment of newborn rats with anti-NGF serum caused a significant decrease in the number of DRG neurons, whereas similar treatment augmented with NGF prevented this loss, demonstrating the importance of NGF in the survival of newborn DRG neurons (Yip *et al.*, 1984). In the adult DRG, neurons have been shown to be NGF-independent *in vivo* as well as *in vitro*. Experiments examining the effects of anti-NGF on recovery after injury by nerve crush showed that there were no differences between injured and uninjured ganglia, demonstrating that the survival of these adult neurons was indeed NGF-independent (Rich *et al.*, 1984; Diamond *et al.*, 1992).

In adulthood, neurons can respond to neurotrophins by undergoing neuritogenesis. NGF receptor signalling pathways are obviously functional in adult neurons, as NGF binding to its receptor may result in activation of signalling pathways such as the phosphatidylinositol 3-kinase (PI3-K) pathway, which is required for neonatal survival (Vogelbaum *et al.*, 1998). Based on studies using sympathetic neurons, it has also been shown that NGF in cultures derived from adult animals is not important for survival, but does play an important role in neurite growth, as use of inhibitors of NGF pathways do not result in cell death (Orike *et al.*, 2001). There is likely interaction between signalling intermediates and potentially through proteins not directly involved in neurotrophin signalling. Therefore, it is imperative to understand the mechanisms underlying adult DRG neurotrophin-independent survival.

1.1.2 Neurotrophin Signal Transduction

Neurotrophin receptors, which are located in the cell membrane, respond to changes in the cellular environment and elicit proper responses. Responses may be quite complicated, as there are many and varied regulatory factors that play a role in the resultant cellular signal. A cell surface glycoprotein receptor generally consists of a ligand-binding extracellular region and a cytoplasmic portion linked via a transmembrane domain embedded within the membrane. One such class of receptors, the Trk family, have an affinity for neurotrophins: TrkA binds NGF and NT-3 (Kaplan *et al.*, 1991; Klein *et al.*, 1991a); TrkB binds BDNF and NT-4/5 (Berkemeier *et al.*, 1991; Ip *et al.*, 1991; Klein *et al.*, 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991); and TrkC binds NT-3 (Klein *et al.*, 1991b; Lamballe *et al.*, 1991). For example, binding of ligand to the NGF receptor TrkA results in the dimerization of two subunits of this receptor via their extracellular components, followed by autophosphorylation that results in the recruitment of specific adaptor proteins to the phosphorylated cytoplasmic domains to initiate intracellular signalling events. The most abundant type of neurotrophin receptor that is expressed in the DRG is high-affinity TrkA, expressed in approximately 35-50% of the neurons (Mu *et al.*, 1993; McMahon *et al.*, 1994; Kashiba *et al.*, 1995). In addition, 5% express TrkB, and 15-20% express TrkC (Kashiba *et al.*, 1995) (see Figure 1.2). When the appropriate ligand binds, the phosphorylated Trk receptor subsequently recruits adaptor proteins resulting in the downstream activation of targets which include Ras,

PI3-K, protein kinas C (PKC) and phospholipase C-γ (PLC-γ) (Kaplan and Stephens, 1994; Kaplan and Miller, 1997).

The PI3-K pathway has been intensely studied over the past decade and is considered to be extremely important in the promotion of survival in PC12 cells and other neuronal cell types, in the central and peripheral nervous systems. Also, this pathway is activated in response to growth factors, such as NGF and IGF (Burgering and Coffer, 1995; Dudek *et al.*, 1997; Andjelkovic *et al.*, 1998; Crowder and Freeman, 1998; Ashcroft *et al.*, 1999; Crowder and Freeman, 2000; Kimpinski and Mearow, 2001; Jones *et al.*, 2003). Once a neurotrophin binds to its receptor (e.g. TrkA), dimerization occurs as well as recruitment of adaptor proteins Grb2 associated binder 2 (GAB) or growth factor related bound protein 2 (Grb2). These adaptor proteins then bind to PI3-K; PI3-K phosphorylates inositol phospholipids to serve as docking sites for specific proteins to initiate signalling cascades. This cascade leads to protein kinase B (Akt) activation through a number of intermediate steps involving integrin linked kinase (ILK), 3-phosphoinositide-dependent protein kinase-1 (PDK1) or 3-phosphoinositide-dependent protein kinase-2 (PDK2) (Osaki *et al.*, 2004). For example, the Akt protein kinase contains a pleckstrin homology (PH) domain that binds these phosphorylated phospholipids to act in the recruitment of Akt to the inner surface of the plasma membrane. Once there, Akt can be phosphorylated by another PH domain containing protein PDK1 (Alessi *et al.*, 1997).

The mitogen activated protein kinase (MAPK) pathway is very important during neurotrophin withdrawal and neuritogenesis. Signal transduction via this pathway

commences upon activation of receptors by neurotrophins with recruitment of Ras to the cell membrane and phosphorylation of mitogen activated protein kinase kinase (MEK), which subsequently phosphorylates MAPK (Virdee and Tolkovsky, 1996; Encinas *et al.*, 1999; Klesse and Parada, 1999; Mazzoni *et al.*, 1999; Xiao and Liu, 2003). Interestingly, MAPK may also be activated through other signalling intermediates such as PKC (Toker, 1998). The signalling mechanisms, such as the PI3-K and MAPK pathways, are much more complicated than the linear diagrams usually depicted in signal transduction pathway illustrations (e.g. Figure 1.3), as crosstalk occurs among various signalling cascades and intermediates.

Neurotrophins act as ligands for two receptors – the Trk receptor, and the low-affinity structurally-unrelated receptor p75 (Rodriguez-Tebar *et al.*, 1990; Rodriguez-Tebar *et al.*, 1992; Chao and Hempstead, 1995; Barker, 1998; Casaccia-Bonnefil *et al.*, 1998; Khursigara *et al.*, 2001). The non-selective p75 receptor is expressed in 90% of TrkA and TrkB expressing neurons, in 50% of TrkC expressing neurons (Wright and Snider, 1995), and can bind neurotrophins and modify signalling by other receptors (Segal and Greenberg, 1996; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001; Huang and Reichardt, 2003; Teng and Hempstead, 2004). The p75 receptor is thought to be involved in apoptotic signalling (Casaccia-Bonnefil *et al.*, 1998). Although NGF can bind TrkA and p75, the role that the p75 receptor plays during DRG neuronal development has yet to be determined; however, it has been shown to play a role in sympathetic neuronal cell death (Carter *et al.*, 1996; Aloyz *et al.*, 1998; Barker, 1998; Miller and Kaplan, 1998). During development of sympathetic neurons, it has been shown that p75 acts independently of TrkA to promote apoptosis, and that TrkA promotes

survival of cells by inhibiting signalling by p75 (Majdan *et al.*, 2001). Studies of PC12 nnr cells, which have no functional TrkA receptors, also show that p75 signalling mediates cell death (Roux *et al.*, 2001) (see Figure 1.3).

1.1.3 Neurotrophin Responsiveness of PC12 Cells

Different cell types respond to stimuli and environmental cues with varying responses. Experiments involving DRG neurons can be difficult due to limitations in dissection, isolation, propagation and in their capability to undergo transfection through traditional lipid-based methods. As an alternate, pheochromocytoma-12 (PC12) cells of adrenal medullary origin have experimental advantages as they endogenously express the TrkA receptor and are responsive to NGF. When cell culture medium is supplemented with NGF, in either low-serum or serum-free medium, they become post-mitotic and acquire a sympathetic 'neuronal' phenotype, as neurites can be seen extending from the cell body, the cell body becomes flattened and they become electrically excitable (Klesse and Parada, 1999). The neurotrophic signalling in PC12 cells, as in other cell types, occurs via the TrkA and p75 receptors, and becoming post-mitotic depends on signal transduction events initiated by the interaction of NGF with p75 receptor (Ito *et al.*, 2003). The signalling cascades in PC12 cells are similar to that of other neuronal cell types and as a result, this cell line is a useful model with which to initially study transduction (Dragunow *et al.*, 2000; Roux *et al.*, 2004; Tischler *et al.*, 2004).

1.2 Stress response

A change in the extracellular environment of a cell can elicit a stress response, which leads to changes in a cell and involves activation of signal transduction pathways. This may be elicited by a number of conditions *in vivo* and *in vitro*, such as oxidative stress, heat, UV radiation, and exposure to certain chemicals. For example, oxidative stress is a common occurrence in mammalian cells which can cause detrimental cellular effects that may affect the organism as a whole or may alter redox state and survival at the cellular level (Berg *et al.*, 2004; Taylor and Crack, 2004). As a result of elicitors of the stress response, the response itself must be a sophisticated system to try and restore homeostasis within cells and body systems. Often such stresses result in induction of heat shock proteins (Koroshetz and Bonventre, 1994; Gabai and Sherman, 2002; Yenari, 2002; Richter-Landsberg and Goldbaum, 2003; Klettner, 2004) (see Figure 1.4).

Ever since the initial discovery of the heat shock response via observation of chromosomal puffs in salivary glands of *Drosophila* (Ritossa, 1962; Lindquist, 1986), considerable effort has gone into the study of the heat shock response. The heat shock system has evolved as a valuable paradigm for the study of the physiological responses to stress. The focus of much of this research has been to elucidate signalling mechanisms and identify important protein interactions.

It is especially important to understand and study responses to stress within the peripheral nervous system, as primary neurons are post-mitotic and, therefore, do not divide and are not replaced. This renders the population of neurons more susceptible to insult, and the mechanisms that exist for coping with stress are critical as these responses

may be the most important line of defence in the preservation of the cell's integrity (Ohtsuka and Suzuki, 2000; Richter-Landsberg and Goldbaum, 2003).

1.3 Heat Shock Proteins (Hsps)

Heat shock proteins (Hsps), or more commonly referred to as "stress proteins', have a dual role – as proteins that respond to stress and as molecular chaperones. Since the discovery of the heat shock response by Ritossa, it has become understood that a heat shock response primarily involves Hsps (Ritossa, 1962). Interestingly, stresses that result in an induction of Hsps may confer tolerance of a cell to further stress that by itself would be lethal (Landry, 1989). A heat stress response involving the induction of Hsps can produce thermotolerance and prevent apoptosis in neuronal cells (Mailhos *et al.*, 1994; Beere, 2001; Lee *et al.*, 2001). Overexpression of Hsps may exert the same effect (Landry, 1989; Huot *et al.*, 1996). It is the balance between the stress response and apoptosis that determines the fate of the cell (Sastry and Rao, 2000).

Many different Hsps have been discovered and divided into families based on their characteristics and structures. Families of Hsps consist of members that may be present in different cellular compartments. For example, the heat shock protein 70 (Hsp70) family consists of members located in the cytoplasm and in the endoplasmic reticulum (Aoki *et al.*, 2001). This family, as well as the heat shock protein 90 (Hsp90) family, require the hydrolysis of ATP for activity and may also require co-factors. For example, Hsp70 requires heat shock protein 40 (Hsp40) as a co-factor (Pratt and Toft, 2003). Other Hsps, such as Hsp27, do not require ATP for its activity and may act without the assistance of cofactors. Primarily, Hsps are known as chaperones, and this is

due to their role in sequestering proteins for proper folding or by targeting proteins for degradation.

Hsps have been studied intensely over the past 40 years, and have proven to be very important in a large number of different cell types and diseases. For example, induction of Hsp chaperones, specifically Hsp70, has been discussed as possible treatments in Parkinson's Disease and Alzheimer's Disease (Kitamura and Nomura, 2003; Klettner, 2004; Franklin *et al.*, 2005), as these are conditions in which there is commonly an over-production or accumulation of misfolded or aggregated proteins. In general, it is known that during the aging process there is a decrease in the constitutive level of chaperones present and that this state may lead to certain deleterious manifestations (Soti and Csermely, 2002a). However, in neurodegenerative diseases, chaperones are often induced in areas of the brain showing degeneration and may play a primary role not only in the chaperone activities that oppose protein aggregation, but also in the prevention of neuronal cell death (Ohtsuka and Suzuki, 2000; Kitamura and Nomura, 2003; Richter-Landsberg and Goldbaum, 2003).

1.3.1 Apoptosis

Cell signal transduction that leads to apoptosis is characterized by events that lead to the activation of caspases (cysteine-dependent aspartate specific protease). Caspases exist as zymogens or procaspases, and are activated once cleaved into small subunits. Activation can occur via two pathways: the intrinsic and extrinsic pathways (Khosravi-Far and Esposti, 2004; Jin and El-Deiry, 2005; Stefanis, 2005). The extrinsic pathway involves the binding of a cell death ligand, such as Fas ligand, to its receptor that triggers

caspase activation, whereas the intrinsic pathway occurs when caspases become activated downstream of mitochondria membrane permeabilization (MMP) and cytochrome c release (Green, 2005; Stefanis, 2005). The initial stages of caspase activation involves caspases 2, 8, 9, 10 and 12, the initiators of apoptosis, activating caspases 3, 6 and 7, the executors of apoptosis (Salvesen and Dixit, 1999; Woolf and Green, 1999). In one of the final steps, caspase 3 cleaves and activates poly (ADP-ribose) polymerase-1 (Garnier *et al.*, 2003), which acts to modify proteins by attaching poly (ADP-ribose) chains to result in depletion of the cell's ATP to, in part, cause apoptosis.

Other signalling molecules, such as the B-cell lymphoma-2 (Bcl-2) family, are known to be involved in the regulation of caspase activation. This family consists of both pro- and anti-apoptotic molecules. Bax and Bak are pro-apoptotic and act upstream of caspases and seem to be involved in mitochondrial caspase cell death pathways (Martinou *et al.*, 1998; Kim, 2005; Kim *et al.*, 2005). Bcl-2 is considered an anti-apoptotic family member, and can bind to and inhibit pro-apoptotic members of the family or indirectly regulate the activity of caspases (Fan *et al.*, 2005). In addition, inhibitors of apoptosis proteins (IAPs) which act as named, and second mitochondrial activator of caspases/direct IAP binding protein with low pH (SMAC/Diablo) may be involved in the caspase pathway (Troy *et al.*, 2002; Tanaka *et al.*, 2004). With the initiation of apoptosis, various signalling cascades are activated that may eventually result in the cleavage and activation of caspases. Other pro-apoptotic genes such as Daxx, c-Jun N-terminal kinase (JNK) and cytochrome c can also be activated to initiate transduction (Mielke and Herdegen, 2000; Tournier *et al.*, 2000; Charette *et al.*, 2001; Kim *et al.*, 2005).

The Hsps bind to and inactivate some of these intermediates and play a role in the maintenance of the active state of anti-apoptotic proteins (Bruey *et al.*, 2000; Mosser *et al.*, 2000; Benn *et al.*, 2002; Paul *et al.*, 2002). Other signalling intermediates are important in response to cellular stresses and insults, such as members of the MAPK family, members of the phosphatidylinositol (PI3-K) pathway and various other proteins that form either complexes with Hsps or become activated with stress. Typically, activation of the PI3-K and MAPK 44/42 (ERK) pathways promote cell survival, whereas the other MAPK family members, JNK and p38MAPK, tend to become activated in response to stress and may be pro-apoptotic (Mielke and Herdegen, 2000).

With regards to stress responses, JNK and p38 MAPK are inter-linked. JNK has been shown to be activated upon stimuli such as hydrogen peroxide, UV radiation and trophic factor withdrawal (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Xia *et al.*, 1995; Crossthwaite *et al.*, 2002; Pantano *et al.*, 2003). JNK also has many downstream transcription factor targets that play a crucial role in determining a cell's fate. The p38MAPK family is known to be an upstream effector that, upon exposure to stressful stimuli, activates transcription factors, and depending on cell type, may either promote cell death or survival. It is a known inducer of Hsp27 phosphorylation (see Figure 1.4 and section 1.3.2.1.4 for further details), and is crucial in preventing apoptosis after various stimuli.

1.3.2 Small Heat Shock Proteins (sHsps)

The small heat shock protein (sHsp) family has been intensely studied, although its role in primary neuronal cell systems, including the DRG, is still largely unknown.

The sHsp family consists of ten members and the majority of studies have focused on Hsp27. Hsp27 has been shown to have many different functions, not only as a chaperone, but also as an integral part of the cytoskeleton and a possible intermediate in pro-survival signal transduction pathways (Lavoie *et al.*, 1993; Landry and Huot, 1995; Lavoie *et al.*, 1995; Mehlen *et al.*, 1996; Kamradt *et al.*, 2001; Gabai and Sherman, 2002).

The family of sHsps, ranging in size from 15-42 kDa, contain an α-crystallin domain of about 80-100 residues in the C-terminal portion of the protein and can form large oligomeric complexes ranging from 150-800 kDa in size (Lambert *et al.*, 1999; Bova *et al.*, 2000; Ganea, 2001). Study of the chaperone function of sHsps has provided evidence that they bind more than one protein per oligomeric complex. The sHsps will detect and sequester proteins that may denature during heat shock or other conditions. These remain bound until favourable conditions allow their refolding by other Hsp families or degradation by the ubiquitin-proteasome system (Ehrnsperger *et al.*, 1997) (see Figure 1.5).

1.3.2.1 Hsp27

There are three main functions of one member of the sHsp family, Hsp27. Hsp27 (the murine form is referred to as Hsp25) is one of the most intensely studied sHsps, due to discovery of its importance in multiple cellular functions, such as modulation of the cytoskeleton and as a factor in maintaining cellular survival.

1.3.2.1.1 The Cytoskeleton

There have been ongoing studies for more than a decade investigating the relationship between Hsp27 and the cytoskeleton. The cytoskeleton is an integral part of mammalian neurons consisting of microtubules, microfilaments and neurofilaments, and within the peripheral nervous system these components are crucial to maintaining growth processes and a healthy cellular environment (da Silva and Dotti, 2002; Dehmelt *et al.*, 2003). Hsp27 has been shown to interact with cytoskeletal components and, in particular, actin, (Miron *et al.*, 1988; Miron *et al.*, 1991; Lavoie *et al.*, 1993; Landry and Huot, 1995; Lavoie *et al.*, 1995; Huot *et al.*, 1996; Guay *et al.*, 1997) in addition to neurofilaments and microtubules (Perng *et al.*, 1999; Mounier and Arrigo, 2002). Hsp27 is known to 'cap' actin, which prevents the addition of more actin molecules than are normally required for growth and expansion of the actin filaments. The capping of actin by Hsp27 is a method to stabilize the cytoskeleton, as may be necessary after stress (Miron *et al.*, 1991; Benndorf *et al.*, 1994; Arrigo, 2000). When nonphosphorylated levels of Hsp27 are decreased, the actin cytoskeleton may become unstable and disorganized (Horman *et al.*, 1999; Williams *et al.*, 2005). Once phosphorylated, however, Hsp27 is freed from actin and the addition of actin monomers is able to proceed (Benndorf *et al.*, 1994; Landry and Huot, 1995; Guay *et al.*, 1997; Gerthoffer and Gunst, 2001; Gusev *et al.*, 2002; Mounier and Arrigo, 2002; Pichon *et al.*, 2004).

Supporting the importance of Hsp27 in axonal growth is the finding that in familial peripheral axonopathies (such as Charcot-Marie-Tooth disease and distal hereditary motor neuropathy [DHMN]), gene deletions of Hsp27 and related Hsp22 are

causative in producing the manifestations of these conditions (Evgrafov *et al.*, 2004; Irobi *et al.*, 2004).

1.3.2.1.2 Role of Hsp27 In Apoptosis

Hsp27 has been the focus of many studies in an attempt to determine other possible functions, such as in the prevention of apoptosis (Gabai and Sherman, 2002). Cells can acquire thermotolerance due to the induction of Hsps, including Hsp27, by application of a sublethal heat stress. Studies attempting to understand the mechanism of protection via thermotolerance have investigated how Hsp27 interacts with other proteins and whether heat shock proteins converge with anti-apoptotic signalling pathways. For example, Hsp27 and Akt, an important anti-apoptotic protein found in many cell types, have been shown to form a complex (Konishi *et al.*, 1997; Murashov *et al.*, 2001; Rane *et al.*, 2003) (also see Chapter 3) . Hsp27 also inhibits signalling intermediates known to promote apoptosis, such as cytochrome c (and its release from mitochondria), caspases and Daxx (Mehlen *et al.*, 1996; Garrido *et al.*, 1999; Charette and Landry, 2000; Charette *et al.*, 2000; Samali *et al.*, 2001; Paul *et al.*, 2002).

At the time I began my thesis work, little was known about Hsp27 and its role in the survival of DRG neurons, as most investigation into its potential in the prevention of apoptosis had been performed in non-neuronal cells. However, some studies had shown that its upregulation after peripheral nerve injury promoted survival of injured neurons (Costigan *et al.*, 1998), as well as the discovery that Hsp27 overexpression correlates with rescue from NGF-withdrawal induced apoptosis of DRG postnatal cultures (Lewis *et al.*, 1999; Wagstaff *et al.*, 1999). Although there have been numerous studies on the

responsiveness of DRG neurons to neurotrophins, no differences have been found between DRG cell populations and Hsp expression. Hence, further investigation was needed to determine the role of Hsp27 in DRG neuronal survival.

1.3.2.1.3 Chaperone Function

Studies of the function of Hsp27 have focused upon apoptosis and the cytoskeleton mechanism, and it seems that the functions involved in these processes cannot be studied in isolation from its role as a chaperone. Chaperones are proteins that are considered highly conserved across species and play roles in events such as protein folding and apoptosis. Consequently, chaperones are considered part of the fundamental cellular machinery that aids in many of its functions. A variety of insults to a cell can often result in specific responses that are suitably adapted to deal with the outcome – whether or not the cell can repair itself or eventually succumb to the insult depends on the functionality of its machinery; in many cases, chaperones may act during at least one part of the response process. Chaperones tend to aid in maintaining a 'favourable' environment in which proteins can fold properly and remain stable (Soti and Csermely, 2002b; Walter and Buchner, 2002).

Proteins must be properly folded into their three-dimensional structures to function. Although first described as a spontaneous process, protein folding is a complex process that involves other proteins. Ultimately, proteins achieve their lowest energy state only upon proper folding. As proteins have specific functions, their production and activity must be regulated (Zhang *et al.*, 2002). A misfolded or non-native protein has exposed hydrophobic regions that tend to bind to each other and possibly form insoluble

aggregates. Formation of non-native proteins may interfere and hinder normal cellular processes such as transcription, translation or protein degradation. Such aggregation is seen in neurodegenerative Alzheimer's disease and manifested as senile plaques (Kopito, 2000). In direct opposition to this process, chaperones can prevent protein aggregation and facilitate protein folding into its proper three-dimensional structure (see Figure 1.5). In addition, during its life span a protein may undergo a number of conformational changes or modifications that are also facilitated or assisted by chaperones (Soti and Csermely, 2002b; Walter and Buchner, 2002).

Most Hsps are classified as chaperones, and as a result, play an important role in defence against injury, in non-neuronal and neuronal cells. *In vitro* studies have elucidated the importance of Hsps in response to injury by inducing their expression or causing overexpression via introduction of exogenous Hsp. The importance of Hsps in injury have also been studied *in vivo* using transgenic animals (Latchman, 2005). Although Hsp70 has been the most widely studied Hsp, Hsp27 is also known for its cell protective role, and the mechanisms of its action will be discussed in the following section.

The chaperone function of Hsp27 depends upon its formation into large oligomers and the ability to prevent aggregation of unfolded proteins (Jakob *et al.*, 1993; Merck *et al.*, 1993). In addition, a study using 1H NMR spectroscopy has determined that the C-terminal extension of the Hsp27 protein structure is required for its chaperone activity (Lindner *et al.*, 2000).

Hsp27 may act in concert with other chaperones to gather and sequester unfolded proteins so that proper folding may occur via other heat shock proteins (Ehrnsperger *et*

al., 1997) (see Figure 1.5). Following stress, such as oxidative stress, Hsp27 interacts with and inhibits intermediate signalling components that may promote cell death and it acts in a chaperone capacity to aid in the cell's resistance to stress and damage (Rogalla *et al.*, 1999). More commonly, the chaperone function of Hsp27 has been studied for its effects in neurodegenerative disease. For example, Hsp27 reduces the effects of oxidative stress, a by-product of Huntington's disease (Wyttenbach *et al.*, 2002), and is increasingly found in the Parkinson's and Alzheimer's diseased brain (Renkawek *et al.*, 1993; Renkawek *et al.*, 1999). The exact molecular mechanism by which Hsp27 prevents damage and acts as a chaperone are still not completely elucidated and requires much further examination.

1.3.2.1.4 Structure and Function of Hsp27

Many studies have attempted to determine the role of Hsp27 in cellular survival and maintenance of the cytoskeleton via mechanisms such as treatment with chemicals, overexpression of Hsp27 and transgenic models. However, other attempts at understanding the importance of Hsp27 have been focused on its structure and the role of regions within its protein sequence. The N-terminal portion of Hsp27 contains a WDPF domain with some non-conserved residues, followed by a short variable sequence. One sequence of interest that is common to all sHsps is the α-crystallin domain, a sequence of ~80 residues located at the protein's carboxy terminus (Gusev *et al.*, 2002). The α-crystallin domain is well conserved, unlike the NH_2 region (Lambert *et al.*, 1999; Theriault *et al.*, 2004) (see Figure 1.6 (A)). As discussed previously, Hsp27 has the

ability to form multimeric structures, up to 800 kDa in molecular weight, and it has been shown that oligomeric structures occur due to interactions between α-crystallin subunits (Koteiche and McHaourab, 2002).

However, post-translational modifications of Hsp27, such as phosphorylation, disrupts oligomeric structures and results in their reduction to a monomeric form (Mehlen and Arrigo, 1994; Lambert *et al.*, 1999; Rogalla *et al.*, 1999). Human Hsp27 can be phosphorylated at 3 serine residues (S15, S78 and S82), unlike rodent Hsp27, which is only phosphorylated at 2 sites (S15 and S86) (Landry *et al.*, 1992; Mehlen and Arrigo, 1994). A few kinases have been suggested to phosphorylate Hsp27, including mitogen activated protein kinase activated protein-kinase 2 (MAPKAP-K2) via p38 MAPK, a stress response pathway, (Guay *et al.*, 1997; Dorion and Landry, 2002) (see section 1.3.2.1.4 for further details), as well as cAMP-dependent kinase and PKC α and δ (Meier *et al.*, 2001; Bitar, 2002). In addition, these phosphorylation sites appear to be important for cellular survival, as triple phosphorylation mutants (S15A, S72A and S82A) of human Hsp27 were reported to be unable to protect DRG neurons from injury (Benn *et al.*, 2002). The phosphorylation of Hsp27 has become one of the most studied posttranslational modification in sHsps with regards to its role in the dynamics of the cytoskeleton and response to stress (Welch, 1985; Stokoe *et al.*, 1992; Landry and Huot, 1995; Guay *et al.*, 1997).

In summary, the function of Hsp27 is related to its structure and potential modifications, either post-translationally or via formation of oligomers. In addition to the role that phosphorylation plays in various processes, it is still unclear as to which

phosphorylation site is most important for particular cellular responses and maintenance or whether the same kinase(s) phosphorylates the different sites (Theriault *et al.*, 2004).

1.3.3 Potential Paradox in Cell Stress Signalling

Signal transduction pathways are quite complex, as stimuli may exert an effect that results in the activation of multiple pathways and proteins. Stimuli that result in cell stress may result in apoptosis or a response that results in repair and rescue. The paradox, then, is what factors act determine whether a cell lives or dies in response to stress?

Once initiated, apoptosis leads to the self-destruction of a cell and activation of the caspase cascade, which may begin after mitochondrial damage and release of cytochrome c, or by activation of death receptor pathways (Beere, 2001). As discussed previously, Hsps are able to interact and inhibit this apoptotic cascade at either the level of caspases or cytochrome c release. Heat shock may activate many pathways: in particular the JNK and p38MAPK pathways, which also play a role in other cellular conditions such as differentiation. The role of activation of the JNK pathway is controversial, as it has been reported to exert both pro- and anti-apoptotic effects (Chen and Tan, 2000; Davis, 2000). Its pro-apoptotic effects have been suggested to occur via downstream activation of cytochrome c (Tournier *et al.*, 2000), but its survival promoting effects are not yet fully understood. As well, p38 MAPK is structurally similar to JNK and is also activated in response to heat shock. However, activation of p38 MAPK appears to contribute to cell survival, as downstream of p38MAPK is MAPKAP-K2, which once activated phosphorylates Hsp27 (see Figure 1.4). This may be important in exerting a chaperone effect and preventing apoptosis (Dorion and Landry, 2002). Hsp27 interaction with Akt

gives precedent to the concept that Hsp27 may interact on many levels in both pro- and anti-apoptotic pathways (Park *et al.*, 2005).

In the DRG neurons, a balance of these proteins and signalling intermediates may be necessary to determine whether a cell succumbs to stress. It may be that convergence of both the apoptotic and stress responses may coordinate the cellular response to stress and determine the fate of the cell. Understanding the role of Hsp27 is imperative to understanding the response of cells to stress, as it is possible that Hsp27 may exert effects upon multiple pathways to determine cell fate.

1.3.4 Regulation of Hsp Expression

Regulation of protein expression can occur at several levels, which includes transcription, translation and posttranslational modifications. As discussed above, Hsp27 can be regulated by a posttranslational modification, phosphorylation. However, studies have also shown that Hsp27 gene expression is regulated at the transcriptional level by transcription factors that may bind to specific regions in the Hsp27 promoter region and induce its expression.

1.3.4.1 HSF-1 and the heat shock response

The heat shock response and the subsequent induction of heat shock proteins are perhaps the most crucial component in maintaining a healthy cellular environment in response to stress. Although there may be a number of factors that cause elevated Hsp27 gene expression, many studies have focused on HSF-1, a member of the heat shock factor (HSF) transcription factor family. This family consists of HSF-1, HSF-2, HSF-3 and

HSF-4, each with different proposed functions (Pirkkala *et al.*, 2001). Since their discovery, HSFs have been implicated in other cellular processes, such as embryonic development (Rallu *et al.*, 1997). HSF-1 exists constitutively as a phosphorylated monomer that is unable to bind DNA (Cotto *et al.*, 1996), and after heat stress, activation of HSF-1 involves the initial trimerization of HSF-1 via leucine zippers (Rabindran *et al.*, 1993) with an increase in molecular mass (Baler *et al.*, 1993; Sarge *et al.*, 1993; Goodson and Sarge, 1995), as well as inducible phosphorylation (Cotto *et al.*, 1996). This causes HSF-1 to activate transcription of downstream targets (Sarge *et al.*, 1993; Goodson and Sarge, 1995; Ahn and Thiele, 2003), and phosphorylation may actually prolong this activation (Wu, 1995; Xia and Voellmy, 1997; Jolly *et al.*, 1999; Tonkiss and Calderwood, 2005). Activation of Hsp genes then depend upon the nuclear translocation of HSF-1 and binding of its DNA binding domain to the promoter region on the hsp gene (HSE – heat shock element) (Wu, 1984; Mosser *et al.*, 1988; Xiao and Lis, 1988) (see Figure 1.6(B)).

The HSE is highly conserved among many species throughout evolution, and consists of sequences nGAAn and nTTCn, of which there are multiple copies (Amin *et al.*, 1988; Xiao and Lis, 1988; Sarge and Morimoto, 1991; Baler *et al.*, 1993). These sequences are involved in the induction of hsp gene transcription (see Figure 1.7), and has been shown to be present in both Hsp27 and Hsp70 gene promotors (Mosser *et al.*, 1988; Cooper *et al.*, 2000). Activation of hsp genes after heat shock is only regulated by HSF-1 (Trinklein *et al.*, 2004). Present in avian cells, HSF-3 is activated after heat stress, but only after exposure to severe heat, unlike HSF-1 which is activated in response to a milder heat stress (Tanabe *et al.*, 1997).

HSF-1 is thought to be regulated positively by other signalling intermediates, such as by DAXX (Boellmann *et al.*, 2004), and negatively by phosphorylation by glycogen synthase kinase (GSK-3β) (at serine 303), PKC (at serine 363), and ERK1 (at serine 307) (Chu *et al.*, 1996; Chu *et al.*, 1998; Tonkiss and Calderwood, 2005). At the time I began my research in this area, there were no reports of the role of HSF-1 in regulation of Hsp gene transcription in neurons. A recent review notes that in the CNS, the levels of HSF-1 and induction of Hsps are low compared to non-neuronal cells, and suggests that this is possibly one of the reasons why neurons have a compromised ability to upregulate Hsps (Tonkiss and Calderwood, 2005). As so little was known about Hsp27 regulation and the role of HSF-1 in this process, this provided the impetus for the experiments described in Chapter 5.

1.3.4.2 Alternate regulators of Hsp27

1.3.4.2.1 Estrogen

Estrogen is a steroid hormone that binds to one of two receptors: estrogen receptor (ER)α, or ERβ. ERα was the first estrogen receptor to be identified. ERβ, on the other hand, was discovered in the last decade and its function is still relatively unknown (Osborne *et al.*, 2001; Hanstein *et al.*, 2004). ERs become activated by binding of ligand, and once this occurs, the receptors are able to homodimerize and bind to other molecules, such as PI3-K (Simoncini *et al.*, 2000).

Estrogen receptors are involved in the signalling of many pathways, and have the ability to activate such intermediates as PI3-K (Simoncini *et al.*, 2000) or become

activated by Akt, a downstream effector of the PI3-K pathway (Martin *et al.*, 2000) . The ER has also been linked to stress activated pathways, such as the JNK/SAPK pathway, as the ER can be activated by JNK and p38 MAPK (Lee *et al.*, 2000; Feng *et al.*, 2001). Therefore, understanding the relationships between estrogen, its receptor and other signalling intermediates may lead to a better understanding of this receptor pathway and its role in other processes within the cell.

A potential relationship between ER and Hsp27 has been examined. The levels of Hsp27 and ER were correlated to each other in many cell types, such as MCF-7 cells, and treatment with 17β-estradiol caused an induction in Hsp27 gene expression (Dunn *et al.*, 1993; Porter *et al.*, 1996). Subsequently, an estrogen response element (ERE) was identified in the promoter region of Hsp27 (Porter *et al.*, 1996) (see Figure1.6 (B)). Additionally, 17β-estradiol was also found to increase protein levels of Hsp27 in cardiac myocytes (Voss *et al.*, 2003) and in the brain after ischemia (Lu *et al.*, 2002). Studies have also examined the relationship between estrogen and other Hsps, such as Hsp90 and Hsp70, and have found that ovariectomy produces an increase in Hsp90 and Hsp70, in addition to ER (Jayachandran and Miller, 2002). In addition, it has been reported that activation of other signalling intermediates, such as nuclear factor-κB (NF-κB), is necessary for the estrogen induced induction of Hsp72 in cardiomyocytes to prevent hypoxia and that its activation occurs prior to HSF-1 activation (Hamilton *et al.*, 2004).

Estrogen receptors are located in many tissues and organs, including the brain. DRG neurons express both ERα and ERβ (Sohrabji *et al.*, 1994), and as it is also present during development, it has been suggested that estrogen and ERs are important for the

development and survival of DRG neurons. Consistent with this, administrating 17β-estradiol after NGF withdrawal rescued a proportion of cells from apoptosis (Patrone *et al.*, 1999). Although both ERα and ERβ are found in DRG, ERβ is more highly expressed in female and male DRG neurons (Taleghany *et al.*, 1999). Due to the relative complexity of estrogen signalling and its interactions with intermediates involved in apoptosis, it will be valuable to determine its role in the DRG and in relation to Hsp27 and stress responses.

1.3.4.2.2 NF-κB

Investigation of the role of NF-κB within the DRG has become an important area of study because of its involvement in a variety of responses to extracellular cues. In the DRG, NF-κB has been reported to be present in 45% of the neurons where it becomes "activated" after sciatic nerve injury (Ma and Bisby, 1998; Schafers *et al.*, 2002; Schafers *et al.*, 2003). Also reported is the presence of TNF in freshly dissociated cultures and in nerve injury, which can result in the activation of NF-κB (Fernyhough *et al.*, 2005). Developing neurons also depend on NF-κB for survival when exposed to cytokines (Middleton *et al.*, 2000). However, its role in neurotrophin signalling appears to be cell-type specific, as stimulation with NGF seems to have no effect on NF-κB activation in DRG neurons, unlike in PC12 cells and sympathetic neurons (Wood, 1995; Maggirwar *et al.*, 1998).

Traditionally, NF-κB, characterized as being involved in the inflammatory and immune response, is activated upon stimulation by various signals such as cytokines (e.g.

interleukin-1β [IL-1β]) and oxidative stress. NF-κB, consisting of two subunits (p50 and p65 or RelA), is sequestered in the cytoplasm by IκB and upon stimulation by various agents IκB is phosphorylated and results in the release of NF-κB. The p50 and p65 subunits of NF-κB are then free to translocate to the nucleus and bind to its consensus sequence on target genes (Rothwarf and Karin, 1999; Karin and Ben-Neriah, 2000).

More recently, studies investigating the role of NF-κB have shown that in neurons NF-κB may be acting as a modulator of cell survival. Many of these studies use TNF as the activator of NF-κB, and found that TNF protects hippocampal neurons against various harmful agents (Cheng *et al.*, 1994; Barger *et al.*, 1995). Likewise, use of NF-κB inhibitors has confirmed its anti-apoptotic role in neuronal cells. One inhibitor, SN50, has been shown to cause cell death in DRG neurons (Fernyhough *et al.*, 2005), as well as in sympathetic neurons (Maggirwar *et al.*, 1998; Yu *et al.*, 1999). The mechanisms by which NF-κB exerts its anti-apoptotic effects are not known, although it is proposed that inhibitors of apoptosis (IAPs) genes may be induced by NF-κB (Chu *et al.*, 1997), and that TNF stimulation may activate Bcl-2 and Bcl-x via NF-κB (Tamatani *et al.*, 1999).

There is a potential link between the heat shock response and NF-κB activation/inhibition. IκB has been studied for its role in inhibition of NF-κB, as studies have shown increased expression of IκB and inhibition of its phosphorylation after heat stress (Shanley *et al.*, 2000; Kohn *et al.*, 2002; Malhotra, 2002; Lee *et al.*, 2004; Pittet *et al.*, 2005). There are reports of NF-κB inhibition after heat shock, using models such as cultured lung epithelium (Wong *et al.*, 1997a) and A549 cells (Wong *et al.*, 1997b);

however, these are cell types that implicate NF-κB as playing a role in apoptosis. The effect that heat shock has on NF-κB in DRG neurons is not known.

1.4 Hypothesis and Specific Aims:

The major question that I wished to investigate was: What factors are important in neurotrophin-independent survival of adult DRG neurons?

When I began my thesis work, this question remained unanswered despite significant research from several different perspectives, although hypotheses were beginning to emerge. Due to existing literature on this subject, I later focused on Hsp27 as being a possible answer to the question at hand. At that time, there were four reports that lead me to study Hsp27:

1. Hsp27 was constitutively expressed within rat sensory neurons (Plumier *et al.*, 1997)
2. Hsp27 was reported to play a role in sensory neuron survival (Costigan *et al.*, 1998; Lewis *et al.*, 1999; Wagstaff *et al.*, 1999)

My experiments and results encompass the first studies of Hsp27 in our laboratory and further attempts to understand the signalling mechanisms involved in neurotrophin-independent survival of adult DRG neurons. The contents of Chapter 2 are published in Brain Research (Dodge *et al.*, 2002) and Chapter 3 is published in Journal of Neurochemistry (Mearow *et al.*, 2002). Chapter 4 and part of Chapter 5 is a manuscript published in Molecular Brain Research (Dodge *et al.*, 2006). The remainder of Chapter 5 is unpublished work.

The hypotheses underlying my studies are as follows:

Hypothesis 1

Constitutive activation of certain signalling intermediates are necessary for adult DRG neurotrophin-independent neuronal survival.

Specific Aims for Hypothesis 1:

1. To identify whether adult DRG neurons require NGF for survival.
2. To identify constitutively active pathways contributing to survival.

Hypothesis 2

Hsp27 contributes to neurotrophin-independent survival of adult DRG neurons.

Specific Aims for Hypothesis 2

1. To determine the role of Hsp27 in neurotrophin-independent survival of adult DRG neurons and determine the mechanism of action of Hsp27 using neuronal cell lines.
2. To confirm the function of Hsp27 in primary neurons and to determine whether induction by physiological mechanisms influences survival.
3. To examine factors important in regulating expression of Hsp27.

1.5 Figures

Figure 1.1: Timeline showing stages of rat DRG neuronal development and the neurotrophins that are necessary for cellular survival. Also shown is expression of Trk receptors during development (Ernfors *et al.*, 1993; Mu *et al.*, 1993; Acheson *et al.*, 1995 Memberg and Hall, 1995; Bennett *et al.*, 1996; Phillips and Armanini, 1996; Molliver an Snider, 1997; Molliver *et al.*, 1997).

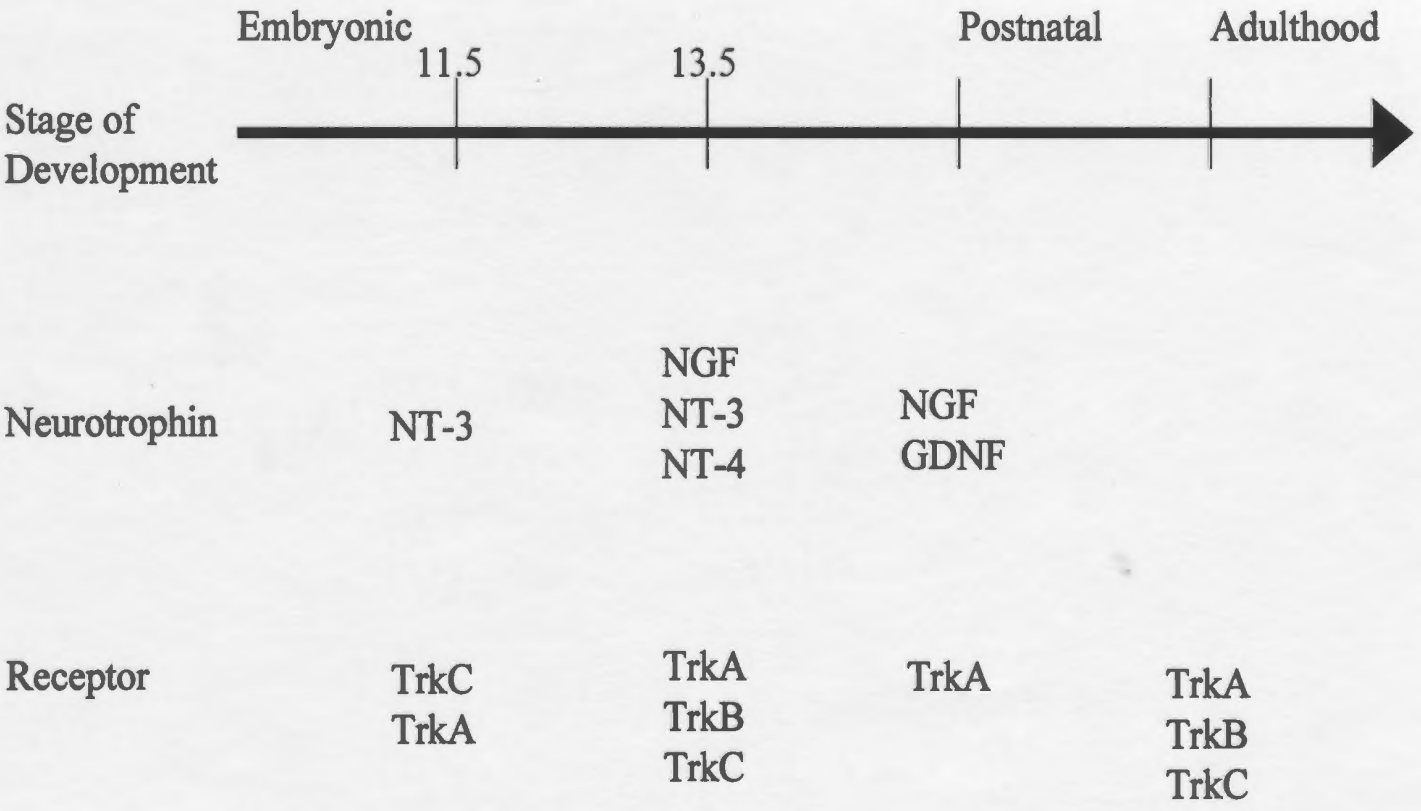

Embryonic
11.5
13.5
Postnatal
Adulthood
Stage of Development
Neurotrophin
NT-3
NGF
NT-3
NT-4
NGF
GDNF
Receptor
TrkC
TrkA
TrkA
TrkB
TrkC
TrkA
TrkA
TrkB
TrkC

Figure 1.2: Proportion of adult DRG neurons expressing Trk receptors (Adapted from Michael *et al.*, 1997).

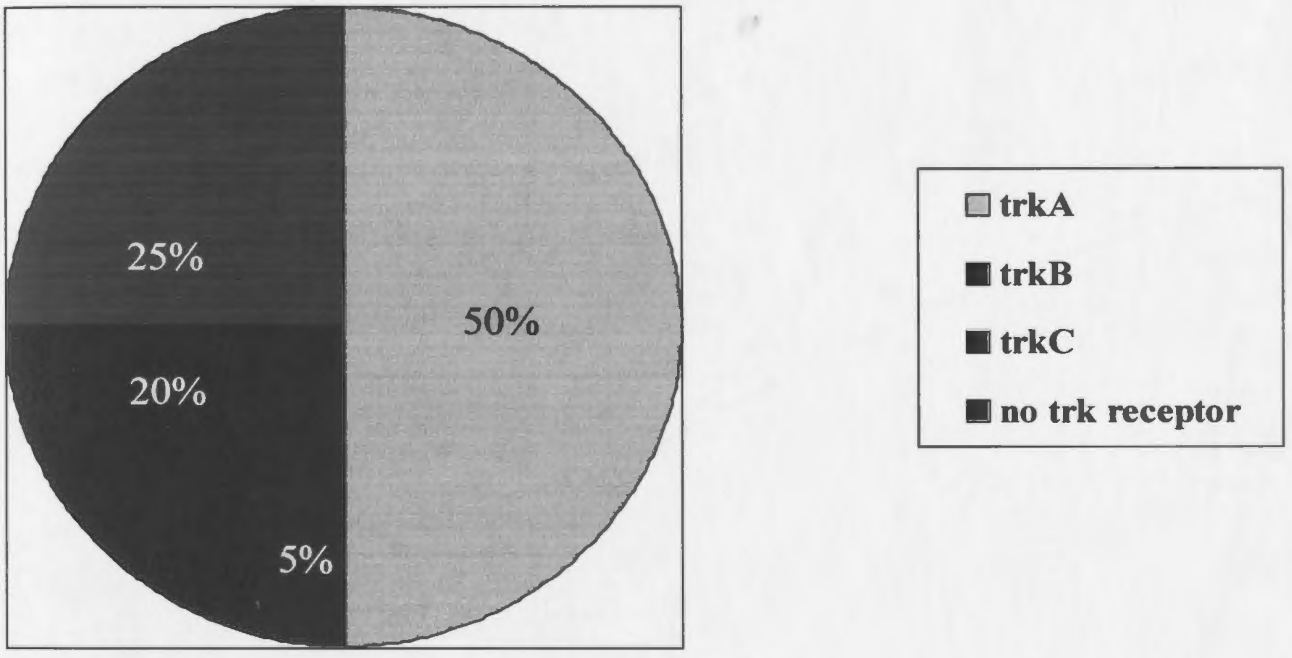

25%
50%
20%
5%
trkA
trkB
trkC
no trk receptor

Figure 1.3: Proposed signal transduction pathways activated by NGF via TrkA and by p75 (Klesse and Parada, 1999; Kaplan and Miller, 2000; Roux *et al.*, 2001).

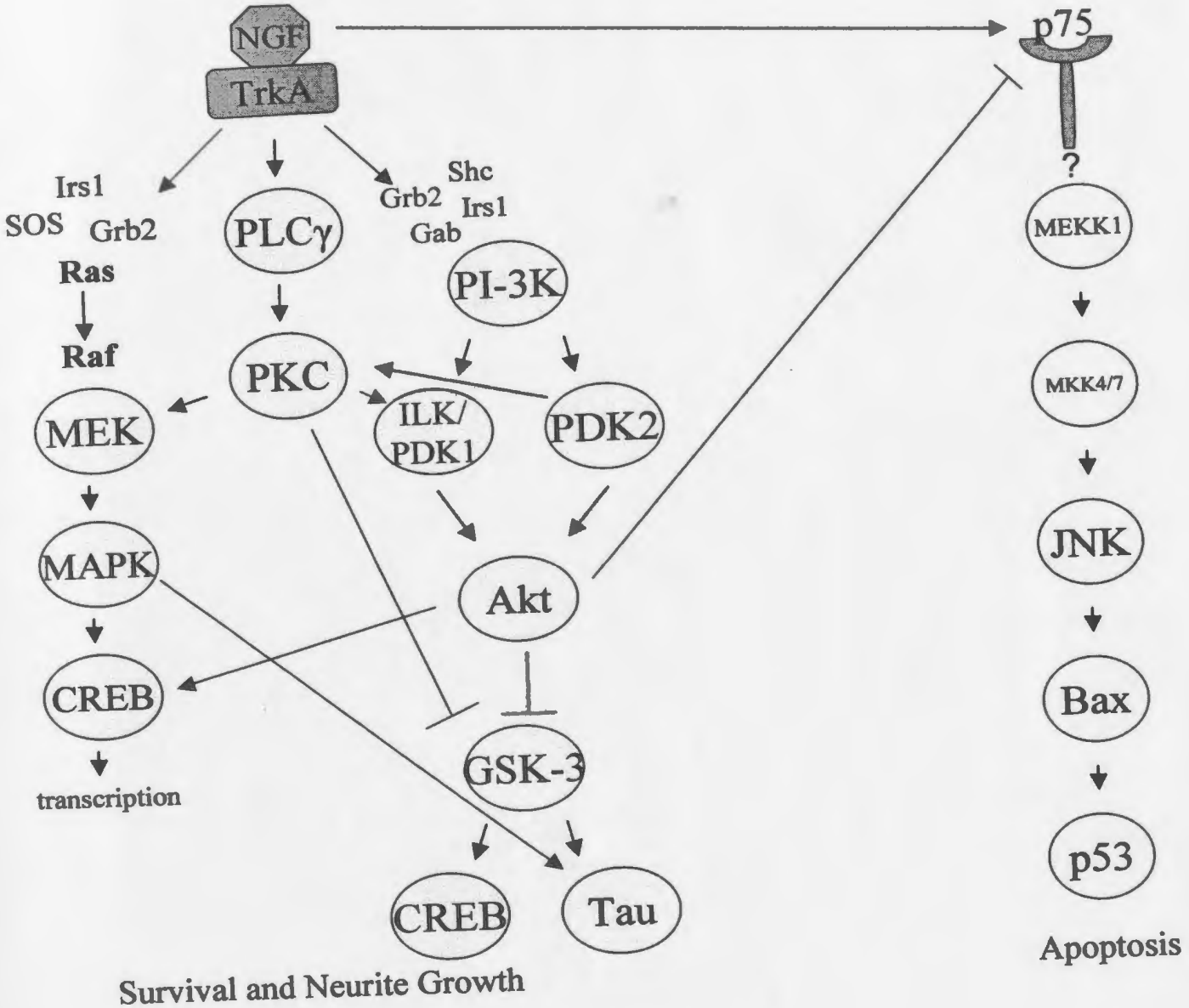
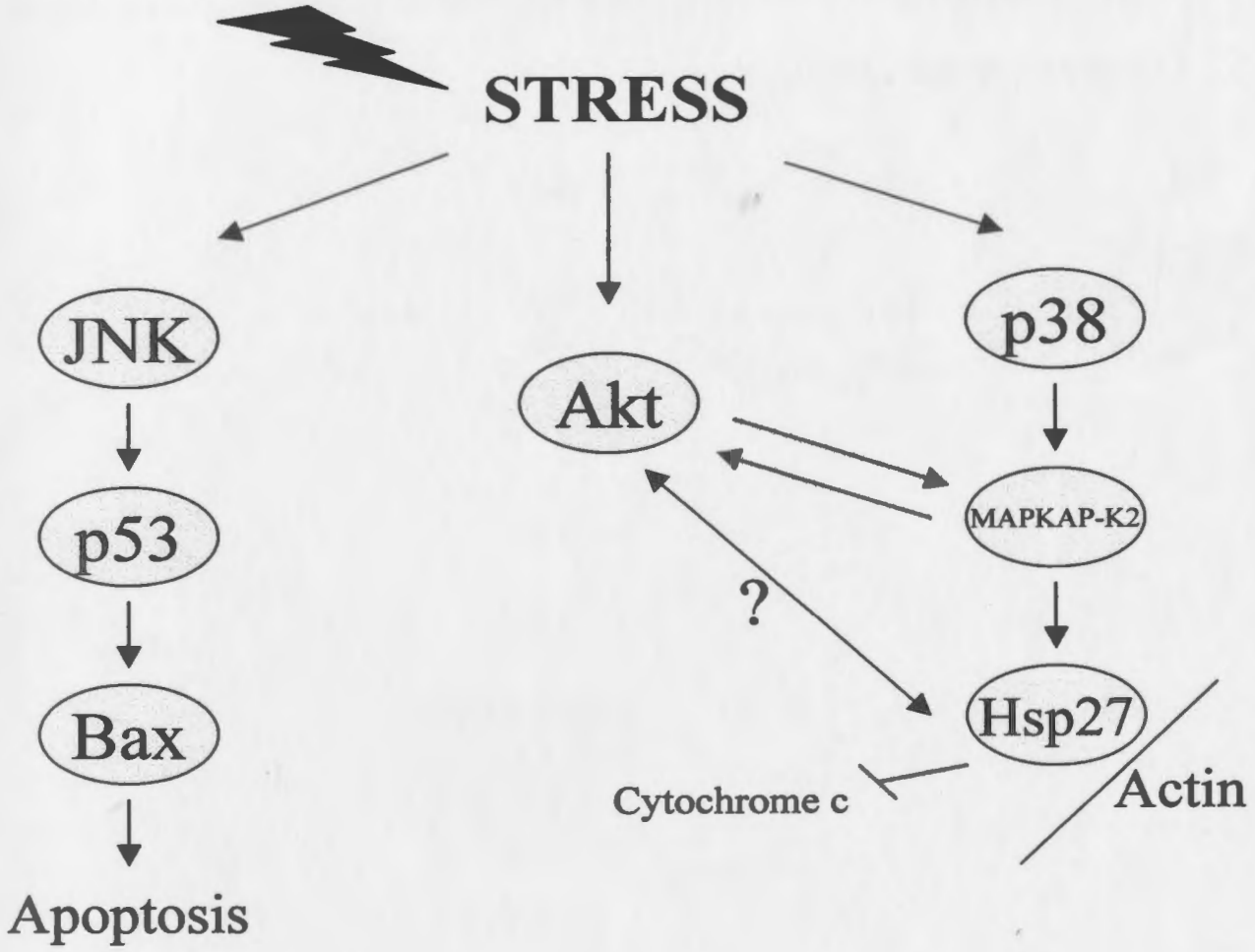

NGF
TrkA
p75
?
Irs1
SOS
Grb2
Ras
Raf
MEK
MAPK
CREB
transcription
PLCγ
PKC
Shc
Grb2
Irs1
Gab
PI-3K
ILK/
PDK1
PDK2
Akt
GSK-3
CREB
Tau
Survival and Neurite Growth
MEKK1
MKK4/7
JNK
Bax
p53
Apoptosis

Figure 1.4: Proposed signalling intermediates involved in response to stress (Derijard *et al.*, 1994; Xia *et al.*, 1995; Guay *et al.*, 1997; Gabai and Sherman, 2002).


STRESS
JNK
p53
Bax
Apoptosis
Akt
p38
MAPKAP-K2
?
Hsp27
Cytochrome c
Actin

(A)
(B)
(C)
Heat shock
Aggregation
Hsp70
(D)
Hsp27

Figure 1.5: Schematic demonstrating the role of Hsps in prevention of aggregation of misfolded proteins. (A) indicates a properly folded protein, which under stress such as heat shock, becomes unfolded (B) and tends to aggregate (C). Hsp27 will sequester these aggregated proteins (D) and allow proper refolding by other Hsps, such as Hsp70 (Ehrnsperger *et al.*, 1997).

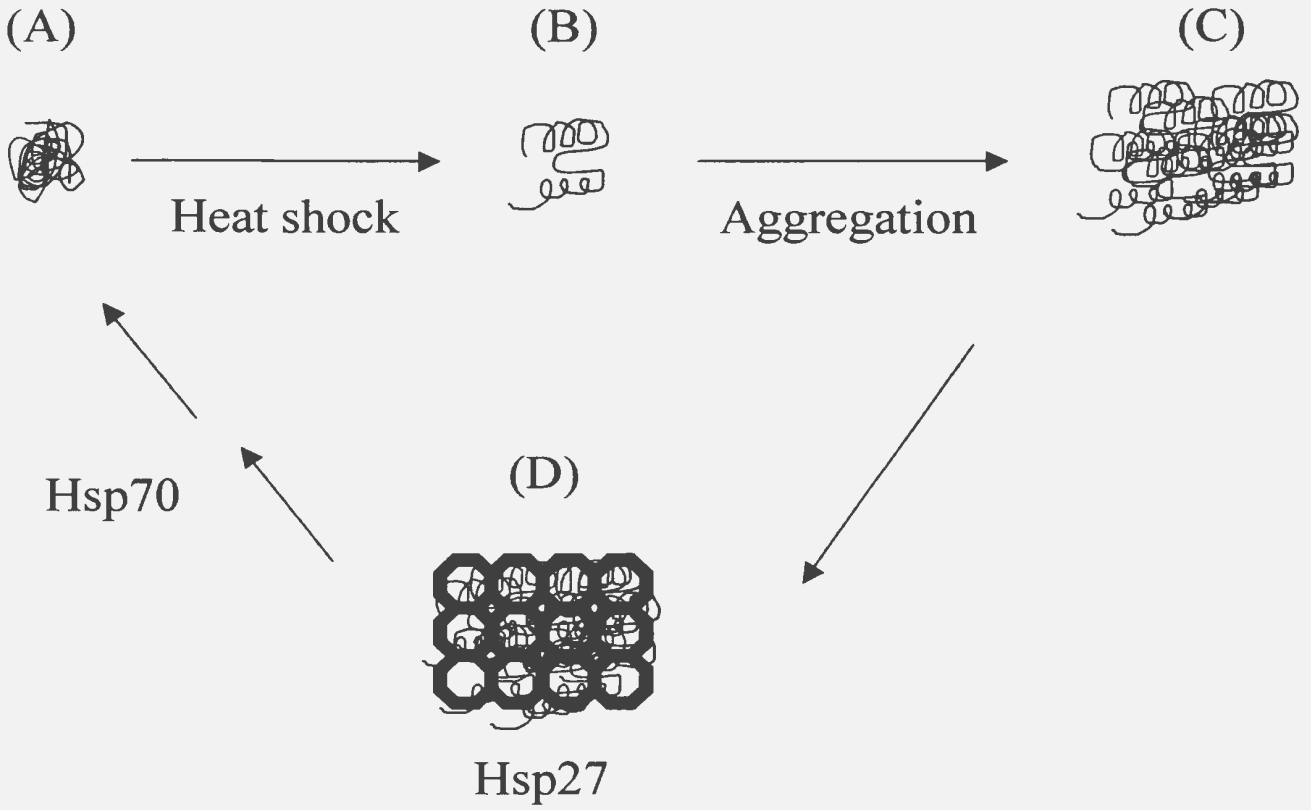

(A)
(B)
(C)
Heat shock
Aggregation
Hsp70
(D)
Hsp27

Figure 1.6: (A) Proposed domains located in the Hsp27 protein structure (Adapted from Gusev *et al.*, 2002). (B) Location of ERE and HSE within the Hsp27 amino acid sequence. Also shown are locations of exons E1, E2 and E3. (Adapted from Uoshima *et al.*, 1993, Gaestel *et al.*, 1993 and Cooper et al., 2000).

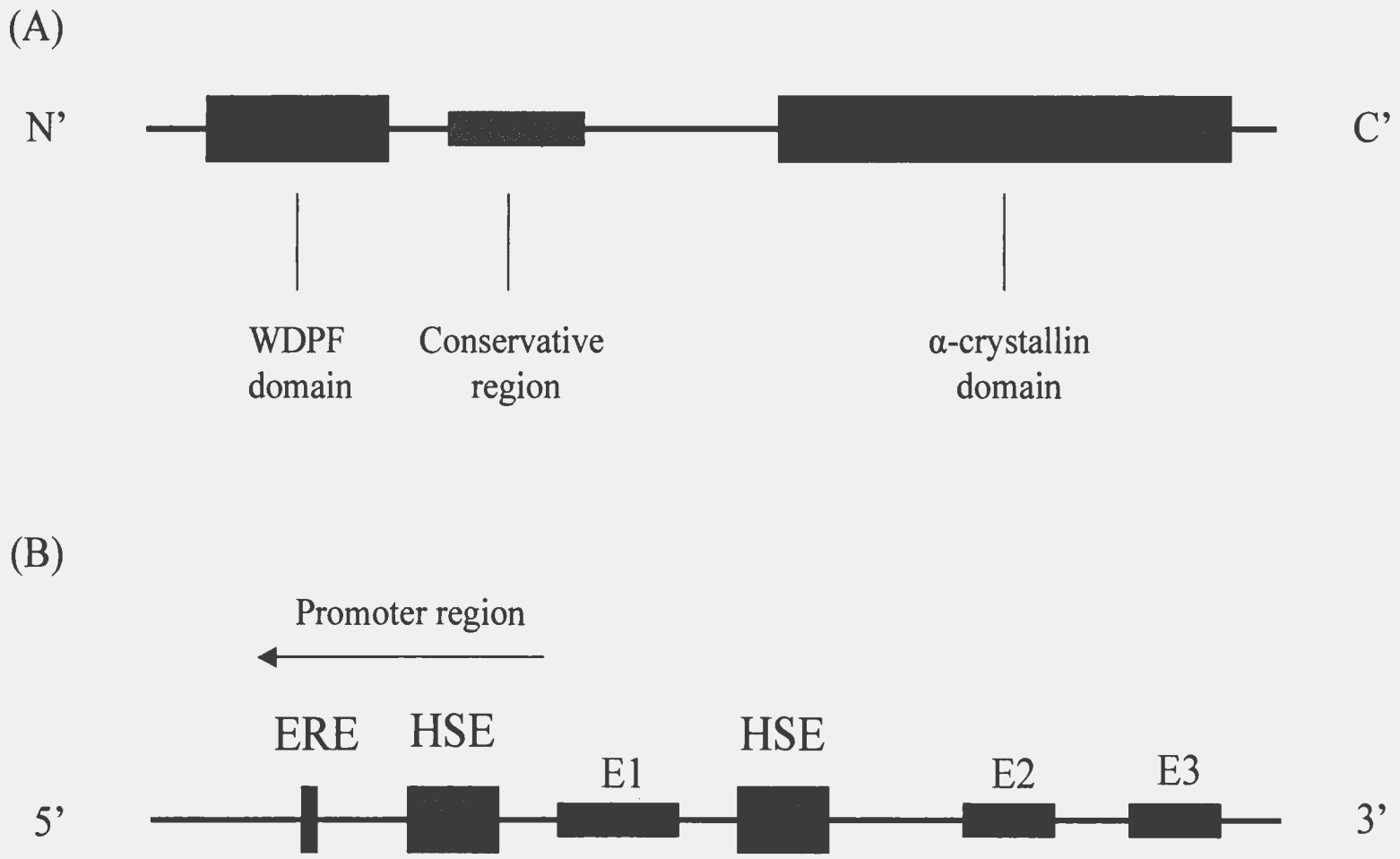
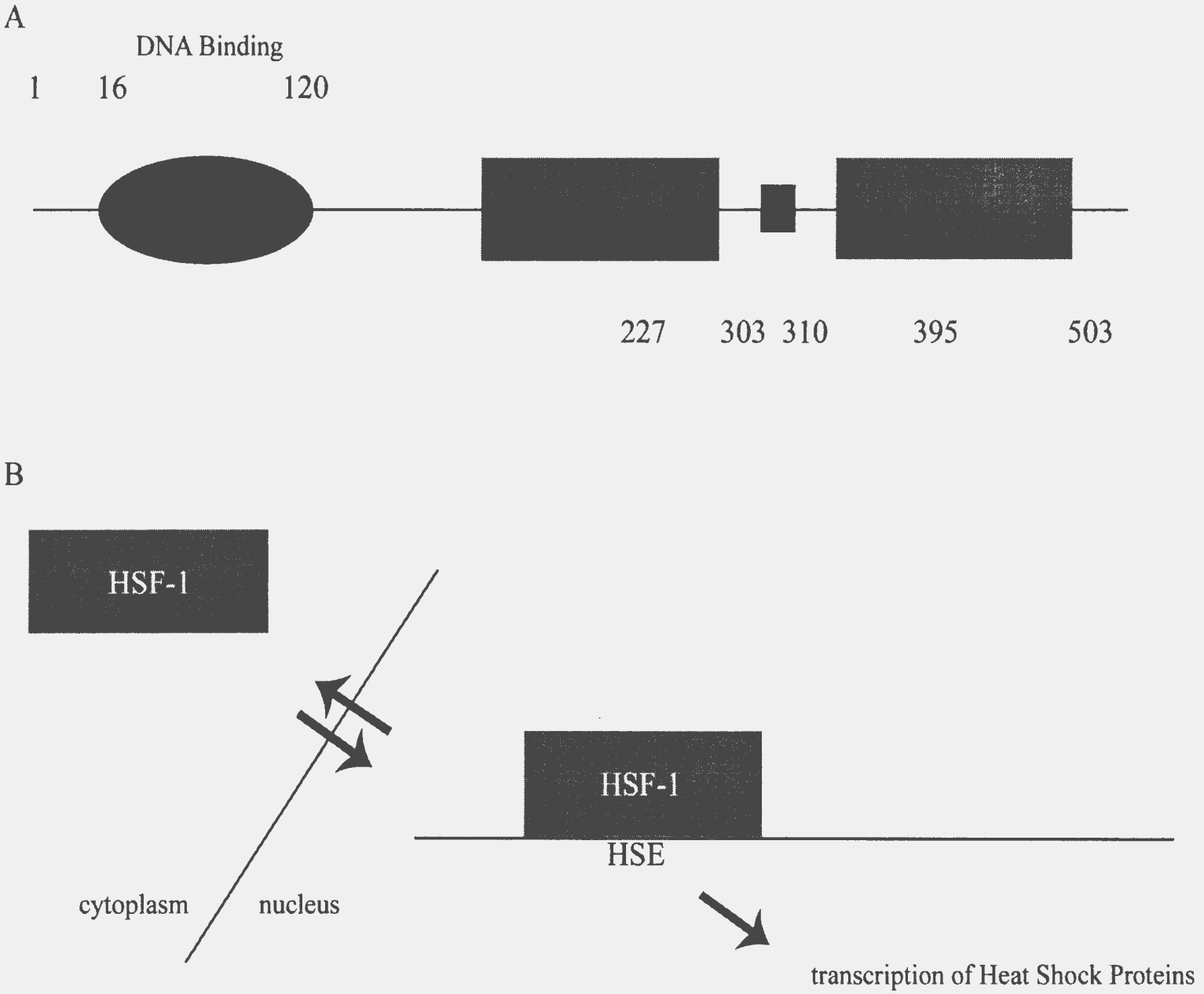

(A)
N'
C'
WDPF domain
Conservative region
α-crystallin domain
(B)
Promoter region
ERE
HSE
HSE
E1
E2
E3
5'
3'

Figure 1.7: (A) Schematic showing domains within the HSF-1 amino acid sequence. (B) Proposed mechanism of induction of Hsps via HSF-1 (Adapted from Morimoto *et al.*, 1998).


A
DNA Binding
1
16
120
227
303
310
395
503
B
HSF-1
HSF-1
HSE
cytoplasm
nucleus
transcription of Heat Shock Proteins

Co-Authorship Statement

Chapter 2, published in Brain Research, was written by myself, and all experiments, preparations and data analysis were completed by me, with the assistance of Dr. Mearow. Masuma Rahimtula provided assistance for animal dissections and some experimental preparation.

Chapter 3, written by Dr. Mearow, contains experiments performed by myself and Dr. Mearow, as we worked in collaboration to complete this work. I completed some of the cell culturing and immunoprecipitation experiments as well as much of the western blotting. Again, experiments were assisted by Masuma Rahimtula. HSPC cell lines were generated by Chuck Yeggapan.

Chapters 4 and 5 were written by myself and all experiments, with the exception of real-time PCR and DNA-binding assays were completed solely by myself. I was assisted with real-time PCR by Sherri Rankin and Cliff Guy, and by Jinguo Wang for the DNA-binding assays. Masuma Rahimtula and Budd Tucker provided technical assistance.

*CHAPTER 2

FACTORS CONTRIBUTING TO NEUROTROPHIN-INDEPENDENT SURVIVAL OF ADULT SENSORY NEURONS

2.1 Introduction

Mechanisms contributing to programmed cell death or apoptosis in neural cells have been extensively studied in the developing and neurotrophin-dependent nervous system. Sensory neurons of the dorsal root ganglia (DRG) are dependent upon neurotrophins such as NT-3 and NGF for their embryonic differentiation and development, although by the late embryonic period, the majority of these neurons are dependent upon NGF for their survival (White *et al.*, 1996). Neurons cultured from the embryonic and early postnatal period show a requirement for NGF for their maintained survival *in vitro*. During the first postnatal week there is a switch in the neurotrophin responsiveness such that 40-50 % of the originally NGF-dependent neurons now respond to GDNF (Molliver *et al.*, 1997). However, also within this postnatal period, the DRG sensory neurons lose their dependence upon trophic factors for survival and an increasing population of neurons survive in serum-free medium in the absence of added trophic factors (Virdee and Tolkovsky, 1996). The majority of postnatal DRG neurons are considered to be independent in terms of survival, although trophic factors are still required for neuritogenesis (Kimpinski *et al.*, 1997; Lindsay, 1998; Vogelbaum *et al.*, 1998).

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* **This chapter has been published in:** Dodge, M.E., Rahimtula, M., Mearow, K.M. (2002) Factors contributing to neurotrophin-independent survival of adult sensory neurons. *Brain Research*, 953: 144-56.

The molecular mechanisms underlying this change from survival dependence to independence are not fully understood. There are a number of intracellular signalling pathways activated in response to NGF binding to its receptors, Trk and p75, including the Ras→ mitogen activated protein kinase (MAPK), the phosphoinositide 3-kinase (PI3-K) and protein kinase C (PKC) pathways (Kaplan and Stephens, 1994; Kaplan and Miller, 1997). Activation of PI3-K by both Ras-dependent and independent means is a major determinant of neurotrophin-dependent cell survival in many cell types, including both sympathetic and sensory neurons (Philpott *et al.*, 1997; Crowder and Freeman, 1998; Klesse and Parada, 1998; Mazzoni *et al.*, 1999). In contrast, the contribution of the activation of MEK and MAPK via Ras is not as clear cut with some studies indicating a role in survival and others suggesting that it is not required for survival (Borasio *et al.*, 1993; Nobes and Tolkovsky, 1995; Creedon *et al.*, 1996; Virdee and Tolkovsky, 1996; Klesse and Parada, 1998; Mazzoni *et al.*, 1999; Orike *et al.*, 2001b).

Our previous results with embryonic and neonatal DRG sensory neurons have shown that the downstream activation of Akt by PI3-K is required for NGF-dependent survival, while MAPK plays a minor role. Inhibition of PI3-K by LY294002 or expression of a dominant-inhibitory form of Akt resulted in decreased survival by 48 h, while inhibition of MEK and MAPK activation using PD98502 or U0126 had little effect on decreasing neuronal survival (Mearow *et al.*, 1998).

In addition to the PI3-K and Ras-MAPK pathways, binding of NGF to Trk also results in the activation of PLCγ, which acts to increase intracellular Ca and protein kinase C (PKC) activation via production of inositol triphosphates and diacylglycerol

(Kaplan and Stephens, 1994). PLCγ activation appears to play more of a role in promoting neuritogenesis in PC12 cells (Stephens *et al.*, 1994). Although the role of PKC in promoting neurotrophin-dependent cell survival is not as well studied, inhibition of some forms of PKC does lead to cell death (Battiani, 2001).

Most of the work examining the requirement of various signalling intermediates on neuronal survival has been done in developing and neurotrophin-dependent neurons. There have been several recent studies that investigated signalling pathways in more mature neurons. Adult sympathetic neurons acquire NGF-independence, and the PI3-K pathway has been shown to be required for maintained survival *in vitro* in the absence of NGF (Orike *et al.*, 2001b). In another study, embryonic DRG neurons grown in culture for 21 d were able to survive NGF withdrawal; unlike the sympathetic neurons, these neurons were resistant to PI3-K inhibition, although NGF was still present in the cultures (Vogelbaum *et al.*, 1998).

In the present report we have used young adult rat DRG neurons to study the effects of inhibition of signalling pathway intermediates on survival in the absence of NGF, with our primary objective being to determine what mechanisms are important in the neurotrophin-independent survival of these sensory neurons. We have assessed the influence of pharmacological inhibitors using cell survival assays combined with the evaluation of protein expression studies to help understand the specifics of signal transduction and apoptosis in primary sensory neurons.

2.2 Materials and Methods

2.2.1 Cell Culture

Primary DRG neurons were dissected from young adult (4-5 week old) male Sprague-Dawley rats and cultured as described previously (Kimpinski *et al.*, 1997; Kimpinski *et al.*, 1999). In brief, ganglia were extracted from all spinal cord levels and incubated in 0.25 % collagenase (Invitrogen/Life Technologies, Burlington, Ontario) for 45 min at 37°C. Next, they were incubated in 0.25% trypsin (Invitrogen) for 20 min at 37°C, and the ganglia were then dissociated by a series of manual triturations using polished pasteur pipettes. The cell suspension was centrifuged at 1000 rpm and the resulting cell pellet was suspended in serum-free Neurobasal medium (Invitrogen) supplemented with B27 additives and antimitotics (cytosine arabinoside, 10 μM). Cells were plated at a density of approximately 500-1000 cells per well of a 96-well plate for survival assays, and at 5000-7000 neurons per well of a 12-well plate for protein analysis. Media was supplemented with 50 ng/mL NGF (Cedarlane Labs, Hornsby, Ontario) where appropriate.

2.2.2 Culture Treatments

Stock concentrations of all inhibitors were made in dimethyl sulfoxide (DMSO). Cells were fed with fresh medium and 48 h after plating, medium was supplemented with various inhibitors added to appropriate wells. Cells were fed every 2 d afterward, supplementing media with inhibitors and/or NGF where appropriate. In some experiments, anti-NGF IgG (500 ng/ml) or control rabbit IgGs (500 ng/ml) (Cedarlane

Labs, Hornby, Ont, Canada) were added to the medium. This anti-NGF antibody has been previously characterized and has been shown to neutralize the activity of purified mouse NGF (200 ng/ml) half-maximally at 100 ng/ml and completely at 500 ng/ml (Coughlin and Collins, 1985; Diamond *et al.*, 1992; van der Zee *et al.*, 1995).

Pharmacological inhibitors LY 294002, chelerythrine chloride, U0126, bisindolylmalemide I, and rottlerin were obtained from Calbiochem (San Diego, CA). Lithium chloride was obtained from Fisher Scientific (Nepean, Ontario). Cultures to be treated with the Ras N17 adenovirus were fed with fresh media, and 48 h after plating, media was supplemented with the appropriate multiplicity of infection (MOI) of adenovirus. Cells were allowed 24 h for adenoviral expression, confirmed by the expression of the green fluorescent protein (GFP) tag on the RasN17 construct. Inhibitors were added to some of the infected cultures 24 h after expression, and then left for another 24 h before Cell Titer 96 Aqueous Assay (Promega, Madison, WI) was performed.

2.2.3 Neuronal Survival/MTS Assay

Cultures in 96-well plates were maintained for various time periods with appropriate inhibitors and/or NGF present in media. Cell survival was measured using the Cell Titer 96 Aqueous Assay (Promega, Madison, WI), according to manufacturer's protocol. Medium volume in 96-well plate cultures was adjusted to 100 μL, and 10 μL/well of the MTS (3-(4,5-dimethylhiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and PMS (phenazine methosulfate) solutions added. Plates

were incubated at 37°C for 1.5 h and the absorbance of the soluble formazan product was read at 490 nm with an ELISA plate reader.

In addition to the MTS assay, the Trypan Blue exclusion method was also used to estimate cell survival in the experiments investigating whether and how long DRG neurons could survive in the absence of NGF. Live cells are able to exclude Trypan Blue, while dying cells lack this ability. The medium was replaced with Hanks Balanced Salt Solution and the cells incubated in Trypan Blue (0.4 %) for 1 min, after which time the wells were carefully washed with fresh HBSS and the cells examined under bright field and phase contrast optics. The number of blue neurons out of a total of 100 neurons per well was counted (3 wells per condition at each time point) and the data then expressed relative to the control condition.

2.2.4 Western Blotting

12-well plates were used, and cells either underwent no additions (control condition), or were exposed to one of the following: 25 μM LY294002, 10 μM U0126, 10 μM chelerythrine chloride, 1μM bisindolylmaleimide I, 10 μM rottlerin, or 10 μM U73122 (Calbiochem, California) for one hour. After 1 h, medium containing 50 ng/mL NGF (for a final concentratinon of 25 ng/ml) was added to each well of all conditions and left for 15 min. Medium was aspirated and cells were removed in ice-cold tris-buffered saline (TBS) with sodium vanadate (200 mM). Three wells were pooled for each experimental condition. Cell pellets were lysed with lysis buffer (10 % glycerol, 1% NP40, aprotonin, sodium vanadate, sodium fluoride, and one protease inhibitor cocktail

tablet (Roche Scientific, Laval, QC). Following determination of protein concentrations, 50 μg of protein per lane was electrophoresed on 10 % SDS polyacrylamide gels. After transfer, the blots were blocked in 3 % milk/TBST (Tris-buffered saline and Tween-20 at 0.2 % v/v) and incubated with primary antibodies overnight at 4°C. Following incubation with appropriate HRP-tagged secondary antibodies (Chemicon International, Temecula, CA), bands were visualized with enhanced chemiluminesence (ECL; Amersham) and exposed to x-ray film. Primary antibodies used were pAkt, Akt, p44/42 MAPK, pGSK-3β, GSK-3β, pp70S6K, pPKCδ (Cell Signalling, Mississuaga, Ontario), and ERK (Santa Cruz Biotechnology, Santa Cruz, CA).

In order to address the possibility that there was endogenous NGF in cultures that did not have added NGF we used an immunoprecipitation method. NGF was immunoprecipitated from 1-2 ml of culture medium from plates cultured with or without 50 ng/ml of NGF. The anti-NGF antibody described above was used for this purpose. The precipitated samples were electrophoresed on a 12 % acrylamide gel, along with known amounts of NGF (serial dilutions - 10 ng, 5 ng, 1 ng, 100 pg, 10 pg and 1 pg) and then probed with the same anti-NGF antibody and detected as above. The antibody was only able to detect as high as 10 pg of the loaded NGF standard.

2.2.5 Preparation of the Recombinant Adenoviral DNA

Original cDNA of dominant negative Ras N-17 was purchased from CLONTECH Laboratories Inc. This cDNA was digested with the appropriate restriction enzymes and ligated into the plasmid pIRES2-EGFP (CLONTECH Laboratories Inc). Subsequently,

the cDNA insert plus the IRES-EGFP cassette was isolated and gel-purified, and ligated into the adenoviral pShuttle vector according to manufacturer directions (CLONTECH laboratories Inc) and from this a recombinant adenovirus was selected which expressed the Ras N-17 construct. Infection of neurons and HEK293 cells with this virus results in an inhibition of the Ras-MAPK pathway assessed by phosphorylation of MAPK in response to either NGF or IGF (data not shown).

2.2.6 Statistical Analysis

Data represent the mean of at least 3 experiments, with 4-6 internal replicates in each experiment, and are expressed as percentages relative to control values (which were taken to represent 100 %). Data were plotted as mean ± SEM, and significance was determined using one-way ANOVA, followed by post-hoc testing with Dunnett's or Bonferroni's test for multiple comparisons.

2.3 Results

2.3.1 Sensory neurons survive in the absence of NGF

Neurons from young adult rats were plated in 96-well plates either in the absence of NGF, plus NGF (50 ng/ml), anti-NGF antibody (IgG) (500 ng/ml) or control IgG (500 ng/ml) and their survival followed over a 2 wk period. Survival was assayed using two techniques, Trypan Blue exclusion and MTS assay as outlined in the Materials and Methods. Quantitation of Trypan Blue exclusion in the presence or absence of NGF, anti-NGF and control IgGs was undertaken on the first day after plating and every second

day thereafter. The number of blue neurons out of a total of 100 neurons per well were counted (3 wells per condition at each time point) and the data then expressed relative to the control condition (Figure 2.1A). In these cultures, if the data are presented as the ratio of blue cells to those excluding trypan blue an initial 15-20 % loss of cells due to the dissociation protocol is observed, although the percentage of surviving neurons remains relatively constant thereafter. However, the data are presented relative to the control values for direct comparison with the MTS assay. In Figure 2.1B, the results of the MTS assays are presented for the same culture conditions and time points. Since each time point has its own control, the data are expressed relative to the control for that time point. As can be observed, there is no difference between any of the experimental conditions, and the estimation of survival is comparable between the two different assays. The addition of NGF does not have any detectable influence on neuronal survival. We also tested the effect of anti-NGF to preclude the possibility that there was some endogenous NGF being released into the culture medium even in the absence of added NGF. As seen in Figure 2.1, the addition of anti-NGF did not influence the survival of the cells over the time course examined. In addition, we tried to estimate the amount of endogenous NGF that might be present in these cultures, using immunoprecipitation, and while we could detect NGF in the medium of the treated cultures, we could not detect NGF in the non-NGF or anti-NGF treated cultures (data not shown, level of sensitivity was $\geq$ 10 pg/ml). A recent report indicating that young adult neurons could survive in the absence of NGF over a relatively short period of time (4 d), also estimated the amount of NGF in non-NGF or anti-NGF treated cultures to be negligible at 0.7 + 0.3 pg/ml (Hall *et al.*, 2001).

2.3.2 Effect of inhibitors on the PI3-Kinase Pathway

The PI3-Kinase pathway has been implicated as a crucial pathway contributing to cell survival (see Figure 2.2 for a summary of potential signalling pathways). To test its importance, primary neurons were plated in 96-well plates and exposed to the inhibitor LY294002 (IC_{50}=10 μM) at concentrations of 10 μM, 25 μM, and 50 μM in the presence or absence of NGF. Survival was assessed using a colorimetric assay of survival (MTS Live-Dead Assay, Promega Corp.). Data were expressed relative to the control condition (no inhibitor) and the results are presented as the mean ± standard error of the mean (SEM) in Figure 2.3. Exposure to 10 μM LY294002 in the presence of NGF caused little or no change in cell survival at 24, 48 or 72 h and was not followed for a further time course. In the presence of NGF, a higher concentration of LY294002 (25 μM) caused about a 25 % decrease in survival after 24 h, 48 h, and 72 h ($p<0.001$). When LY294002 was administered at concentrations of 50 μM, significant decreases in cell survival were also observed at all time points ($p<0.001$). In the absence of NGF, LY294002 also inhibited cell survival ($p<0.001$ and 0.05), although NGF did not appear to have an effect on maintaining neuronal survival as there was no difference between the survival of cells in the presence or absence of NGF. These effects were both time-dependent and dose-dependent, although at the higher concentrations there appears to be a threshold reached in terms of influence on survival.

2.3.3 Effect of inhibitors of MAP Kinase pathway

Our previous work had indicated that the Ras-MAPK pathway was not critical for survival in neurotrophin-dependent neonatal DRG neurons (Mearow *et al.*, 1998). Here we tested the importance of the MAPK pathway in the survival of adult neurons by adding the MEK inhibitor U0126 to cell cultures at various concentrations. The effect of U0126 on the survival of neurons in the presence or absence of NGF is illustrated in Figure 2.4. As expected we observed little or no cell death in the presence of NGF, although there was an increase in apparent survival at 72 h with 10 and 50 μM concentrations of inhibitor (Figure 2.4A); in the absence of NGF (Figure 2.4B), again there is little difference compared to the control condition. Similar effects with the MEK inhibitor PD98059 have been previously reported with embryonic neurons, although these authors did not comment on these observations (Klesse and Parada, 1998). We have consistently observed this apparent increase in survival in the presence of the MEK inhibitor, which may be related to the increased phosphorylation of Akt that we detect in these conditions (see Section 2.3.6) (Jones *et al.*, 2003). These results further support the notion that MAPK is not a major determinant of cell survival in these neurons.

2.3.4 Effect of inhibitors of the PKC pathway

PLCγ is another target activated by NGF, and has been shown to play a role in neurotrophin dependent differentiation and survival (Kaplan and Miller, 1997). In preliminary experiments we found that inhibition of PLCγ with the selective inhibitor U73122 had no significant effect on survival (data not shown), and thus did not

investigate it any further. However, one other signalling intermediate that can be activated via IP3 and DAG production subsequent to PLCγ activation is PKC and we were interested in determining the influence of PKC on survival. The role of PKC in neurotrophin signalling is not well understood. Studies have reported that PKC is involved in neuronal differentiation and survival although the contribution of the different isoforms is not clear (Zirrgiebel *et al.*, 1995; Wert and Palfrey, 2000; Wooten *et al.*, 2000). We used several different inhibitors of PKC in our studies, including chelerythrine chloride, bisindolylmaleimide I and rottlerin. Chelerythrine chloride is a potent competitive inhibitor of the conserved catalytic sites of PKC (IC_{50} = 0.66 μM) and its effects on cell survival are presented in Figure 2.5. In the presence of NGF (Figure 2.5A), the lowest concentration of chelerythrine used (1 μM) had no significant effect on survival. However, at the higher concentrations, there was a clear inhibitory influence, with survival decreasing to less than 50 % by 3-4 d of treatment at 10 and 50 μM concentrations ($p<0.001$). In the absence of NGF (Figure 2.5B), there was no further increase in the effect of chelerythrine on survival and although there appears to be increased survival in the presence of NGF, these results were not significantly different.

Another PKC inhibitor, bisindolylmaleimide I, acts by binding competitively to the ATP-binding site of PKC (IC_{50} = 87 nM). This inhibitor had no significant effects on neuronal survival (Figure 2.6A). We employed a third inhibitor, rottlerin, which is a selective inhibitor of the PKCδ isozyme (IC50 = 3-6 μM for PKCδ; IC50 = 30-40 μM for PKCα and PKCβ), but again this inhibitor had little effect on neuronal survival in our assays (Figure 2.6B). The data for both these inhibitors are from cultures in the absence

of NGF. Since we were primarily interested in the survival in the NGF-free cultures, we did not carry out parallel experiments in the presence of NGF, particularly in cases where there was little effect in the absence of NGF.

Thus our data suggest that there is some differential contribution of PKC isoforms to the survival of these adult neurons; however, we are not able to determine which PKC class is the major determinant.

2.3.5 Involvement of other intermediates

Since our results had shown contribution of PI 3-K and PKC to survival, we then investigated the potential involvement of other regulators of these pathways. In other studies we have employed adenoviruses expressing either dominant inhibitory or constitutively active forms of Ras to further examine the contribution of Ras to signal transduction (Mearow *et al.*, 1998) (unpublished observations). We have constructed adenoviruses expressing a dominant-negative RasN17 with an EGFP epitope tag. Because Ras can also activate PI 3-K in addition to its actions on Raf → MAPK, we examined whether the RasN17 would influence survival in the adult neurons. In embryonic neurons, this same construct results in about 50 % loss of neurons by 48 h in the presence of NGF (Mearow *et al.*, 1998) (unpublished observations).

Neurons were infected with the virus 2 d after plating and survival assessed at 48-96 h later; a control EGFP adenovirus was used to assess viral toxicity. As shown in Figure 2.7A, expression of N17 (detectable due to the co-expression of the EGFP) resulted in 25-30 % decrease in survival in the absence of NGF ($p<0.001$). These results

are similar to those obtained with LY294002, suggesting that RasN17 may be acting via the PI 3-K pathway to inhibit survival.

There are at least two intermediates downstream from PI3-K known to be important in cell survival. Akt is a key component being required for survival in a number of cell types, including DRG neurons (Kaplan and Miller, 2000), although since there are no commercially available selective inhibitors we were unable to selectively test the role of this intermediate in survival in these experiments (but see below).

Another intermediate is glycogen synthase kinase (GSK3). Activation of PI 3-K and subsequently Akt leads to the phosphorylation of GSK3, which unlike many other kinases, results in its inactivation. When PI 3-K is inhibited, GSK3 becomes dephosphorylated and activated, and has been reported to have a pro-apoptotic role in various cell types (Frame and Cohen, 2001; Grimes and Jope, 2001). GSK3 can be inhibited by LiCl, shown to act as a competitive inhibitor of GSK3 α and β (Klein and Melton, 1996; Stambolic *et al.*, 1996; Grimes and Jope, 2001). Thus to investigate whether the effects of PI 3-K inhibition were modulated via increased activity of GSK3, we treated the cells with LiCl alone and plus LY294002 or RasN17 and carried out the survival assays. The results of these experiments are presented in Figure 2.7B-D.

In preliminary experiments, lithium had no effect on the survival compared to the control situation at 24 and 48 h (data not shown). However, in this series of experiments, we observed that there was about a 20 % decrease in survival compared to the controls; the reason for this is not clear, although it may relate to the role of GSK3 in cellular metabolic processes such as glucose and glycogen regulation. When we examined the

effect on the combination of lithium plus LY294002 or plus the RasN17, we found little protective effect, which was not what we would have expected if GSK3 activity was playing an important role in the effects of PI 3-K inhibition on survival. We are currently constructing adenoviruses to express wild-type and constitutively active GSK3 to further study the role of GSK3 in the survival of DRG neurons.

2.3.6 The effect of the inhibitors on the activation of signalling intermediates

In order to confirm that the inhibitors used did have the expected effects on their respective targets (despite apparent lack of effect in the biological assays) we carried out immunoblotting experiments. Neurons were plated for 24 h in the absence of NGF and then treated with the appropriate inhibitors for 1 hr prior to 15 min stimulation with NGF. This short-term stimulation with NGF has been shown to result in the phosphorylation of downstream signalling intermediates and is used to assess the activation state of the kinases (Kaplan and Miller, 1997, 2000; Kimpinski and Mearow, 2001). Neuronal lysates were then collected and subjected to SDS-PAGE electrophoresis. The resulting blots were probed with phospho-specific antibodies against phospho-PKCδ, phospho-Akt, phospho-GSK and phospho-MAPK, followed by blotting with antibodies to detect total protein levels. Results from representative blots are presented in Figure 2.8. In the absence of NGF, these adult neurons show detectable levels of the phospho-forms of most of the intermediates examined (first lane in all the blot strips). We observe this routinely in our cultures and suggest that this is indicative of a constitutive or basal level of activation, which in itself could be important for both survival and neuritogenesis

(Kimpinski *et al.*, 1999; Kimpinski and Mearow, 2001). However, the addition of NGF to the cultures results in the increased phosphorylation of the targets (second lane). When the neurons are pre-incubated with the inhibitors prior to the NGF stimulation, the upregulation of the appropriate targets is blocked. Thus in Figure 2.8A, in the presence of LY294002, the NGF-induced phosphorylation of Akt is blocked; the effect on the phosphorylation of GSK is much more modest. Similarly, in the presence of U0126, phosphorylation of MAPK is completely inhibited. Reprobing of the same blots with Akt, GSK and MAPK demonstrate that there is no loss of expression of these proteins. Although we did not have antibodies to non-phospho-PKC, these are the same blots probed with Akt, GSK and MAPK, indicating that there is no difference in protein loading that would account for the loss or decrease in the phospho-specific signal. For PKC, we used an antibody against the delta isoform as we had originally hypothesized based on the literature that this would be the isoform important for NGF-stimulated events. NGF clearly results in phosphorylation of PKCδ (Figures 2.8A and B), and this appears to be inhibited by all the inhibitors used in panel A. The U73122 compound is a selective inhibitor of PLCγ, which might be expected to also inhibit activation of the downstream PKC. PDK1 has also been reported to phosphorylate PKC (Toker, 1998) and since PDK1 is downstream of PI 3-K, LY294002 inhibition of PI 3-K could result in PDK1 inhibition as well. In panel B, the effects of the selective PKC inhibitors are presented, and it can be seen that chelerythrine is most effective in inhibiting the phosphorylation of PKCδ. While this antibody is reported to be selective for PKCδ (Cell Signalling Technologies), it is possible that it does cross-react with the other isoforms of

PKC. The lack of influence of the supposedly PKCδ-selective inhibitor rottlerin could also be explained by the fact that the antibody is directed against only one of the phosphorylation sites and this site may not be inhibited by the rottlerin. Thus it is possible that the lack of effect of rottlerin and bisindolylmaleimide on the survival of neurons could be due to incomplete inhibition. However, it is clear that inhibition of the phosphorylation of PKCδ by chelerythrine chloride correlates with its inhibitory effect on neuronal survival.

2.4 Discussion

In this report we have investigated potential mechanisms contributing to the neurotrophin-independent survival of young adult rat DRG neurons. Our results show that adult DRG neurons can survive in the absence of NGF for at least 2 wks and that the addition of NGF has no obvious influence on enhancing survival. We then investigated the effect of inhibiting various components of signal transduction pathways known to be activated by NGF and reported to play a role in neuronal survival (see Figure 2.2). We find that of the signalling intermediates activated in these cells, PI3-K and PKC appear to be more important in promoting this survival, since inhibition of PI3-K with LY294002 or PKC with chelerythrine both resulted in significant decrease in survival. Our immunoblotting experiments provide confirmatory evidence that the inhibitors were, for the most part, having the expected effects on their respective signalling intermediates.

2.4.1 The role of PI 3-K and its downstream targets in neuronal survival

Studies with embryonic and neonatal sympathetic or DRG sensory neurons have pointed to the importance of the PI 3-K in NGF-dependent survival. The survival-promoting activity of PI 3-K is thought to be primarily mediated by Akt, one of its downstream targets (Philpott *et al.*, 1997; Crowder and Freeman, 1998; Klesse and Parada, 1998; Mearow *et al.*, 1998; Mazzoni *et al.*, 1999; Vaillant *et al.*, 1999).

Fewer studies have investigated what signalling pathways are required for, or at least contribute to, the neurotrophin-independent survival of mature DRG sensory neurons. A recent report demonstrates that adult superior cervical ganglion (SCG) neurons are able to survive in the absence of NGF for at least 5 d and thus display NGF-independence (Orike *et al.*, 2001b). However, these neurons show a similar requirement for PI3-K as the NGF-dependent neurons, in that treatment with LY294002 results in an 80 % loss of survival by 24 h in low-density cultures (Orike *et al.*, 2001a).

DRG neurons also acquire independence from NGF for their survival in the early postnatal period, such that adult DRG neurons can survive in the absence of added neurotrophins, as we and others have shown (Kimpinski *et al.*, 1997; Lindsay, 1998; Vogelbaum *et al.*, 1998). In the latter report, rodent embryonic E15 DRG neurons were plated and maintained in culture in the presence of NGF for up to 21d (21 DIC), at which point the neurons were equivalent to 2 wk old or P14 neurons (Vogelbaum *et al.*, 1998). When these neurons were treated with LY294002 in the presence of NGF, it was found that the 21 DIC neurons were less sensitive to the effects of the inhibitor than neurons grown in culture for 5 or 10 d; that is, there was less cell death in the older neurons than in the younger ones.

Our results suggest that PI 3-K activation plays a role in maintaining survival in the absence of NGF in DRG neurons, and that this is likely to be at least partly mediated by the downstream activity of Akt. Although we did not directly interfere with Akt activation (there are no available specific inhibitors and we did not use dominant negative constructs of Akt), the inhibition of PI3-K with LY294002 clearly resulted in the concomitant inhibition of the activation of Akt, as observed on the immunoblotting experiments. It should also be noted that in our cultures we routinely observe a basal level of phosphorylation of Akt, along with other intermediates like MAPK and GSK, which can be further increased by the addition of NGF. This points to a constitutive level of activation of these pathways that could be contributing to the survival in the absence of added NGF or other growth factors.

We also examined whether inhibition of the activity of GSK3 might mitigate the inhibitory effects of PI3-K inhibition, either by LY294002 or expression of the dominant negative RasN17. GSK3 has a complex role in cellular metabolism, but has been reported to exert a pro-apoptotic effect in various cell types (Frame and Cohen, 2001; Grimes and Jope, 2001; Woodgett, 2001). Lithium has been shown to act as a competitive inhibitor of GSK3 with a half-maximal effect at 1 mM, although at higher concentrations it can also inhibit other enzymes (Klein and Melton, 1996; Stambolic *et al.*, 1996). In our experiments we did not see any protective influence of LiCl in attenuating the cell death seen with either LY294002 or RasN17, and in fact at longer incubation times observed that LiCl itself resulted in decreased survival. The reason for this is not clear, although it is possible that at the longer incubation times (especially with the viral infections) the inhibition may have more to do with the role of GSK3 in

metabolic processes rather than in the regulation of cell death (Frame and Cohen, 2001; Grimes and Jope, 2001). However, interestingly lithium has also been reported to inhibit NGF-induced upregulation of PKCα in PC12 cells (Li and Jope, 1995).

Although we did not examine the effect of LiCl on the inhibition (i.e., dephosphorylation) of GSK3 in this study, in other experiments investigating signalling required for NGF-dependent neuritogenesis we have observed that LiCl (3-5 mM) over a 24 h treatment period results in both the dephosphorylation of GSK3 and phosphorylation of Akt (Jones *et al.*, 2003). Similar results have been reported by others (Chalecka-Franaszek and Chuang, 1999; Grimes and Jope, 2001).

2.4.2 The role of MEK and MAPK activation in survival

Another major pathway that is activated by NGF binding to its receptor, Trk, is the Ras→ MAPK pathway. Unlike the PI 3-K pathway, this pathway does not play a major role in NGF-dependent survival of sympathetic or sensory neurons (Borasio *et al.*, 1993; Creedon *et al.*, 1996; Klesse and Parada, 1998; Mearow *et al.*, 1998; Mazzoni *et al.*, 1999). Our results, like those of Orike and colleagues (Orike *et al.*, 2001a), provide further evidence that MAPK is not required for neurotrophin-independent survival of sympathetic and sensory neurons.

2.4.3 PKC and neuronal survival

The protein kinase C (PKC) family is categorized into 3 subgroups, reflecting differential activation by calcium and lipid second messengers. The conventional isotypes

(PKCα, β, γ) require both calcium and diacylglycerol (DAG), the novel isotypes (PKCδ, ε, η, θ) require DAG, but not calcium, while the atypical forms (PKCξ, λ) respond to phosphoinositides (Toker, 1998; Parekh *et al.*, 2000). Signalling pathways activated by receptor binding of agonist (including growth factors) act via PLC and the subsequent production of DAG, IP3 as well as alterations in intracellular calcium levels (Toker, 1998; Parekh *et al.*, 2000). The role(s) of PKC in regulating multiple cellular processes are the subject of intensive investigation and clearly comprises a complex series of regulatory events. In terms of its role in neuronal survival, there are a number of studies pointing to both positive and negative regulation, which seems to also depend upon the cell type and the nature of the extracellular stimulus (eg., oxidative stress, growth factor deprivation, amyloid-induced toxicity) (Battiani, 2001). For example, BDNF activation of PLCγ and the downstream activation of PKC was shown to be required for the survival of cerebellar granule cells, with inhibition of PKC by calphostin C resulting in neuronal apoptosis (Zirrgiebel *et al.*, 1995). NGF-withdrawal induced apoptosis was blocked by treatment with bisindolylmaleimide (Tanaka and Koike, 2001). The specific isoforms involved in these effects were not identified, since the inhibitors used were not subclass-selective. However, other studies have reported that PKCα, ε, ξ and λ play a role in inhibiting cell death, while PKCβ1 and PKCδ appear to act in a pro-apoptotic manner (Deacon *et al.*, 1997; Gubina *et al.*, 1998; Li *et al.*, 1999; Battiani, 2001; Maher, 2001). Thus the results from a variety of studies suggest that the activities of multiple PKC isoforms, each differentially activated, contribute to the regulation of cellular survival.

In our experiments, we used several different inhibitors of PKC and only one of these, chelerythrine, resulted in significant loss of neuronal survival. The PKCδ-selective inhibitor, rottlerin, had no detectable influence on cell survival. Although PKCδ was phosphorylated in both the control and NGF-stimulated cells, this activation did not seem to contribute negatively to cell survival; the antibody used detects phosphorylation of Thr505 in the activation loop domain, one of the sites required for subsequent PKC activity (Parekh *et al.*, 2000). These results suggest that either PKCδ is not involved in survival signalling in these adult DRG neurons, or alternatively that there are other pathways that counteract the potential negative effect of PKCδ. Since neither chelerythrine nor bisindolylmaleimide are isoform-selective inhibitors, we are unable to determine exactly which form of PKC is most important in the maintenance of neurotrophin-independent survival.

The unexpected deleterious effects of lithium, as noted above, could also result from its influence on PKC. Lithium inhibits PKC activation, potentially by altering the availability of inositol phospholipids (Manji and Lenox, 2000; Detera-Wadleigh, 2001), or via its inhibition of PDK-1, a key regulatory enzyme necessary for the activation of both PKC and Akt (Toker, 1998; Parekh *et al.*, 2000). So, in addition to its ability to inhibit GSK3 activity, lithium also influences other signalling pathways important for cell survival.

2.4.4 Other contributions to survival?

In our experiments we rarely saw complete loss of survival assayed by either the Trypan Blue or MTS method of quantitation. Because both assays rely on estimation of survival on a population basis compared to a control condition, it was not possible to determine whether there were particular neuronal phenotypes that were resistant to the different inhibitors. However, similar results have been observed in other studies examining survival of sympathetic and sensory neurons under various conditions. While NGF withdrawal from embryonic or neonatal neurons resulted in the most extensive loss of survival with residual survival percentages ranging from 20-40 %, use of the various methods to inhibit PI 3-K or MAPK signalling generally resulted in less loss of survival (40-60 % remaining survival) (Creedon *et al.*, 1996; Philpott *et al.*, 1997; Crowder and Freeman, 1998; Klesse and Parada, 1998; Mazzoni *et al.*, 1999; Vaillant *et al.*, 1999). This suggests that overlapping or convergent signalling plays a role in promoting the survival of various classes of neurons, including depolarization, cAMP or extracellular matrix activated signalling pathways.

2.4.5 Summary

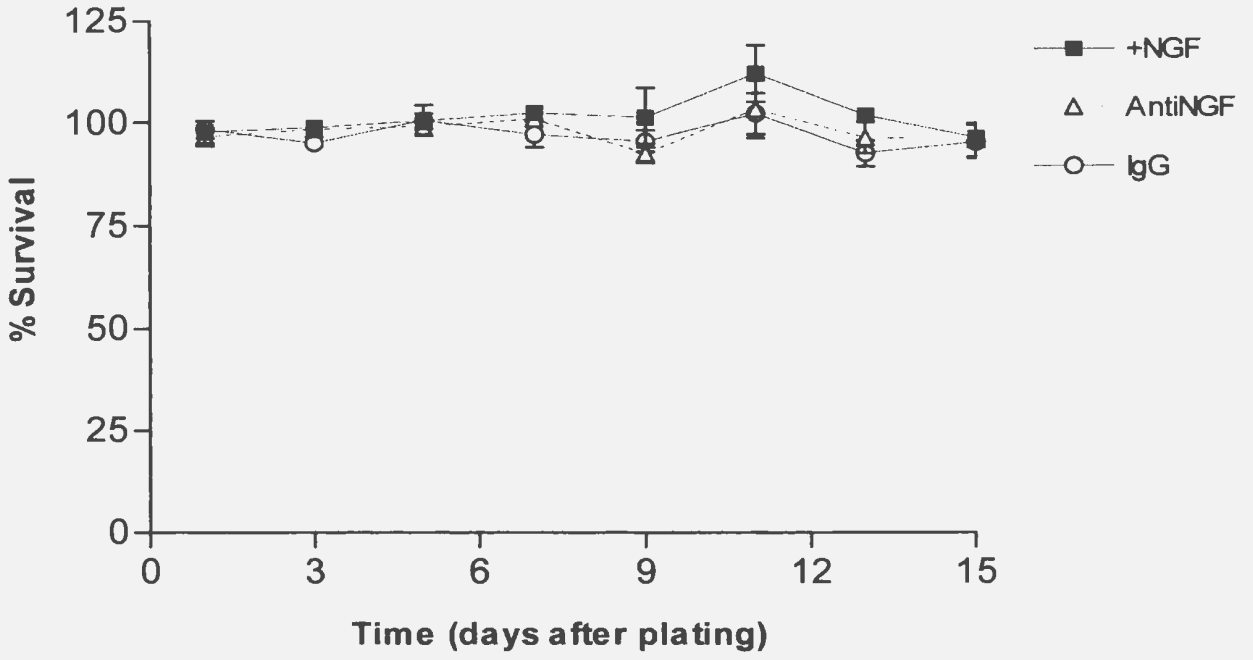
Our results point to a role of PI 3-K and PKC in maintaining the neurotrophin-independent survival of adult sensory neurons. However, one potential caveat of this and all *in vitro* studies is that the neurons have been subjected to axotomy and as such the apparently constitutive activation may be a stress or injury related response. While *in vivo* studies indicate that generally adult sensory neurons can survive after injury or after anti-NGF treatment, the role of PI 3-K, PKC or MAPK signalling *in vivo* in this regard

has not been well studied. While we have focused our investigation on components of the so-called "survival" pathway (Kaplan and Miller, 2000; Miller and Kaplan, 2001), it is possible that there is some level of pro-apoptotic signalling in these neurons in the absence of sustained neurotrophin or other growth factor dependent signalling. Further investigation is required to address these possibilities and to determine if PI 3-K and PKC play any role in inhibiting potential pro-apoptotic signalling.

2.5 Figures

Figure 2.1. Adult neurons survive for up to 2 wks in the absence of nerve growth factor (NGF). Cells were plated in serum-free medium with or without NGF (50 ng/mL), with rabbit anti-NGF IgG (500 ng/ml), and control rabbit IgG (500 ng/ml). (A). At 1 d after plating, the percentage of live neurons was counted by the Trypan Blue exclusion method and subsequently this was repeated every 2 d up to 15 d *in vitro*. Data are expressed as mean ± SEM (n=3), relative to the control. (B). Survival was also estimated using the MTS assay as outlined in the text as a comparison with the Trypan Blue method. Data are expressed as the mean ± SEM (n=4) relative to the control (no additives) for each time point.

A


125
100
75
50
25
0
% Survival
0
3
6
9
12
15
Time (days after plating)
+NGF
AntiNGF
IgG

B

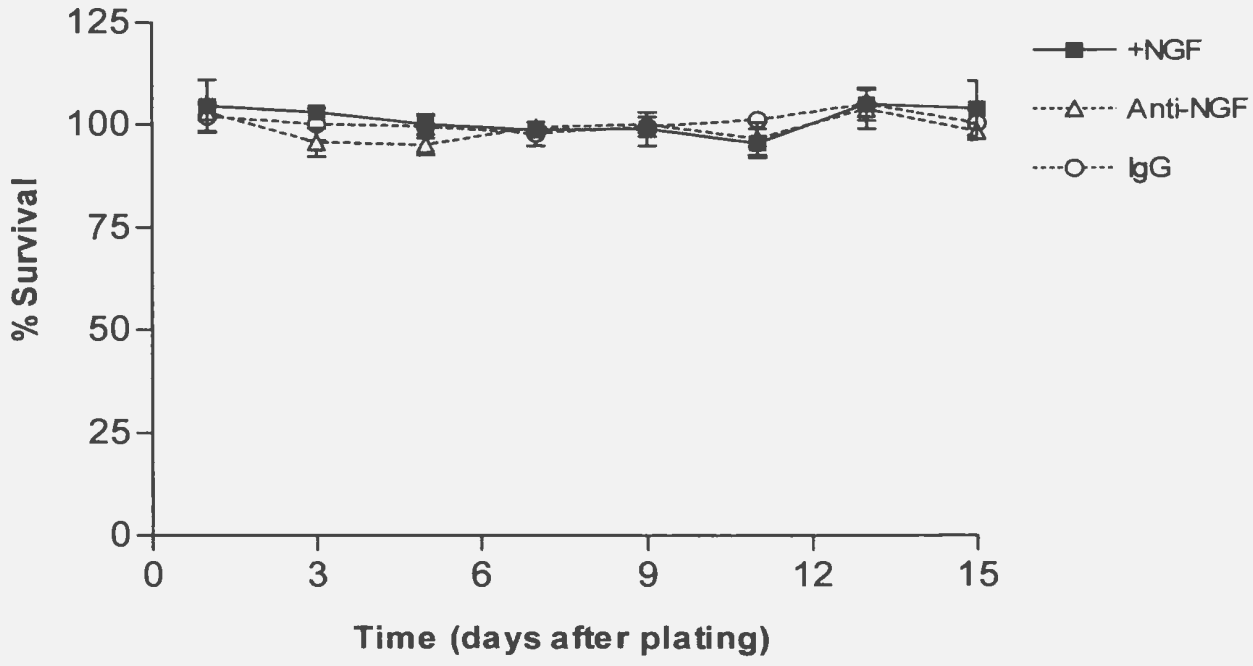

125
100
75
50
25
0
% Survival
0
3
6
9
12
15
Time (days after plating)
+NGF
Anti-NGF
IgG

Figure 2.2. Schematic outline of potential signalling intermediates involved in neuronal survival. Signalling intermediates known to be activated by NGF-Trk signalling are shown. In addition, the sites of action of the pharmacological inhibitors employed in this study are also indicated (-). Our results suggest that signalling via the PI 3-K→Akt pathway contributes to survival in adult DRG neurons even in the absence of NGF; the possible downstream targets or effectors have not been defined. In addition, our results indicate that PKC plays a role in promoting survival although the effectors of this pathway are not clear. The Ras→MAPK pathway has little effect on survival in our model, although it may play a role in other aspects of sensory neuron function.

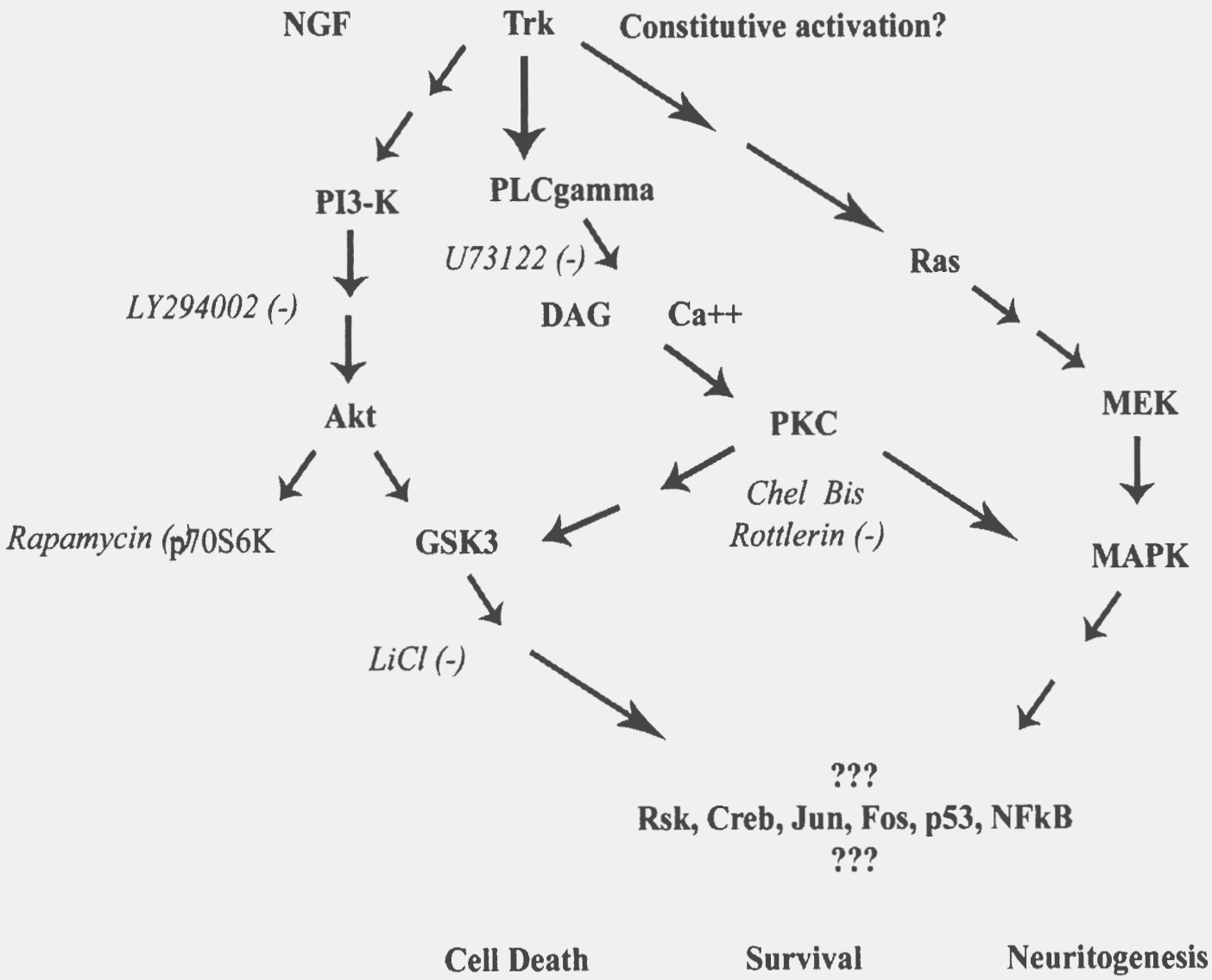
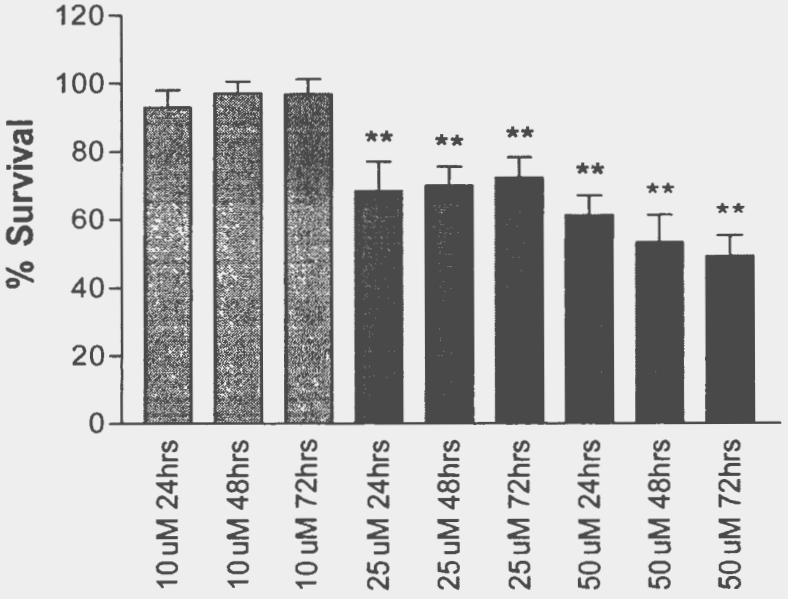

NGF
Trk
Constitutive activation?
PI3-K
PLCgamma
U73122 (-)
Ras
LY294002 (-)
DAG
Ca++
Akt
PKC
MEK
Chel Bis
Rottlerin (-)
Rapamycin (p70S6K
GSK3
MAPK
LiCl (-)
???
Rsk, Creb, Jun, Fos, p53, NFkB
???
Cell Death
Survival
Neuritogenesis

Figure 2.3. The effect of PI 3-Kinase inhibition on cell survival. Cells were treated with medium containing the indicated concentrations of LY294002 in the presence (A) or absence (B) of 50 ng/mL NGF. At 24 h, 48 h and 72 h after addition of the inhibitors, survival was assessed by the MTS Live-Dead assay (as outlined in Material and Methods). Data are expressed as percentages of the controls (wells with no inhibitor added) (mean ± SEM, n=3-5). *$p<0.05$; **$p<0.001$.

A


% Survival
120
100
80
60
40
20
0
**
**
**
**
**
**
10 uM 24hrs
10 uM 48hrs
10 uM 72hrs
25 uM 24hrs
25 uM 48hrs
25 uM 72hrs
50 uM 24hrs
50 uM 48hrs
50 uM 72hrs

B

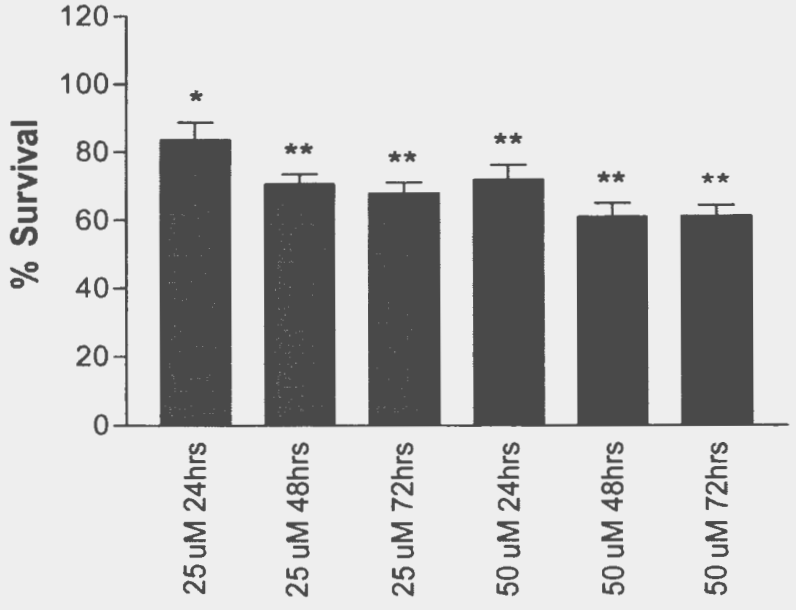
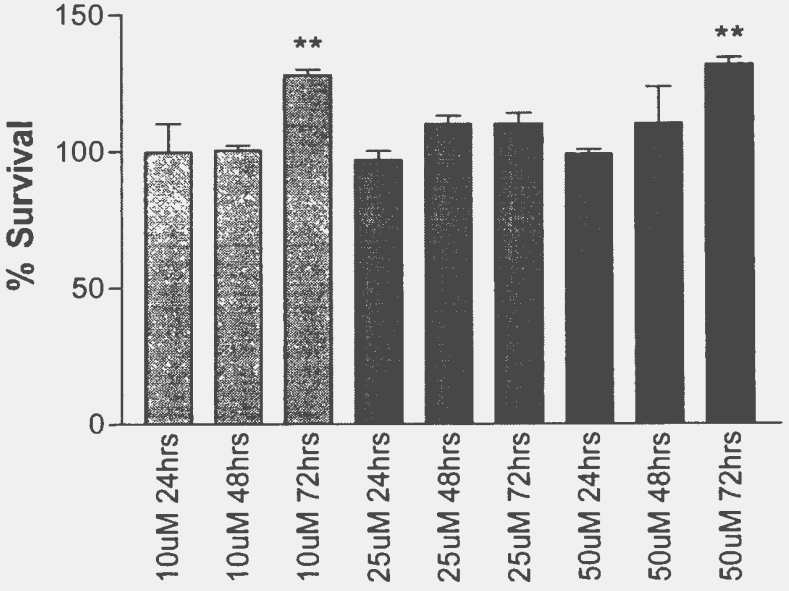

% Survival
120
100
80
60
40
20
0
*
**
**
**
**
**
25 uM 24hrs
25 uM 48hrs
25 uM 72hrs
50 uM 24hrs
50 uM 48hrs
50 uM 72hrs

Figure 2.4. The effect of MAP Kinase inhibition on cell survival. Cells were treated with medium containing the indicated concentrations of U0126 in the presence (A) or absence (B) of 50 ng/mL NGF. At 24 h, 48 h and 72 h after addition of the inhibitors, survival was assessed by the MTS Live-Dead assay (as outlined in Material and Methods). Data are expressed as percentages of the controls (wells with no inhibitor added) (mean ± SEM, n=3-5). **$p<0.001$.

A


150
100
50
0
% Survival
**
**
10uM 24hrs
10uM 48hrs
10uM 72hrs
25uM 24hrs
25uM 48hrs
25uM 72hrs
50uM 24hrs
50uM 48hrs
50uM 72hrs

B

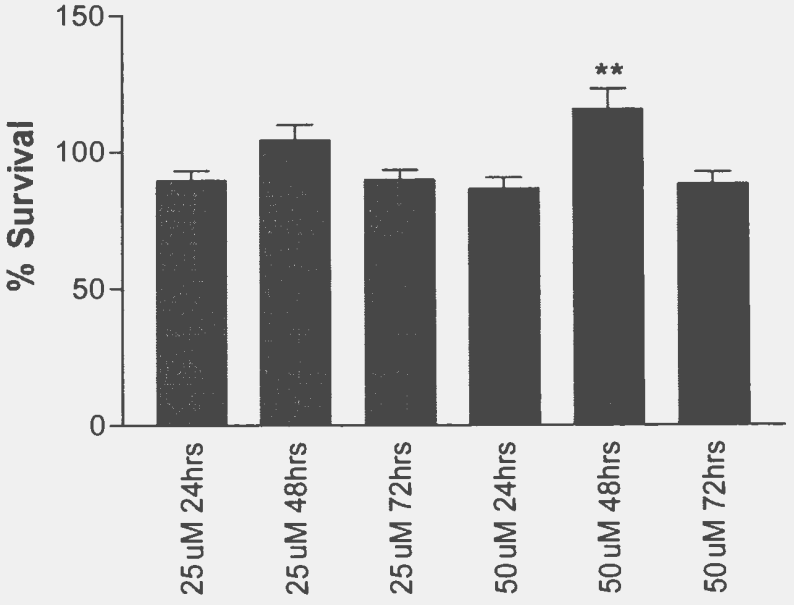
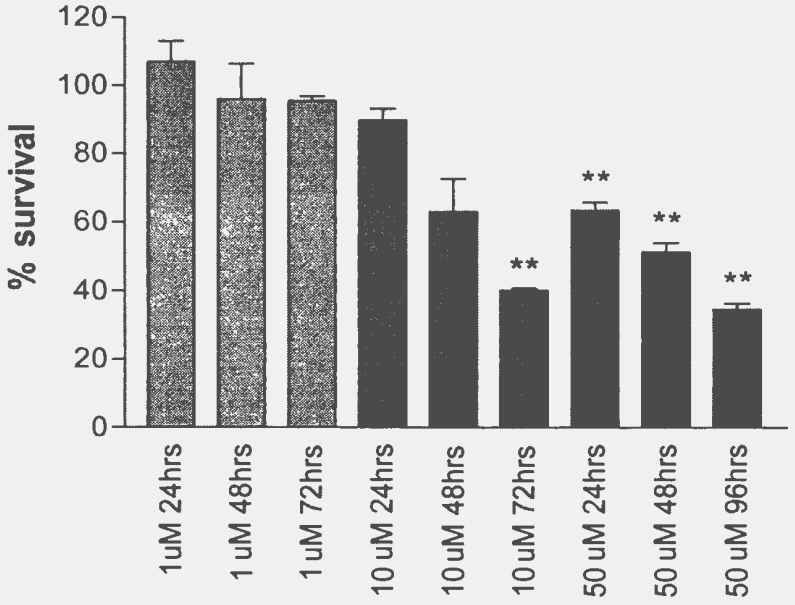
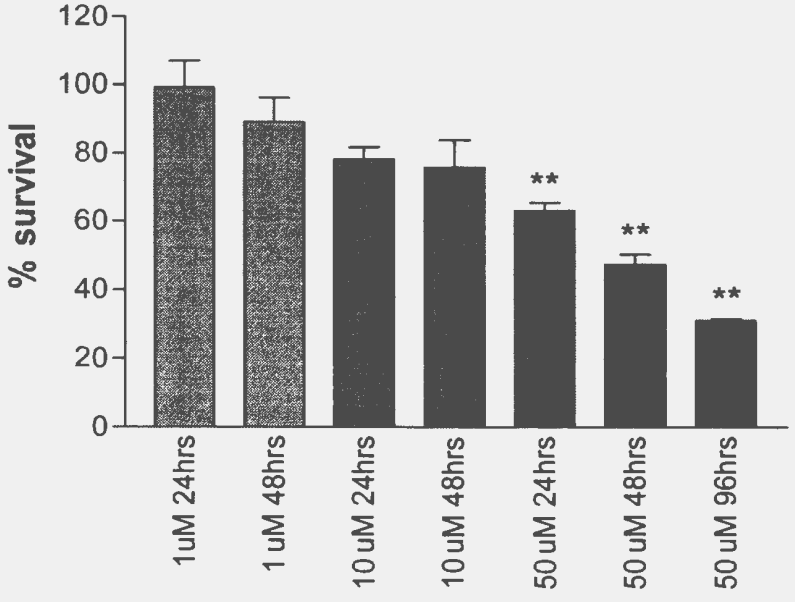

150
100
50
0
% Survival
**
25uM 24hrs
25uM 48hrs
25uM 72hrs
50uM 24hrs
50uM 48hrs
50uM 72hrs

Figure 2.5. The effect of PKC inhibition with chelerythrine on cell survival. Cells were treated with medium containing the indicated concentrations of chelerythrine chloride in the presence (A) or absence (B) of 50 ng/mL NGF. At 24 h, 48 h, 72 h and 96 h after addition of the inhibitors, survival was assessed by the MTS Live-Dead assay (as outlined in Material and Methods). Data are expressed as percentages of the controls (wells with no inhibitor added) (mean ± SEM, n=3-5). **$p<0.001$.

A


% survival
120
100
80
60
40
20
0
**
**
**
**
1uM 24hrs
1 uM 48hrs
1 uM 72hrs
10 uM 24hrs
10 uM 48hrs
10 uM 72hrs
50 uM 24hrs
50 uM 48hrs
50 uM 96hrs

B


% survival
120
100
80
60
40
20
0
**
**
**
1uM 24hrs
1 uM 48hrs
10 uM 24hrs
10 uM 48hrs
50 uM 24hrs
50 uM 48hrs
50 uM 96hrs

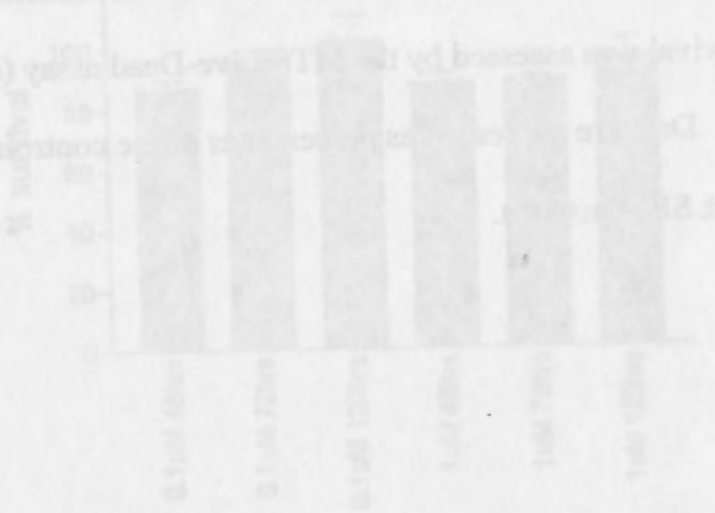
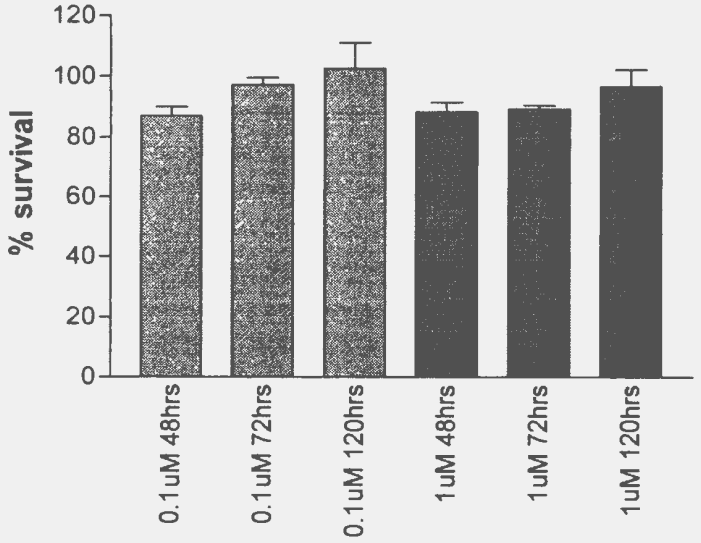


Figure 2.6. The effect of other PKC inhibitors on cell survival. Cells were treated with medium containing the indicated concentrations of Bisindolylmaleimide I (A), or Rottlerin (B), in the absence NGF. At 48 h, 72 h and 120 h after addition of the inhibitors, survival was assessed by the MTS Live-Dead assay (as outlined in Material and Methods). Data are expressed as percentages of the controls (wells with no inhibitor added) (mean ± SEM, n=3-5).

A


% survival
120
100
80
60
40
20
0
0.1uM 48hrs
0.1uM 72hrs
0.1uM 120hrs
1uM 48hrs
1uM 72hrs
1uM 120hrs

B

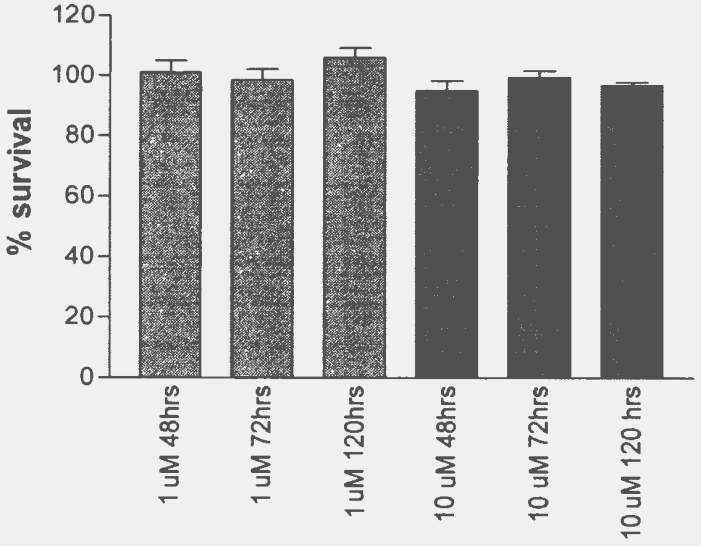

% survival
120
100
80
60
40
20
0
1 uM 48hrs
1 uM 72hrs
1uM 120hrs
10 uM 48hrs
10 uM 72hrs
10 uM 120 hrs

Figure 2.7. The involvement of other intermediates in neuronal survival. Cells were infected with a RasN17 adenovirus as outlined in the Methods and survival assessed at 48 h, 72 h and 96 h after infection. In addition, the effect of LiCl alone on survival was assessed (B), and in combination with either RasN17 (C) or LY294002 (D). Data are expressed as percentages of the controls (wells with no inhibitor added in (B, D) or with control adenovirus added (A, C) (mean ± SEM, n=3-5). *$p<0.05$; **$p<0.001$.

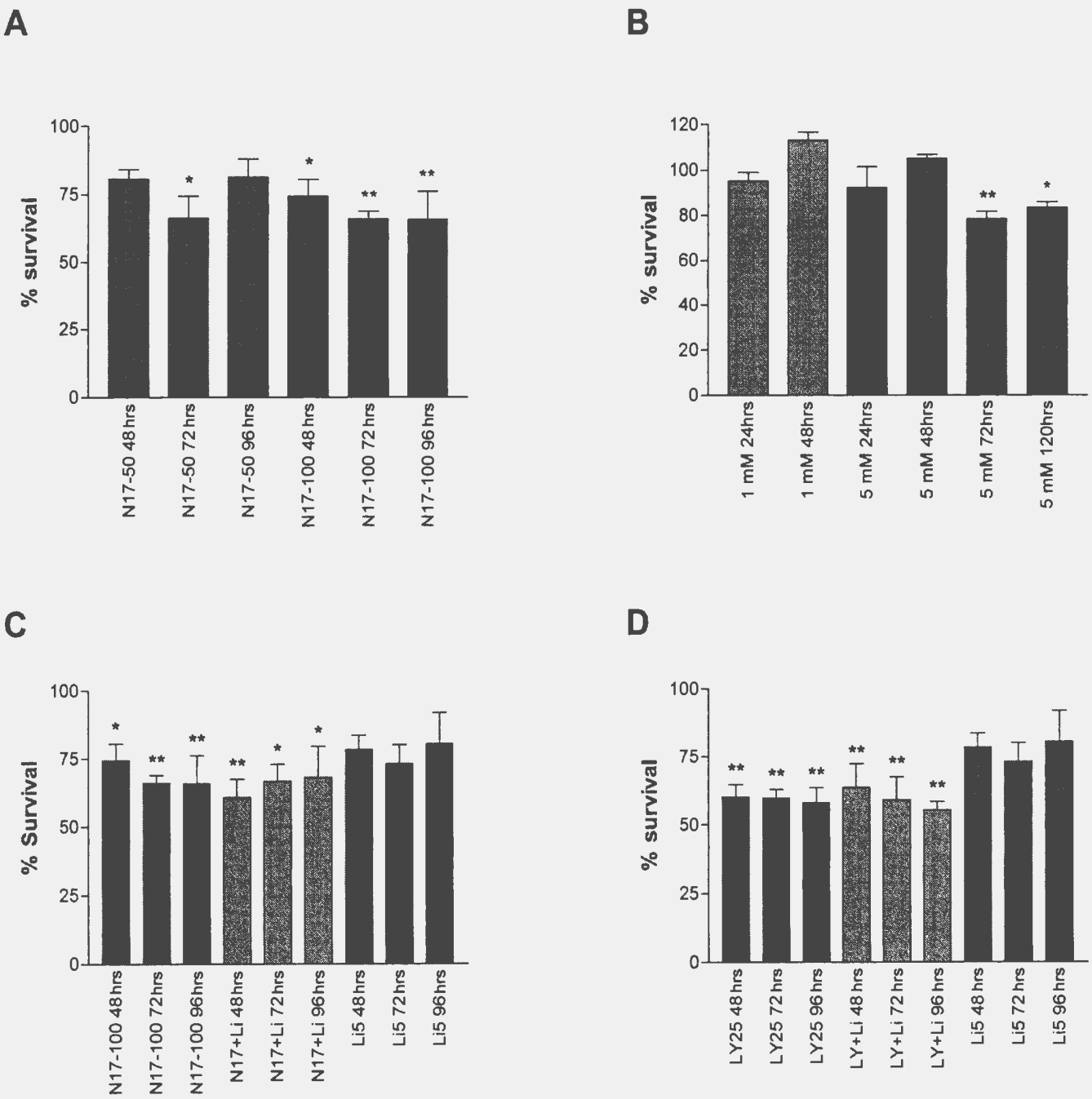
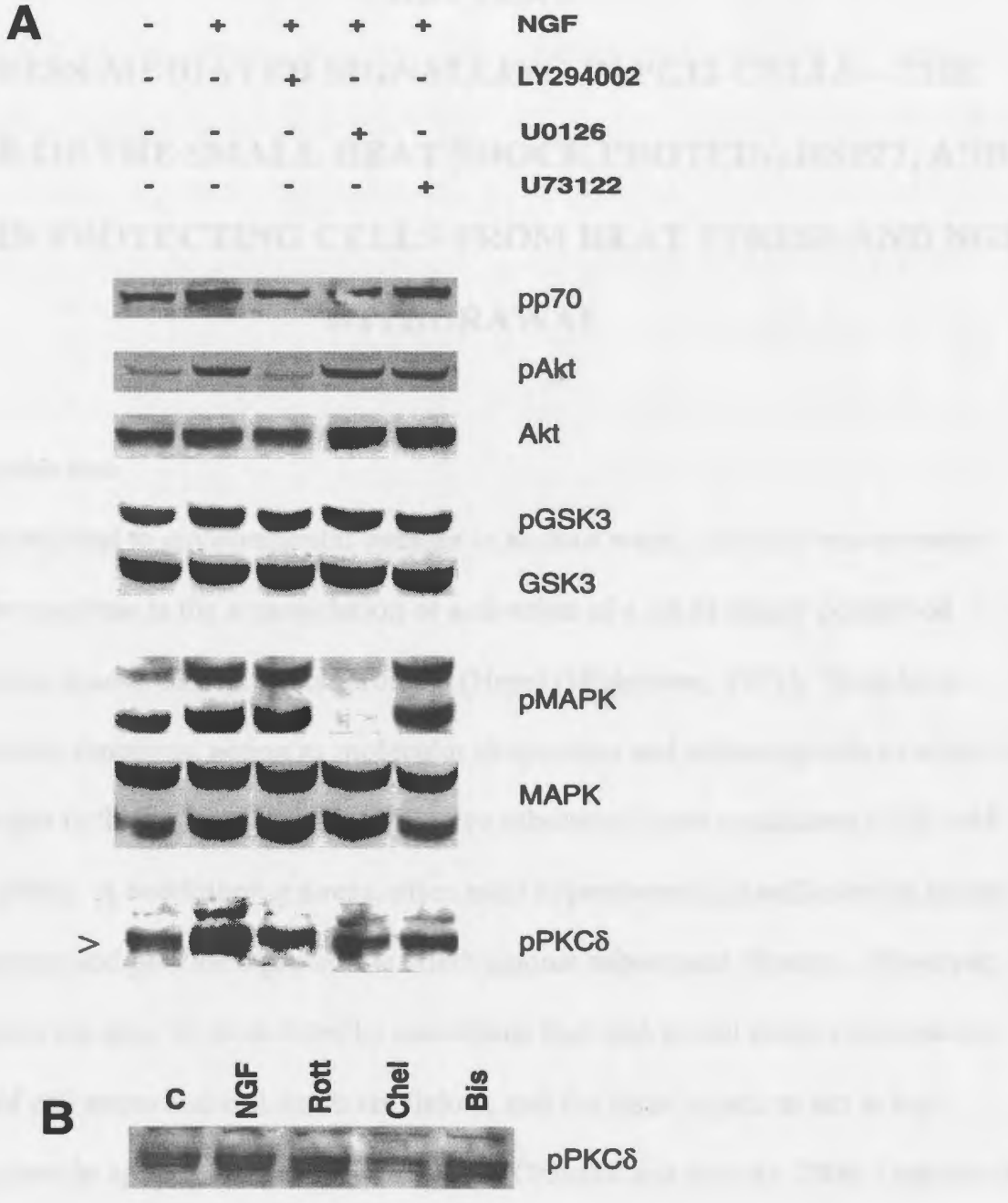

A
% survival
100
75
50
25
0
N17-50 48 hrs
N17-50 72 hrs
N17-50 96 hrs
N17-100 48 hrs
N17-100 72 hrs
N17-100 96 hrs
B
% survival
120
100
80
60
40
20
0
1 mM 24hrs
1 mM 48hrs
5 mM 24hrs
5 mM 48hrs
5 mM 72hrs
5 mM 120hrs
C
% Survival
100
75
50
25
0
N17-100 48 hrs
N17-100 72hrs
N17-100 96hrs
N17+Li 48 hrs
N17+Li 72 hrs
N17+Li 96 hrs
Li5 48 hrs
Li5 72 hrs
Li5 96 hrs
D
% survival
100
75
50
25
0
LY25 48 hrs
LY25 72 hrs
LY25 96 hrs
LY+Li 48 hrs
LY+Li 72 hrs
LY+Li 96 hrs
Li5 48 hrs
Li5 72 hrs
Li5 96 hrs

Figure 2.8. The effect of inhibitors on the activation of signalling intermediates. Cells were treated for one hour with 25 μM LY294002, 10 μM U0126, 10 μM Chelerythrine chloride, 1 μM Bisindolylmaleimide I, 10 μM Rottlerin, or 10 μM U73122 and were sampled immediately after a 15 min NGF (50 ng/mL) stimulation. Equivalent amounts of protein were then electrophoresed and the resulting blots probed with the indicated antibodies. A) Effects of NGF, LY294002, U0126, and U73122 on levels of expression and activation of PI 3-K, MAPK, and PKC pathway intermediates. NGF stimulation results in increased phosphorylation of all intermediates. Treatment with the individual inhibitors results in the blocking of this phosphorylation; eg., LY294002 inhibits phosphorylation of Akt and p70. U0126 blocks activation of MAPK and all inhibit the phosphorylation of PKCδ. In (B) the effects of selective PKC inhibitors on the phosphorylation of PKCδ are presented.


A
- + + + + NGF
- - + - - LY294002
- - - + - U0126
- - - - + U73122
pp70
pAkt
Akt
pGSK3
GSK3
pMAPK
MAPK
pPKCδ
B
C
NGF
Rott
Chel
Bis
pPKCδ

*CHAPTER 3

STRESS-MEDIATED SIGNALLING IN PC12 CELLS – THE ROLE OF THE SMALL HEAT SHOCK PROTEIN, HSP27, AND AKT IN PROTECTING CELLS FROM HEAT STRESS AND NGF WITHDRAWAL

3.1 Introduction

Cells respond to environmental stresses in various ways, although one common feature of the response is the accumulation or activation of a set of highly conserved cellular proteins known as heat shock proteins (Hsps) (Hightower, 1991). Hsps have strong protective functions, acting as molecular chaperones and allowing cells to adapt to gradual changes in their environment and survive otherwise lethal conditions (Jolly and Morimoto, 2000). A conditioning stress, often mild hyperthermia, is sufficient to induce the Hsp response and provide a protective effect against subsequent stresses. However, Hsp expression can also be modulated by conditions that lead to cell death or apoptosis. The events of cell stress and cell death are linked, and the Hsps appear to act at key regulatory points in apoptosis signalling cascades (Ohtsuka and Suzuki, 2000; Garrido *et al.*, 2001).

Exposure of cells to a mild or sublethal stress appears to protect against a subsequent challenge that is, by itself, lethal. So for example, a mild thermal stress has

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* **This chapter has been published in:** Mearow, K.M., Dodge, M.E., Rahimtula, M., Yegappan, C. (2002) Stress-mediated signalling in PC12 cells – the role of the small heat shock protein, Hsp27, and Akt in protecting cells from heat stress and NGF withdrawal. *J Neurochemistry*, 83: 452-62.

been shown to protect neuronal cells against subsequent insults (Mailhos *et al.*, 1994). Hsp70 and Hsp90 appear to be the major proteins induced by stress in the nervous system and their neuroprotective roles have been demonstrated both *in vivo* and *in vitro* (Mailhos *et al.*, 1994; Amin *et al.*, 1995; Lee *et al.*, 2001a; Lee *et al.*, 2001b). In addition, a family of small Hsps, which include the Hsp27 and αB-crystallin, has also been shown to be upregulated by stresses. Hsp27 clearly has protective effects in a variety of non-neuronal cells, and it is becoming clear that it has similar effects in certain classes of neurons as well (Landry *et al.*, 1989; Huot *et al.*, 1996; Costigan *et al.*, 1998; Lewis *et al.*, 1999; Wagstaff *et al.*, 1999; Charette *et al.*, 2000). In the CNS, expression of Hsp27 is primarily glial although it is expressed in some neuronal populations, and upregulation of expression has been observed in several different experimental models including hyperthermia and nerve injury (Hopkins *et al.*, 1998; Bechtold and Brown, 2000; Krueger-Naug *et al.*, 2000). Hsp27 is developmentally regulated in peripheral DRG neurons, and it has been suggested that this may play a role in the decreased vulnerability of mature DRG neurons to heat shock or trophic factor withdrawal (Costigan *et al.*, 1998; Lewis *et al.*, 1999; Wagstaff *et al.*, 1999).

Despite the clear protective influences of the Hsps in neural cells, the mechanisms underlying this effect are not at all clear. Cellular stresses that result in the induction of Hsps also result in the activation of particular signal transduction pathways. The classical stress-activated pathway involves activation of members of the MAP kinase family like the p38 MAPK family (Mielke and Herdegen, 2000). Activation of MAPKAP-K2 by p38 MAPK results in the phosphorylation of Hsp27 (Stokoe *et al.*, 1992; Huot *et al.*, 1995),

which may or may not be required for its protective influence (Lavoie *et al.*, 1995; Preville *et al.*, 1998). However, in addition, thermal or oxidative stress results in the rapid phosphorylation of Akt, a key component of survival-promoting signalling in a wide variety of cell types (Konishi *et al.*, 1997; Shaw *et al.*, 1998; Bijur and Jope, 2000). The interaction between these different signal transduction pathways and the increased expression and protective influence of Hsps are not well understood.

In our efforts to understand more about the role of small Hsps and the signalling pathways activated by extracellular stresses, and why some stresses result in cell death and others convey some measure of tolerance, we have used PC12 cells to examine the protective role of Hsp27 and the contributions or requirement for signalling via p38 MAPK or Akt in this protection. We have used PC12 cells, and a stable cell line expressing an Hsp27-EGFP fusion protein, and observed the effects of this overexpression on both cell survival and biochemical signalling after heat stress and NGF withdrawal. We show that the fusion protein is functional and can protect NGF-differentiated cells from NGF withdrawal-induced apoptosis and also from heat and oxidative stress. We have examined the potential role of p38 MAPK and Akt signalling pathways in the cellular response of the PC12 cells to these stresses and how these interact with Hsp27. Our results indicate that Akt is activated rapidly after heat or oxidative stress, followed by p38 activation. Hsp27 is phosphorylated after the stress and its expression is increased within 3 h after these stresses. Furthermore, we provide evidence for an immunoprecipitable complex formed by Akt, p38 and Hsp27, that appears to be constitutive in PC12 cells.

3.2 Materials and Methods

3.2.1 Reagents

Reagents used in the lysis buffer included glycerol, Nonidet P-40, sodium fluoride and sodium vanadate, PMSF, all obtained from Sigma Chemicals (St. Louis, MO). Protease inhibitor tablets were obtained from Roche Diagnostics (Laval, QC). U0126, LY294002, SB203580 were obtained from CalBiochem (San Diego, CA). The expression vector, pEGFP-C2 was from Clontech Laboratories (Palo Alto, CA), while the hamster Hsp27 cDNA was a kind gift of Dr. J. Landry (Universite de Laval, Quebec City, Quebec, Canada). Lipofectamine 2000 and all cell culture media and supplements were from Invitrogen Corp. (Mississauga, Ont.). The PC12 cell line was originally obtained from the American Tissue Type Collection (ATCC, Manassas, VA.), and maintained in DMEM with 10 % FCS.

3.2.2 Culture and Treatment of Cells

PC12 cells were transfected with the Hsp27/EGFP cDNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen Corp, Burlington, Ont.). The efficiency of positive transfection was estimated from the percentage of green fluorescing live cells to be approximately 40 %. Subsequently, stable transfectants were selected with G418 (Geneticin, 15 ug/ml, Invitrogen). The cell line selected was termed HSPC cells. In experiments, both the parental PC12 line and the HSPC cells were used for comparison. In some experiments, we also used transient transfectants of Hsp27 for comparison with the PC12 cells.

The HSPC and PC12 cells (ATCC, Manassas, VA) were grown at 37°C in DMEM (Invitrogen Corp) supplemented with 10 % fetal bovine serum and penicillin-streptomycin (100 U/ml) in a humidified atmosphere of 95 % air, 5 % CO2. Cells were seeded in either 6- or 12-well plates for experimental manipulations.

For heat shock treatment, medium on the cells was changed to fresh medium 1 h prior to the treatment, and then the plates were exposed to a 45°C water bath for 15 min; in some experiments the cells were pre-incubated in the inhibitors for 1 h prior to heat shock. Subsequently, cells were sampled either within 5 min of heat shock, or allowed to recover at 37°C for various periods of time. At the time of sampling, medium was aspirated from the cells and the cells washed with TBS (tris-buffered saline, pH 7.5) and collected in TBS after scraping with a rubber policeman. Following pelleting of the cells, the cell pellet was suspended in protein lysis buffer (1 % NP40, 10 % glycerol in TBS plus protease inhibitors, 1 mM sodium vanadate, and sodium fluoride). After cell lysis and centrifugation of the lysate at 10,000rpm for 5 min, the supernatants were used to determine protein concentrations using the BCA protein assay (Pierce Chemicals, Rockford, IL.).

3.2.3 Western Blot Analyses

Equivalent amounts of protein (50 ug) were subjected to SDS-PAGE (10 % or 7.5-15 % gradient gels) (Kimpinski and Mearow, 2001). Following transfer to nitrocellulose, the blots were first stained with Ponceau Red to assess the equivalency of protein loading. Following washing with TBS, the blots were subsequently probed with antibodies to Hsp27 (Stressgen, Victoria, BC, or Santa Cruz Biotech, Santa Cruz, CA),

GFP (Santa Cruz Biotech), phosphospecific Akt, phospho-p38 MAPK, Akt, p38 MAPK (NEB/Cell Signalling, Beverley, MA).

We also employed two different antibodies that recognized phospho-Hsp27 – a sheep polyclonal pHsp27 antibody, specific for phosphorylation on the Ser15 site of human Hsp27 was obtained from UBI (Lake Placid, NY), while a second phospho-Hsp27 against the consensus sequence of human Hsp27 was obtained from Santa Cruz Biotech. The latter antibody is reported to also recognize rodent pHsp27; the peptide sequence used for both antibodies differs from the rat and hamster Hsp27 sequence by 2 amino acids. Blots were visualized with ECL reagents (NEN, Boston, MA) and exposure to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC). Blots were digitized with a Cyclone ChemiImager and composite figures prepared using Adobe Photoshop graphics software.

For IEF gels, 15 ug of total protein was diluted in glycerol and electrofocused on precast IEF gels (Ready Gel IEF gels, BioRad Laboratories, Montreal, QC). Gels were run at 100 V for 1 h, 250 V for 1 h and 500 V for 30 min. Gels were then transferred to nitrocellulose in 0.7 % acetic acid at 100 V for 1 h, and the resulting blots probed with anti-Hsp27 (Stressgen Corp). Estimation of pI was determined from IEF standards.

3.2.4 Immunoprecipitation

Immunoprecipitation was carried out using a standard protocol. Equivalent amounts of protein (100 ug) lysates were first pre-cleared with protein A-agarose (Roche Diagnostics) for 1 h at 4°C. The lysates were then incubated with the precipitating antibody (1-5 ug of anti-Hsp27, Stressgen Corp) for 1-2 h at 4°C, followed by addition of

protein A-agarose beads (25 µl of a 1:1 slurry of beads to lysis buffer) and overnight incubation at 4°C. After centrifugation and removal of the supernatant, the IP complexes were washed 3 times with 5x volume of lysis buffer, followed by one final wash with TBS and removal of the wash solution. 50 µl of 2x Laemmli SDS sample buffer were added to the samples, and after heating at 95°C for 4 min, SDS-PAGE (10 % acrylamide) was carried out followed by Western blot analyses with the appropriate antibodies. In several experiments, the supernatant was also subjected to Western blotting to confirm complete IP of the Hsp27 from the lysates (data not shown).

3.2.5 Immunocytochemistry

PC12 and HSPC cells were plated and grown in 16-well chamber slides (Nunc, VWRCanlab, Mississauga, Ont). For immunocytochemistry, the medium was aspirated and the cells fixed with 4 % paraformaldehyde in PBS (phosphate buffered saline), permeabilized and blocked with 0.1 % triton X-100, in TBS with 3 % BSA and subsequently incubated in the primary antibodies overnight at 4°C. Antibodies used were Hsp27 (rabbit polyclonal, Stressgen Corp) and phospho-Hsp27 (UBI). Secondary antibodies labeled with Cy2 or Cy3 (Jackson Immunoresearch Labs, West Grove, PA) or with FITC (Santa Cruz) and conventional fluorescence or scanning laser confocal microscopy were used to visualize immunolabeled cells. For double labeling, cells were first exposed to the pHsp27 primary antibody overnight at 4°C, followed by the appropriate secondary antibody and washing; the second primary antibody (the Hsp27 antibody) was incubated for 2 h at 37°C, followed by the appropriate secondary antibody.

The cells were coverslipped with glycerol for microscopy. Digital images were collected and composite figures prepared using Adobe Photoshop.

3.2.6 MTS Cell Survival Assay

Cell survival was assayed using the Cell Titer 96 Aqueous Assay (Promega Corp, Madison, WI) according to manufacturer's protocol. Medium volume in 96-well plate cultures was adjusted to 100 µl, and 10 µl/well of the MTS (3-(4,5-dimethylhiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-suflophenyl)-2H-tetrazolium) and PMS (phenazine methosulfate) solution added. Plates were incubated at 37°C for 2 h and the absorbance of the soluble formazan product read at 490 nm with an ELISA plate reader.

3.2.7 Statistical Analysis

Data were analyzed for significance using one-way ANOVA, followed by post-hoc testing with Dunnett's or Bonferroni's test for multiple comparisons. Data were expressed as percentages relative to control values (which were taken to represent 100 %). All experiments were replicated a minimum of 3 times, although the IP experiments were performed 5 times.

3.3 Results

3.3.1 Overexpression of Hsp27-EGFP fusion protein in PC12 cells

A cDNA for hamster Hsp27 (Lavoie *et al.*, 1990) was subcloned into the Hind III site of the pEGFP-C2 cloning vector. Following determination of the correct orientation

of insertion, the new construct (Hsp27-EGFP) was transfected into PC12 cells using Lipofectin 2000. The efficiency of transfection was approximately 40 %, as determined by the percentage of brightly fluorescing green cells and subsequently, a stable cell line (HSPC) was selected with geneticin (G148) (Figure 3.1A). As observed with Western blotting analyses these cells expressed a fusion protein of approximately 52 kD (Hsp27 fused to EGFP), which could be identified with either antibodies to Hsp27 or GFP, in addition to the endogenous Hsp27 (Figure 3.1B). In addition, in some experiments, transiently transfected PC12 cells were employed as a comparison with the stable transfectants.

3.3.2 Exposure of PC12 and HSPC cells to heat stress

Cultures of both PC12 and HSPC cells were exposed to a heat stress of 45°C for 15 min, and samples collected 5 min, 1 h, 3 h, 6 h and 24 h after the heat treatment. Equivalent amounts of protein (50 ug) were electrophoresed and probed with antibodies to Akt, p38 MAPK and Hsp27 (Figure 3.2). As shown in Figure 3.2, activation of Akt (assessed by the phospho-specific S473 Akt antibody) occurred rapidly and has already peaked within minutes of the end of the heat stress. In the PC12 cells (Figure 3.2A), this phosphorylation decreased within 1 h and returned to control levels by 3 h. In contrast, phosphorylation of Akt was prolonged in the HSPC cells (Figure 3.2B). Quantitation of the results of several different experiments (n=4-6) indicates that the pAkt levels in the PC12 cells were back to control values by 3 h, while those of the HSPC cells were maintained at significantly increased levels for more than 6 h after the heat shock (Figure

3.2C, pAkt panel, $p< 0.001$). However, the total Akt levels did not differ significantly between the PC12 and the stable or transient transfectants.

There was also a modest increase in phosphorylated p38 MAPK (Figure 3.2A, B), which remained elevated above control levels in both the PC12 and HSPC cells (Figure 3.2C, pp38 panel, $p<0.05$).

With respect to Hsp27, in both the PC12 (Figure 3.2A) and the HSPC (Figure 3.2B) cells there was an upregulation of Hsp27 expression by 3 h after the stress, which continued to increase over the 24 h period (Figure 3.2C, Hsp27 panel, $p<0.001$).

We also examined the influence of several pharmacological inhibitors on the activation of Akt and p38 by heat shock, and representative results are shown in Figure 3.3. In these experiments, the cells were sampled after a 1 h recovery period at 37°C. In the absence of the heat shock, there was little phospho-Akt in either the PC12 (Figure 3.3A) or HSPC cells (Figure 3.3B), which was increased after the heat shock. In the PC12 cells, there was also phosphorylation of p38 observed after the heat shock, while in the HSPC cells there was detectable p-p38 in both the control and heat shocked cells. In the presence of the PI3-kinase inhibitor, LY294002 (10 μM), there was no phosphorylation of Akt detected, indicating that the response requires the upstream activation of PI3-K. The p38 MAPK inhibitor, SB203580 (5 μM), resulted in a modest depression of the activation of p38 MAPK after the heat shock in the PC12 cells, although there was less of an influence on the HSPC cells after heat shock. It should be noted that this inhibitor has an influence on the activity of p38 rather than on its activation. We also

employed the MEK inhibitor, U0126 as an internal control for the effects of the inhibitors, and it had little influence on the parameters examined.

3.3.3 HSP27 is phosphorylated with stress and this results in nuclear localization

Hsp27 is known to be phosphorylated by MAPKAP-K2 subsequent to the activation of p38 MAPK. Most efforts to detect phosphorylation have relied on either isoelectric focusing (IEF) or gel chromatography. We attempted to detect differential phosphorylation of Hsp27 using IEF gels, and found with HS that 3 species were detectable, compared to the 1-2 species in the control specimens (Figure 3.4A); pI values were estimated to be 4.5, 5.1 and 6 using IEF standards, similar to results for other cell types (Landry *et al.*, 1991). In the unstimulated HSPC cells (Figure 3.4B), there were 3 species also detectable, suggesting that overexpression may result in constitutive phosphorylation, perhaps due to decreased dephosphorylation by phosphatases. In addition we were able to detect pHsp27 using a phospho-specific antibody that recognizes the consensus sequence around the Ser15 site in human Hsp27. In order to determine whether this antibody would also recognize the rodent pHsp27-ser15, we used human HEK293 and HeLa cells and compared the response after heat shock to that from PC12 cells. As shown in Figure 3.4C, this antibody is able to recognize both human and rat phosphorylated Hsp27. Furthermore, it is also able to recognize the hamster Hsp27-fusion protein as seen in Figure 3.4D.

Following Hsp27 phosphorylation, it has been reported that the cellular distribution of Hsp27 changes, with nuclear or perinuclear localization being observed

(Arrigo *et al.*, 1988; Nakatsue *et al.*, 1998; Geum *et al.*, 2002). We have used immunocytochemistry with the phospho-Hsp (pHsp)- specific antibody and confocal laser scanning microscopy to examine whether these sorts of changes also occur in PC12 cells and whether there was any difference between the PC12 and the HSPC cells (Figures 3.5A-I, PC12 cells, and Figures 3.5 J-O, HSPC cells). In PC12 cells, in the control conditions most of the pHsp was cytosolic (Figures 3.5A-C), although after HS differential localization was observed (Figures 3.5 D-I). In cells fixed 5 min after the end of the 15 min HS, there was a clear increase in the pHsp staining, both cytoplasmic and with increased nuclear staining (Figures 3.5E, F); there was little or no non-phosphorylated Hsp27 observed in the nucleus (Figures 3.5D, F). After a 1 h recovery period, there was a redistribution in many cells, with pHsp27 localization now being most prominent in the peripheral cytoplasm (Figures 3.5H, I).

In the HSPC cells, we observed that even in the control conditions, there was some nuclear punctate staining for pHsp (Figures 3.5J-L), but we could also detect distinct perinuclear localization in some cells after a 1 h recovery period (Figures 3.5M-O). We suggest that the increased expression of Hsp27 in these cells results in increased phosphorylation and a redistribution of Hsp27 in a manner that allows the cells to respond more readily to potentially lethal stresses.

3.3.4 Hsp27 forms immunoprecipitable complexes with Akt and p38 MAPK

Because of a report indicating that Hsp27 could bind to Akt (Konishi *et al.*, 1997), we undertook immunoprecipitation analyses. For these experiments we used the PC12 cells primarily, as we found that the presence of both the fusion protein and the

endogenous Hsp27 in the HSPC cells made the interpretation of the results somewhat confusing due to the similar sizes of the fusion protein and the IgG heavy chain. We used anti-Hsp27 (Stressgen Corp) for immunoprecipitation, followed by Western blotting with Hsp27, Akt and p38 MAPK antibodies. The immune complexes were electrophoresed and the blots probed with a series of antibodies. As shown in Figure 3.6A, both p38 and Akt were co-immunoprecipitated with the Hsp27. In the lysates from the heat shocked cells, both phospho-Akt and phospho-p38 were detectable; we were unable to detect JNK/SAPK, MAPKAP-K2 or MAPK in these complexes. By 6 h after the stress, the amount of Akt and p38 associated with Hsp27 has decreased. The time course of the pAkt detection seems to be different from that observed with the Western blots (Figure 3.2) where phospho-Akt detected in the total cellular lysates is decreased back to control levels by 3h. However, one possible explanation for this may be that the Akt complexed with the Hsp27 is protected from dephosphorylation and thus in the immunoprecipitates pAkt is detected over a somewhat prolonged time course (see Discussion).

We also examined the effect of treatment with SB203580 (an inhibitor of p38 MAPK activity) on complex precipitation in order to determine whether p38-dependent phosphorylation of Hsp might play a role in the complex formation or dissociation. As shown in Figure 3.6B, there was little observable difference at the early time point, although at the 60 min recovery time point there was less pAkt and Akt, as well as pHsp detectable in the immunoprecipitates. This suggests that p38 activity associated with phosphorylation of Hsp27 may play a role in complex formation, although further investigation is required to confirm this possibility.

3.3.5 HSPC cells are more resistant to NGF-withdrawal

PC12 cells can be differentiated into neuron-like cells by treatment with NGF in low serum or serum-free medium. The differentiated cells become flattened and put out neuritic-like processes. Once differentiated, these cells rely on the presence of NGF for their continued survival; if the NGF is washed out of the medium (and/or neutralized by the use of anti-NGF antisera), the PC12 cells will undergo apoptosis, which is almost complete by 72 h post-NGF withdrawal.

Control PC12 and HSPC cells were treated with NGF to induce differentiation and were cultured in the presence of NGF for 7 d. NGF was removed from the cells and the extent of apoptosis was assessed over the subsequent 72 h. Two methods of assessing cell death were employed. In the first, a colorimetric cell survival assay in which the optical density (OD) values correlate with number of viable cells was used. Figure 3.7 presents the results of the MTS assay experiments. It is clear that the HSPC cells were less vulnerable to the effects of NGF withdrawal than the non-Hsp overexpressing PC12 cells. Additionally, control and experimental cultures were subjected to propidium iodide (PI) staining 24 and 48 h after NGF withdrawal, and the results corresponded with those of the MTS assays (data not shown).

3.3.6 Signalling pathways activated by NGF withdrawal

We then examined the effects of NGF withdrawal on the Akt, p38, and Hsp27 expression. Representative results are shown in Figure 3.8. Interestingly, the presence or absence of NGF did not appear to have any detectable influence on the expression of Hsp27 (Figure 3.8A). Phospho-Akt and p38 were increased after NGF withdrawal in

both the PC and HSPC cells (Figure 3.8A). In the PC12 cells, there was a decrease in detectable pAkt by 48 h, although in the HSPC cells the levels remained elevated. This maintained phosphorylation could be a contributing factor to the prolonged survival of the HSPC cells after NGF withdrawal. As in the undifferentiated cells (see Figure 3.2), the levels of total Akt did not differ between the PC and HSPC cells, although as might be expected in NGF-treated cells, the pAkt levels were higher in both cell types in these experiments compared to non-NGF-treated cells.

We also examined poly (ADP-ribose) polymerase (PARP) as a marker of potential apoptosis; this protein can be cleaved by activated caspases to yield 89 and 24 kD cleavage products. The antibody we used detects both the uncleaved 115 kD and the 89 kD cleavage product. We found that total PARP levels were similar in both cell types, but there were increased amounts of the 85 kD fragment of PARP in the PC12 cells compared to the HSPC cells after NGF withdrawal. However, since there is still significant cell death in the HSPC cells, especially at the later time points (see Figure 3.7) it is not surprising that there were detectable levels of cleaved caspase 3 and PARP.

We also carried out experiments with transiently transfected PC12 cells. The cells were differentiated with NGF prior to transfection and the NGF-withdrawal experiments carried out as described above. In these experiments we also treated the cells with the general caspase inhibitor, peptidyl inhibitor carbobenzoxy-Val-Ala-Asp-fluoromethylketone (zVADfmk) (50 μM), to determine if caspase activation played a role in the PARP cleavage and apoptosis. As shown in Figure 3.8B, the caspase inhibitor did decrease the amount of cleaved PARP detectable, along with caspase 9, and cleaved

caspase 3. There was less detectable cleaved caspase 9 and 3 in the PC12 cells overexpressing Hsp27, compared to the non-transfected PC12 cells. However, it should also be noted that there was not complete inhibition of the caspases in these experiments, nor complete blockade of PARP cleavage. This may be related to the fact that neither differentiation nor cell death in this particular model is synchronous, as well as the fact that the transient transfectants had much lower levels of Hsp27 expression, compared to the stable cell line.

These results support our observations that there was less apoptosis detectable in the Hsp27-overexpressing cells after NGF withdrawal, and further support the interpretation that Hsp27 can protect cells from NGF withdrawal-induced cell death.

3.4 Discussion

In this study we have shown that heat shock treatment of PC12 and HSPC cells results in the rapid activation of Akt and p38 MAPK. Inhibition of PI3-K blocks the activation of Akt, but has no effect on p38 MAPK, and inhibition of p38 has little influence on Akt activation. Hsp27 expression is upregulated in both cell types by 3h after heat shock. Phosphorylation of Hsp27 occurs after heat shock in both PC12 and HSPC cells, although there appears to be an increased level of phosphorylated Hsp27 in basal conditions in the HSPC cells. Heat shock also results in changes in the cellular localization of pHsp27 from primarily cytoplasmic to nuclear and perinuclear sites; the appearance of granules in the nuclei of the cells may correspond to the heat shock granules previously described in other cell types (Arrigo *et al.*, 1988). The overexpression of Hsp27 also protects differentiated HSPC cells from cell death that

normally occurs within 24-48 h after NGF withdrawal. Following NGF withdrawal, pAkt levels remain elevated in the HSPC cells compared to the PC12 cells, similar to what was observed in the non NGF-differentiated cells. Furthermore, we also observed increased PARP cleavage in the PC12 cells compared to the HSPC cells. Immunoprecipitation experiments demonstrated that Hsp27 forms a complex with Akt that also includes p38MAPK. Furthermore, heat shock did not result in a clear dissociation of the complex. Taken together with the observations that phosphorylation of Akt is maintained or prolonged with Hsp27 overexpression, it is possible that Hsp27 is protecting Akt from dephosphorylation.

Heat stress or mild hyperthermia can result in the acquisition of tolerance, the ability of cells to withstand subsequent usually lethal stresses. The mechanisms underlying this phenomenon are subject to much investigation, with tolerance usually ascribed to the increased expression of one or more Hsps (Ohtsuka and Suzuki, 2000; Garrido *et al.*, 2001). However, post-translational modifications such as phosphorylation that modulate Hsp function, as well as increased expression of Hsps are dependent upon intracellular signalling cascades that are activated in response to the various stressors (Rouse *et al.*, 1994; Lavoie *et al.*, 1995; Guay *et al.*, 1997). While p38 is activated by a range of stresses and plays a role in apoptosis (Kato *et al.*, 1999; Mielke and Herdegen, 2000), there is emerging evidence for its involvement in processes that could contribute to survival. One target of p38 is MAPKAP-K2, which phosphorylates Hsp27, phosphorylation which modulates both the chaperone activity and anti-apoptotic effects of Hsp27 (Preville *et al.*, 1998; Rogalla *et al.*, 1999; Charette *et al.*, 2000).

NGF withdrawal from NGF-dependent neural cells often results in apoptosis. Growth factor receptor activation of the PI3-K →Akt and Ras→ MAPK pathways are survival promoting, while p38 activation is associated with neuronal death following growth factor withdrawal (Xia *et al.*, 1995; Harding *et al.*, 2001). In our experiments, p-p38 was increased both by the addition and withdrawal of NGF. In addition, pAkt levels were maintained following NGF withdrawal in the overexpressing PC12 cells. Expression of the endogenous Hsp27 or overexpression of the fusion protein was not altered by NGF. We also found that Hsp27 levels in the NGF-differentiated PC12 and HSPC cells were not different from those in the non-differentiated cells. This is in contrast to what has been observed with Hsp70, wherein NGF-differentiated cells lose their ability to induce Hsp70 in response to stress (Dwyer *et al.*, 1996; Hatayama *et al.*, 1997).

However, as pointed out above, there was still cell death in the overexpressing cells (as seen in the survival assays, and the detection of caspase and PARP cleavage). This is similar to what has been recently reported for the protective effect Hsp105 in PC12 cells, where overexpression of Hsp105 resulted in 10-40 % protection in response to various stressors, but still resulted in significant cell death and significant PARP cleavage (Hatayama *et al.*, 2001).

Several mechanisms have been put forward to account for the antiapoptotic functions of Hsp27 in addition to its chaperone and actin stabilizing activities, including preventing the interaction of the Daxx with Ask1 (Charette *et al.*, 2001), preventing activation of caspases 9 and 3 (Garrido *et al.*, 1999; Pandey *et al.*, 2000), interacting with and inhibiting release of cytochrome c (Concannon *et al.*, 2001; Paul *et al.*, 2002). In our

study we saw less activation of PARP, which was accompanied by a modest decrease in caspase 9 and 3 activation (as detected by the presence of the small cleavage products) in the Hsp-overexpressing cells compared to PC12 cells after NGF withdrawal. We did not, however, undertake experiments to directly determine the influence of Hsp27 overexpression on caspase levels or activation.

While Hsp27 has been shown to exert protective influences in a number of non-neural cellular models, there have been few studies examining its effects or regulation in neural cells. Recent reports have demonstrated that viral transfer of Hsp27 also protects neuroblastoma and primary neonatal DRG neurons against lethal thermal stress, NGF withdrawal and axotomy-induced cell death (Costigan *et al.*, 1998; Lewis *et al.*, 1999; Wagstaff *et al.*, 1999). A number of studies have shown that following hyperthermia, ischemia or neural injury, Hsp27 expression is increased in the CNS in both neurons and glial cells (Bechtold and Brown, 2000; Currie *et al.*, 2000; Krueger-Naug *et al.*, 2000; Akbar *et al.*, 2001; Murashov *et al.*, 2001). However, these studies did not examine the potential mechanisms underlying these effects.

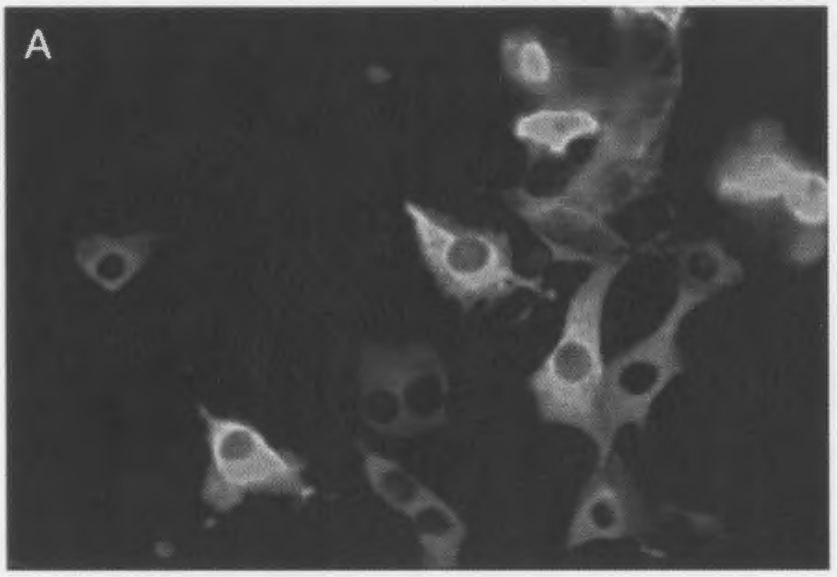
Hsp27 was previously found to form a complex with Akt after heat stress in COS cells, with gradual dissociation during the recovery period (Konishi *et al.*, 1997). These authors suggested that Hsp27 might function as a chaperone to alter the conformation of the enzyme for its activation upon stress stimulation. Further, a recent report indicates that Akt, p38 and Hsp27 form a complex in damaged motor neurons, and these authors too propose that such a complex may maintain Akt in a biologically active conformation (Murashov *et al.*, 2001), although they do not present any data demonstrating Akt activation. Akt has also been shown to be associated with the Hsp90, in such a way that

suggested that Hsp90 acts to stabilize and protect Akt (Sato *et al.*, 2000). Our results also suggest a similar role for Hsp27. Our immunoprecipitation experiments point to the possibility that Akt complexed with Hsp27 may be protected from dephosphorylation or turnover. In addition, our observation of prolonged phosphorylation of Akt in the HSPC cells after heat shock or NGF withdrawal lends more weight to the hypothesis that one role of Hsp27 may be to chaperone Akt, preventing its dephosphorylation and degradation after stress. Perhaps Hsp27 may be acting to prevent activation of proteins such as the caspases, thus inhibiting apoptosis, as well as promoting the stability and activation of Akt, which would enhance the pro-survival pathways. Clearly, further investigation of the interactions between Hsp27 and Akt and their contribution to cellular protection is required.

3.5 Figures

Figure 3.1. Generation of a stable cell line that overexpresses Hsp27/EGFP. (A) Live HSPC cells expressing the Hsp27/EGFP fusion protein are shown; cells were visualized with epifluorescence on an inverted microscope. (B) Western blots showing the expression of the endogenous Hsp27 in the parental PC12 cells (lane 1) and the presence of both the endogenous Hsp27 and the Hsp27/EGFP fusion protein in the HSPC cells (lane 2).

A



B

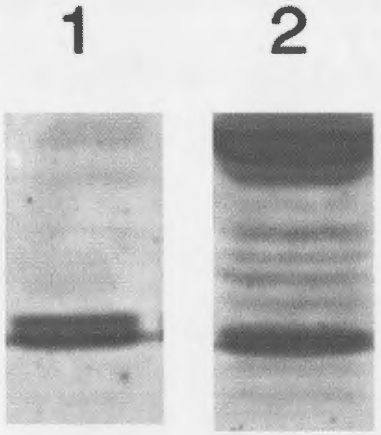
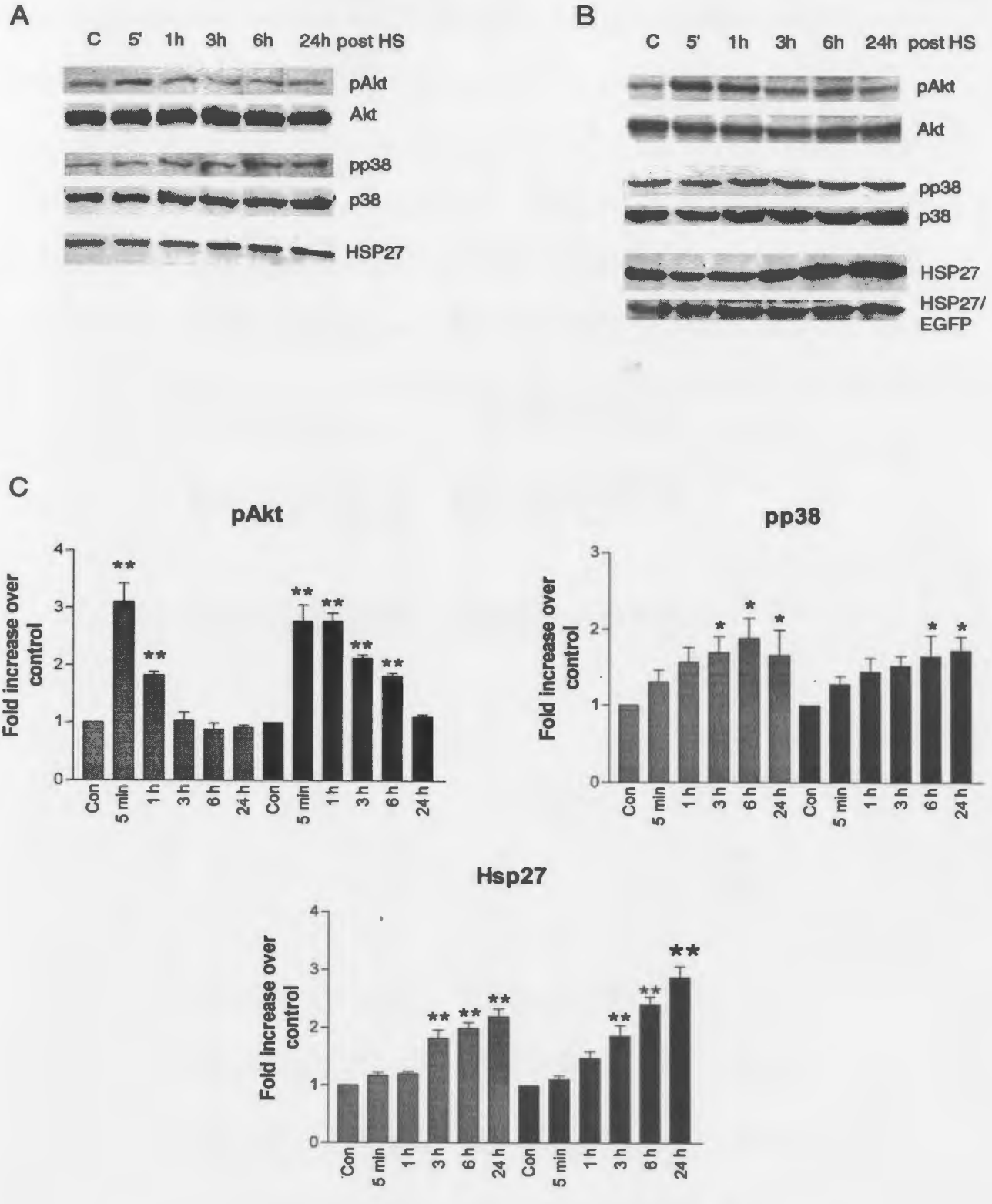


Figure 3.2. Time course of signalling activated by heat shock in PC12 and HSPC cells. Cells were subjected to a heat shock treatment (45°C, 15 min) and subsequently sampled at the indicated times following recovery at 37°C. Lysates (50 ug of total protein) were immunoblotted with the indicated antibodies. Panel A displays the lysates from the PC12 cells and panel B those from the HSPC cells. pAkt refers to Akt phosphorylated at the Ser473 residue, pp38 refers to p38 MAPK phosphorylated at Thr180/Tyr182, while Akt refers to total Akt and p38 refers to p38. The Hsp27/EGFP fusion protein was detected with both the Hsp27 antibody, and anti-GFP antibody (shown here). (C). Blots from several experiments (n=3-5) were subjected to densitometry. The phospho-specific data for pAkt and pp38 were first normalized to the total Akt and p38 for each condition and then the normalized data expressed relative to the control conditions in each experiment. For Hsp27, the experimental data are expressed relative to the control values. In these experiments, data from the HSPC stable cell line as well those from several transient transfection experiments were used for quantitation. Data are expressed as the mean ± SEM. Light bars – PC12 cells; Dark bars – HSPC cells. * $p < 0.05$; ** $p < 0.001$.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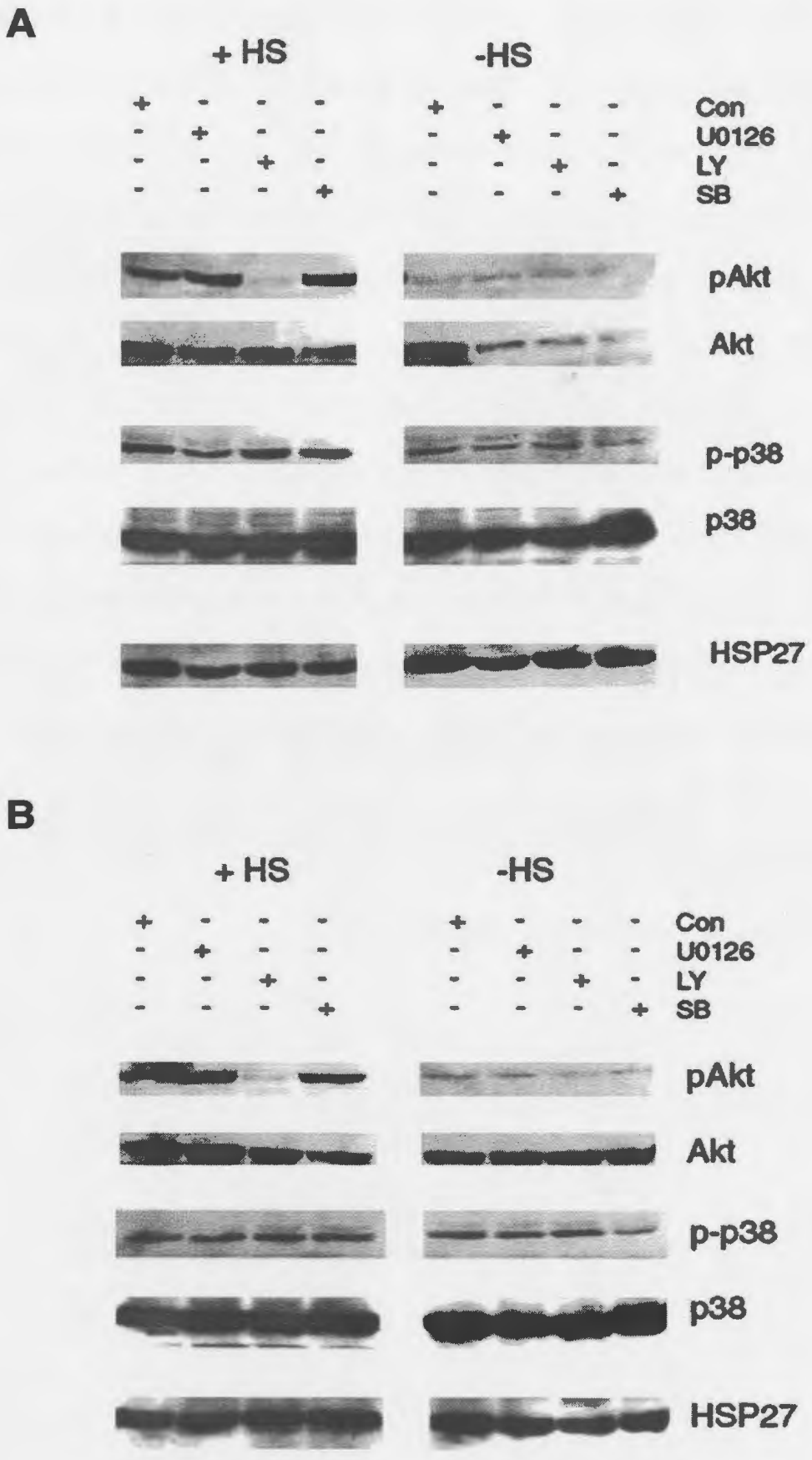
pp38
p38
HSP27
HSP27/ EGFP
C
pAkt
Fold increase over control
Con 5 min 1 h 3 h 6 h 24 h Con 5 min 1 h 3 h 6 h 24 h
pp38
Fold increase over control
Con 5 min 1 h 3 h 6 h 24 h Con 5 min 1 h 3 h 6 h 24 h
Hsp27
Fold increase over control
Con 5 min 1 h 3 h 6 h 24 h Con 5 min 1 h 3 h 6 h 24 h

Figure 3.3. Heat shock activation of Akt is a PI 3-Kinase-dependent process. Cells were preincubated with 10 μM LY294002 (to inhibit PI3-K), 5 μM SB203580 (to inhibit p38 MAPK) or 10 μM U0126 (to inhibit MEK) for 1 h prior to and during the heat shock. Cells were then sampled following a 1 h recovery period at 37°C. Lysates were immunoblotted with the indicated antibodies as described in Figure 1. Panel A – PC12 cells. Panel B – HSPC cells. +HS – lysates from cells exposed to the heat shock; -HS lysates from cells not exposed to heat shock; Con – cells not treated with any of the inhibitors.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+ HS
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+ HS
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Con
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LY
SB
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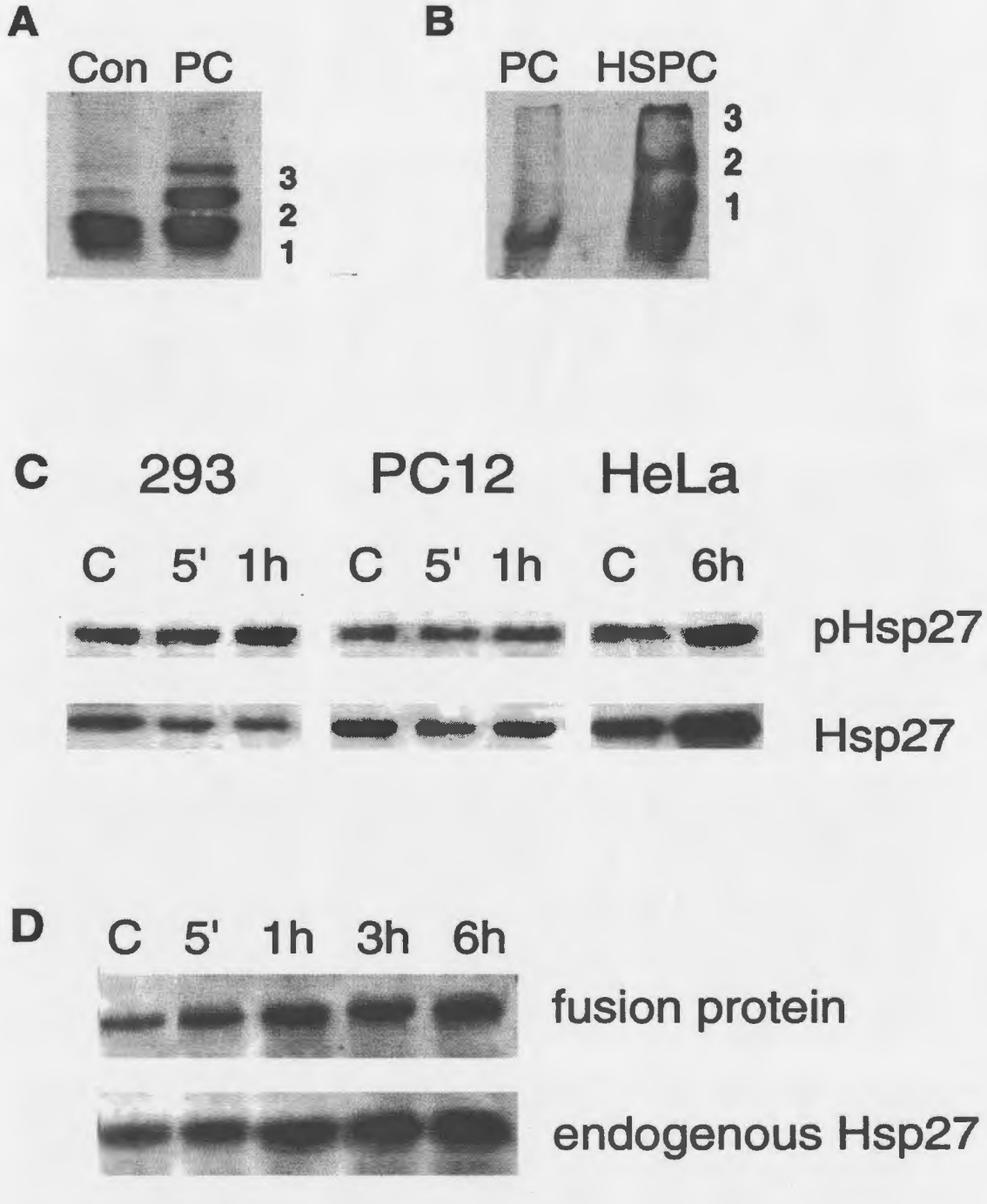
Akt
p-p38
p38
HSP27

Figure 3.4. Heat stress results in phosphorylation of Hsp27. In A and B, lysates (15 ug total protein) were subjected to isoelectric focusing and subsequently immunoblotted with anti-Hsp27. A) lysates from unstimulated PC12 cells (control) and heat-shocked PC12 cells (PC); B) lysates from unstimulated PC12 cells (PC) and unstimulated HSPC cells (HSPC). pI values were estimated from IEF standards. C) Several cell lines were either not treated (C), or subjected to heat stress and sampled after 5 min, 1 h or 6 h of recovery at 37°C. An antibody against phospho-Hsp27 (ser15, UBI) was then used to detect pHsp to test for the specificity of the antibody. HEK293 and HeLa cells are human cell lines, while the PC12 cells are a rodent line; the pHsp antibody detects both the human and rodent phosphorylated Hsp27. The blot was subsequently probed with an Hsp27 antibody (Santa Cruz) that recognizes both the human and rat Hsp27. D) HSPC cells were either not treated (C) or subjected to heat stress and sampled at the indicated times after recovery at 37°C. Anti-Hsp27 (Stressgen) was used to immunoprecipitate the Hsp27 and the Hsp27/EGFP fusion protein, and the blot was then probed with the anti-pHsp27 antibody (UBI), followed by the Hsp27 antibody.


A
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C
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pHsp27
Hsp27
D
C
5'
1h
3h
6h
fusion protein
endogenous Hsp27

Figure 3.5. Heat shock results in changes in the intracellular localization of Hsp27 in PC12 (A-I) and HSPC (J-O) cells. PC12 cells were either not stimulated (A-C) or subjected to heat shock and fixed at 5 min (D-F) or 1 h (G-I) after the heat shock. HSPC cells were either not stimulated (J-L) or subjected to heat shock and fixed at 1 h after the shock. Following immunostaining with pHsp27 or Hsp27 cells were visualized with confocal scanning laser microscopy. PC12 cells were immunostained with anti-Hsp27 (green, A, D, G) or the pHsp27 (UBI) antibody (red, B, E, H); panels C, F, I represent the merged images of the Hsp27 and pHsp27 labeled cells. Arrows in E show nuclear localization; arrows in H, I indicate the cytoplasmic redistribution. HSPC cells were immunostained with anti-phosphoHsp27 (red, J, M). EGFP fluorescence (green, K, N) was used to detect localization of Hsp fusion protein. Panels L and O are the merged images of pHsp27 and Hsp/EGFP. J, M – arrows, Nuclear localization; O –Arrow, perinuclear localization.

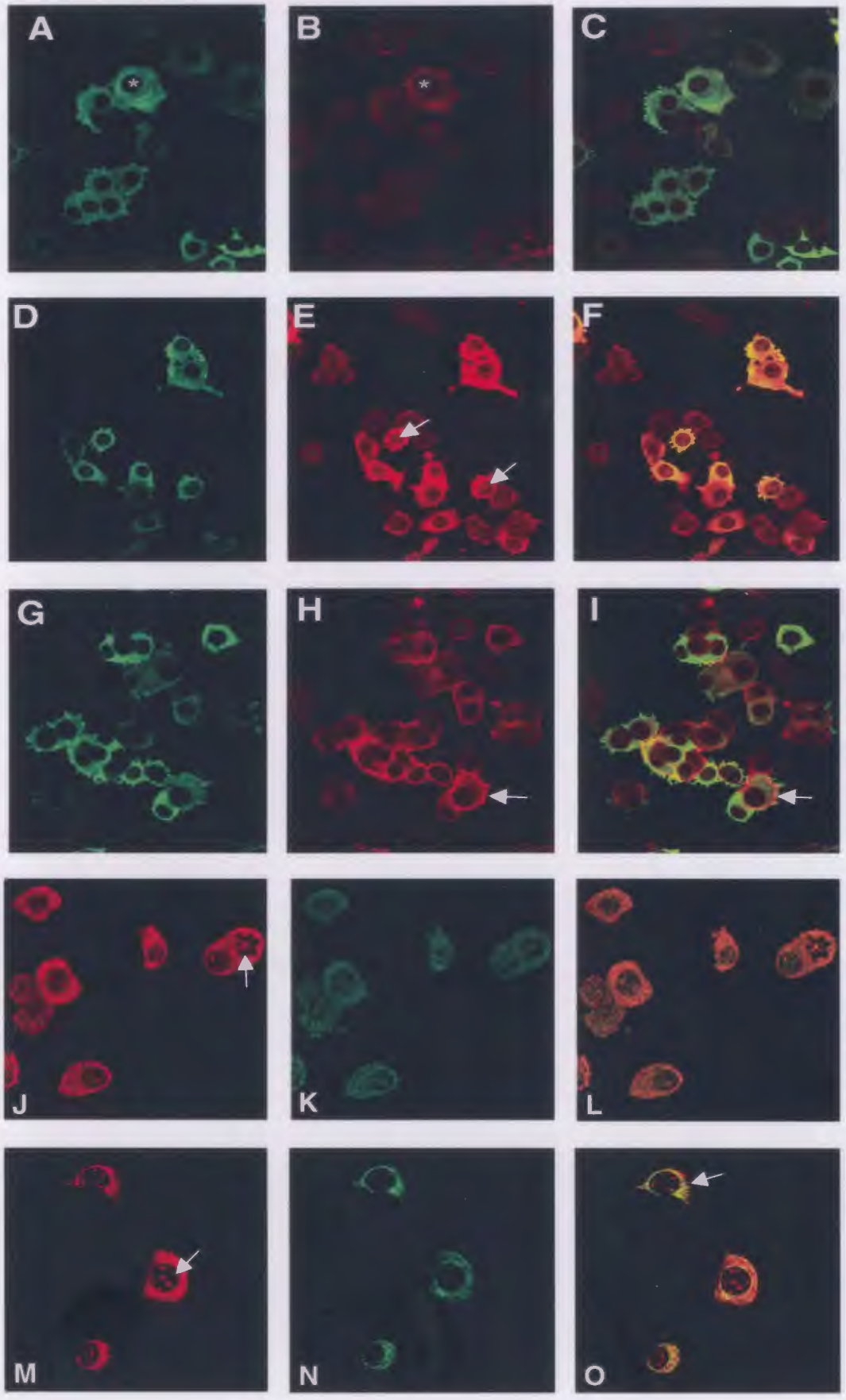
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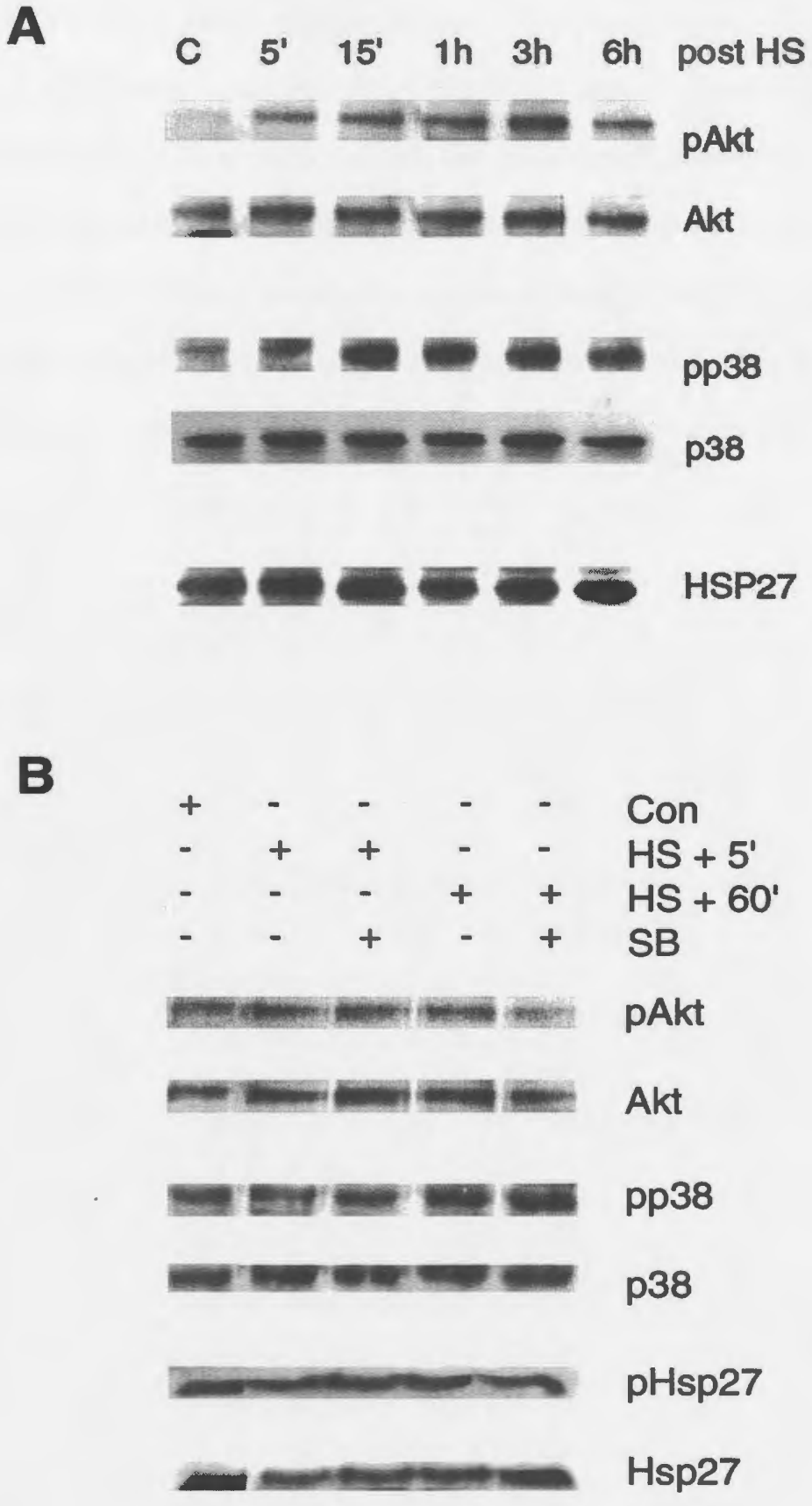
Figure 3.6. Hsp27 forms an immunoprecipitable complex with Akt and p38 MAPK. A) PC12 cells were subjected to heat shock and lysates sampled at the indicated times after recovery at 37°C; C – control non-shocked cells. Equivalent amounts of total protein (100 ug) were subjected to immunoprecipitation with anti-Hsp27 as outlined in Materials and Methods. Immunoblotting with the indicated antibodies was then carried out sequentially. B) PC12 cells were treated with the inhibitor SB203580 (SB) where indicated and then subjected to heat shock and sampled after either 5 min or 1 h recovery at 37°C. Immunoprecipitation with anti-Hsp27 was carried out as above, followed by immunoblotting with the indicated antibodies.


A
C
5'
15'
1h
3h
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pAkt
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pp38
p38
HSP27
B
+ - - - - Con
- + + - - HS + 5'
- - - + + HS + 60'
- - + - + SB
pAkt
Akt
pp38
p38
pHsp27
Hsp27

100
80
60
40
20
0
24
48
72
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**
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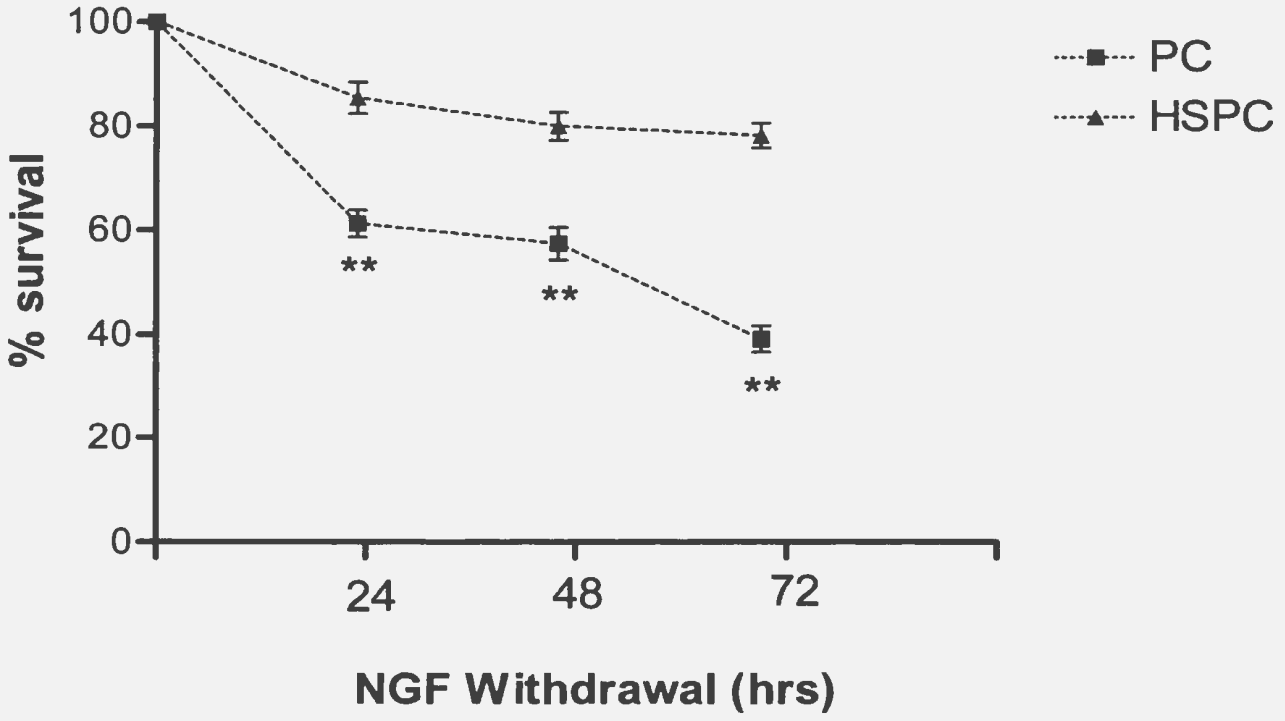
Figure 3.7. Hsp27 overexpression protects differentiated cells from NGF withdrawal-induced apoptosis. PC12 and HSPC cells were differentiated with NGF for 7 d, transferred to 96-well plates for 2 d, and then subjected to NGF withdrawal. Assessment of surviving cell numbers was carried out using MTS cell proliferation/cell survival assay. Data are expressed as a percentage of the control condition (cells maintained with NGF). Each point is the mean $\pm$ SEM of 3 independent experiments, with 8 replicated per experiment (** $p < 0.01$, ANOVA, with Tukey post-hoc test).


100
80
60
40
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0
% survival
**
**
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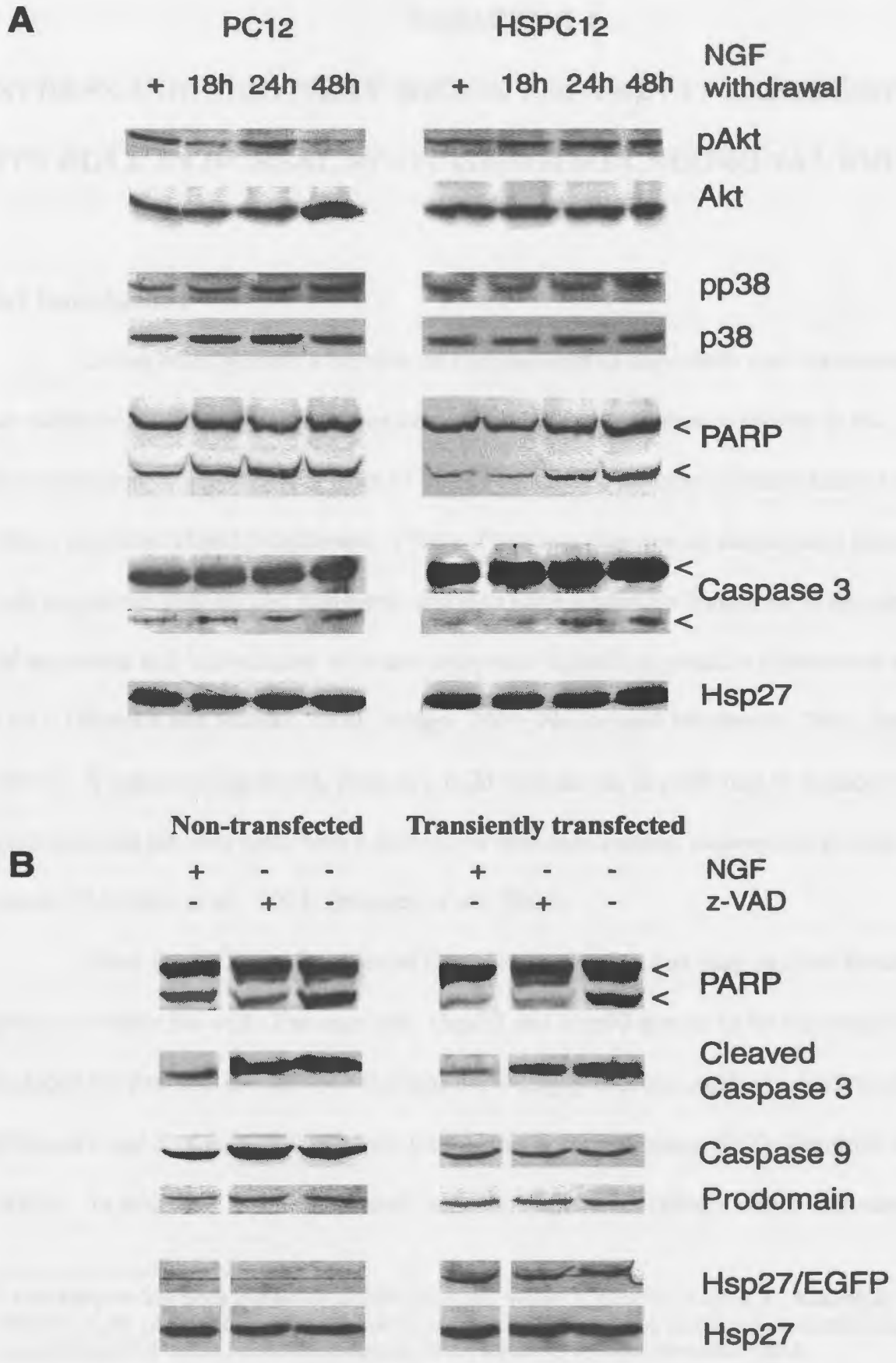
24
48
72
NGF Withdrawal (hrs)
PC
HSPC

Figure 3.8. Hsp27 overexpression maintains Akt phosphorylation and results in less activation of PARP following NGF withdrawal. A). Differentiated PC12 and HSPC12 cells were subjected to NGF withdrawal and lysates prepared at the indicated times after withdrawal; the (+) lanes are lysates from cells maintained in NGF. Lysates were immunoblotted with the Akt, p38 and Hsp27 antibodies as previously described. An antibody that recognizes both the uncleaved and cleaved forms of poly(ADP-ribose)polymerase (PARP) was also used to determine if there was a correlation between this and the extent of apoptosis following the NGF withdrawal. There is increased expression of full length PARP (upper arrowhead), and an increase in detectable levels of the cleavage product (lower arrowhead) in the PC12 cells compared to the HSPC cells. Full length and cleaved caspase 3 were detectable in both cell lines (upper arrowhead - full length caspase 3, lower arrowhead – cleaved caspase 3).

B). Differentiated parental PC12 cells and PC12 cells transiently transfected with Hsp27 treated with the zVAD-fmk (50 μM) were subjected to NGF withdrawal. Cells were sampled at 18 h after NGF withdrawal and the blots probed with the indicated antibodies. Z-Vad treatment partially inhibits the PARP cleavage in both cell lines, although like the stable HSPC line, the transient transfectants display less activation of caspases and PARP cleavage (right panel) than the parental PC12 cells (left panel).


A
PC12
HSPC12
+ 18h 24h 48h
+ 18h 24h 48h
NGF withdrawal
pAkt
Akt
pp38
p38
PARP
Caspase 3
Hsp27
B
Non-transfected
Transiently transfected
+ - -
- + -
+ - -
- + -
NGF
z-VAD
PARP
Cleaved Caspase 3
Caspase 9
Prodomain
Hsp27/EGFP
Hsp27

*CHAPTER 4

STRESS-INDUCED HEAT SHOCK PROTEIN 27 EXPRESSION AND ITS ROLE IN DORSAL ROOT GANGLION NEURONAL SURVIVAL

4.1 Introduction

Living cells possess a number of mechanisms to cope with various stresses such as radiation, oxidants, chemicals or heat, although one common response is the accumulation or activation of a set of highly conserved cellular proteins known as heat shock proteins (Hsps) (Hightower, 1991). Hsps can function as chaperones playing a key role in protein folding and transport, and also have important functions in the prevention of apoptosis and interactions with anti-apoptotic signalling proteins (Morimoto *et al.*, 1997; Ohtsuka and Suzuki, 2000; Arrigo, 2001; Nollen and Morimoto, 2002; Beere, 2004). A conditioning stress, such as a mild heat stress, is sufficient to induce the Hsp response and provide cells with a protective response against subsequent potentially lethal insults (Mailhos *et al.*, 1993; Quigney *et al.*, 2003).

There are different families of heat shock proteins that vary in their function and purpose within the cell. For example, Hsp70 and Hsp90 appear to be the major proteins induced by stress in the nervous system, providing protection against a variety of insults (Ohtsuka and Suzuki, 2000; Richter-Landsberg and Goldbaum, 2003; Franklin *et al.*, 2005). In addition, a family of small heat shock proteins (sHsp), which includes

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* **This chapter has been published in:** Dodge, M.E., Wang, J., Guy, C., Rankin, S., Rahimtula, M., Mearow, K.M. (2006) Stress-induced heat shock protein 27 expression and its role in dorsal root ganglion neuronal survival. Brain Research Molecular Brain Research in press, corrected proof.

Hsp25/27 and α-crystallin, have been shown to be upregulated by stress. Hsp27 has protective effects against heat shock, oxidative stress and other models of cellular injury in a variety of cell types, including neurons (Landry *et al.*, 1989; Lewis *et al.*, 1999; Wagstaff *et al.*, 1999; Benn *et al.*, 2002; Zourlidou *et al.*, 2004; Arrigo *et al.*, 2005; Latchman, 2005).

Dorsal root ganglion (DRG) sensory neurons are known to be dependent upon neurotrophins for their embryonic development and differentiation. During this early embryonic period their dependence is based upon neurotrophins such as NT-3 and NGF; however, dependence is shifted primarily to NGF during late embryonic development (Ruit *et al.*, 1992; Memberg and Hall, 1995; White *et al.*, 1996; Molliver and Snider, 1997; Vogelbaum *et al.*, 1998). It is well established that early neonatal DRG neurons are dependent on NGF for survival, and when NGF is withdrawn from culture, these neurons die via an apoptotic mode of cell death, likely by inhibition of pro-survival signalling (eg., PI3K→ Akt), and activation of pro-apoptotic genes c-Jun and Bax (Deckwerth *et al.*, 1996; Tong *et al.*, 1996; Datta *et al.*, 1997; Vogelbaum *et al.*, 1998).

On the other hand, adult DRG neurons do not require NGF for survival and survive up to two weeks in culture in the absence of NGF (Dodge *et al.*, 2002). The mechanism by which developing DRG neurons become less sensitive to harmful stimuli as they progress to adulthood is the subject of much study and a number of molecular candidates, including Hsp27, have been suggested (Benn *et al.*, 2002; Walsh *et al.*, 2004; Fernyhough *et al.*, 2005). For example, it has been reported that the developmental regulation of Hsp27 may play a role in the decreased vulnerability of mature DRG

neurons to trophic factor withdrawal (Costigan *et al.*, 1998; Lewis *et al.*, 1999; Benn *et al.*, 2002). Hsp27 is constitutively expressed in subpopulations of motor and sensory neurons in the adult rat nervous system (Plumier *et al.*, 1997), and overexpression of exogenous Hsp27 in neonatal DRG neurons protects them from cell death due to both peripheral nerve injury and NGF withdrawal (Lewis *et al.*, 1999; Wagstaff *et al.*, 1999). Based upon our previous work (Mearow *et al.*, 2002) and that of others noted above, we were interested in further examining whether the intrinsic differences in expression of Hsp27 were a key factor in the NGF-independence of adult DRG neurons, and determining whether a physiologically relevant manipulation might be sufficient for neuronal protection. Our results confirm that constitutive expression of Hsp27 is minimal in neonatal neurons, whereas levels are significantly higher in adults both in cultures and intact DRGs. In addition, a stress consisting of non-lethal heat shock results in increased phosphorylation and expression of endogenous Hsp27 in both neonatal and adult neurons, and this upregulation of Hsp27 in neonatal cultures prior to NGF withdrawal rescues these neurons from cell death normally seen by NGF withdrawal alone.

We have also used siRNA specific for Hsp27 and examined its effects on both Hsp27 expression and neuronal survival in adult and neonatal neurons. Decreased Hsp27 expression correlated with increased cell death in adult neurons and pretreatment with siRNA inhibited the heat-shock induction of Hsp27 and protection against cell death in the neonatal neurons.

4.2 Materials and Methods

4.2.1 Cell Culture

Primary DRG neurons were dissected from young adult (4-5 wks old) or postnatal day 2 (P2) Sprague-Dawley rats. Animals were anaesthetized with Somnotol and sacrificed by decapitation. The usage of animals was approved by the Institutional Animal Care Committee, St. John's, NL, Canada. DRG neurons from young adults were cultured as described previously (Dodge *et al.*, 2002). In brief, ganglia were extracted from all spinal cord levels and incubated in 0.25 % collagenase type II (Invitrogen/Life Technologies, Burlington, Canada) for 45 min at 37°C, and then incubated with 0.25 % trypsin (Invitrogen) for 20 min at 37°C. The ganglia were dissociated by a series of manual triturations using polished Pasteur pipettes. The cell suspension was centrifuged at 1000 rpm and the resulting cell pellet was suspended in serum-free Neurobasal medium (Invitrogen) supplemented with B27 additives and antimitotics (10 μM cytosine arabinoside, Sigma, St. Louis, MO, USA). P2 neurons were cultured similarly with the following changes: DRGs were not incubated with collagenase but were incubated with trypsin for 45 min at 37° C, followed by incubation in DNAse A (10 mg/mL) for 5 min at room temperature. Dissociation was by manual trituration and suspension of cells was in serum-free Neurobasal medium supplemented with B27 additives, N_2 and antimitotics. Cells were plated at a density of 5000-7000 cells per well of a 12-well plate for protein analysis. Cells to be plated on culture slides were centrifuged on a 30 % Percoll (Amersham Biosciences, Quebec) gradient and plated at a density of 200-300 cells per

well. Medium was supplemented with 50 ng/mL NGF (Cedarlane Labs, Hornsby, Canada) where appropriate.

4.2.2 Cell Culture Treatments

For NGF withdrawal experiments, P2 DRG neurons were plated with medium supplemented with 50 ng/mL NGF. For protein experiments, cultures were left for 3-4 d to allow for elimination of non-neuronal cells. Cells were then washed three times over a period of 3-4 h to withdraw NGF from the medium, leaving cells in an NGF-free environment; medium was also supplemented with anti-NGF IgG (500 ng/mL). Protein was collected at various time points afterwards. For culture slides and survival assays, NGF withdrawal was performed by the same procedure and survival was assayed at various time points afterwards by either propidium iodide or TUNEL labelling methods. Controls for these experimental conditions were cultures maintained in medium containing NGF.

For heat shock treatment, culture plates were immersed in a 45°C water bath for 15 min. Cells were allowed to recover for varying intervals after heat shock and sampled for protein analysis or fixed for immunocytochemistry.

siRNA was introduced into cells using the AMAXA nucleofection system (AMAXA Inc, Maryland, USA). DRGs were dissociated by incubating with dispase (2 mg/mL collagenase type II; 5 mg/mL protease, Sigma; 0.1 mg/mL Dnase, Sigma) for 45 min at 37°C, followed by manual triturations with a polished Pasteur pipette. Cells to be plated in 12-well plates were spun down at 500 rpm for 5 min and the pellet resuspended in

AMAXA nucleofector solution to which negative control siRNA or Hsp27 siRNA was added. The suspension was then placed in a cuvette, and transfected using program G-13. Cells to be plated in glass chamber slides were first subjected to a 30 % Percoll gradient before transfection. Negative control siRNA tagged with Alexa Fluor 546, and two Hsp27 siRNA sequences were transfected into neurons at a concentration of 2 μM each. Target sequences for silencing Hsp27 gene expression were: 5'UCA CUG GCA AGC ACG AAG A3' and 5'GAG UGG UCU CAG UGG UUC A3'; the siRNA was synthesized and purchased commercially (Dharmacon, Colorado, USA). Negative control siRNA was purchased from Qiagen (Mississauga, Canada), target sequence 5'AAT TCT CCG AAC GTG TCA CGT3'. After transfection with the AMAXA system, cells were incubated in medium containing 10 % FCS (Invitrogen) for 10 min, then plated in medium void of FCS and allowed to recover for 24 h.

4.2.3 Western Blotting

Culture medium was aspirated and cells were removed with ice-cold Tris-buffered saline (TBS) with sodium vanadate (200 mM). Cell pellets were suspended in protein lysis buffer consisting of 10 % glycerol, 1 % Nonidet P-40, sodium vanadate (200 mM), sodium fluoride (200 mM), and one protease inhibitor cocktail tablet (Roche Scientific, Laval, QC). Cell lysates were then centrifuged at 10,000 rpm for 10 min. For experiments involving subcellular fractionation, protein was extracted using the Subcellular Proteome Extraction Kit (Calbiochem, La Jolla, CA). Protein concentrations were determined using the BCA protein assay (Pierce Chemicals, Rockford, IL, USA).

Equivalent amounts of protein (30 ug) were electrophoresed on 10 % SDS-polyacrylamide gels. Following transfer to nitrocellulose, blots were stained with Ponceau Red to assess the equivalency of protein loading. Following washing with TBS, the blots were blocked in 3 % milk-TBST (Tris-buffered saline and Tween 20 at 0.2 % V/V) and probed with polyclonal antibodies to pHsp27 (1:800, Affinity Bioreagents, CA), Hsp70 (1:10,000, Stressgen, Victoria, B.C), Hsp27 (1:2000, Stressgen), MAPK (1:1000, Santa Cruz Biotechnologies, California), pAkt (1:1000) and Akt (1:1000, Cell Signalling, Mississauga, Canada). Blots from several experiments were quantified using densitometry.

4.2.4 Immunocytochemistry

Adult and neonatal neurons were plated on 16-well glass chamber slides (Nunc, VWR Canlab, Mississauga, ON, Canada). The medium was aspirated and cells were fixed with 4 % formaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1 % Triton-X and blocked with 10 % goat serum in PBS. Immunocytochemistry on whole DRG sections involved extracting whole DRGs from adult and neonatal rats, and freezing them in tissue freezing media (TBS, Triangle Biomedical Sciences, Durham, N.C.) at -70°C until ready to use. DRGs were cryosectioned in 8 μm (for adults) and 16 μm (for neonates) slices, and sections were placed onto glass slides. The sections were fixed in 4% formaldehyde, permeabilized in 0.2 % Triton-X and blocked in 10 % goat serum in PBS. Subsequently, cells and sections were incubated with a polyclonal primary antibody to Hsp27 (1:250, Stressgen) or pHsp27ser15 (1:100, Affinity Bioreagents) overnight at

4°C. Secondary antibodies labelled with Cy2 or Cy5 (1:100, Jackson Immunoresearch Laboratories, West Grove, PA, USA) and conventional fluorescence or scanning laser confocal microscopy was used to visualize the immunolabeled cells.

4.2.5 Cell Survival Assays

Cell survival was assessed in 16-well chamber slides. For propidium iodide (PI) labelling, medium was aspirated and PI was added to wells at a concentration of 10 ug/mL. PI was then washed off with Hanks Balanced Salt Solution (Invitrogen) and the cells fixed using an immunocytochemistry protocol. Cell survival was determined from four different fields in each well, counting the number of cells that excluded PI, and then calculating the values into a percentage of survival relative to control values. In some experiments we employed TUNEL labelling (In situ Cell Death Detection Kit, POD, Roche Applied Science, Indianapolis, USA). This involved fixing of cells in 4 % formaldehyde in PBS, permeabilization with 0.1 % Triton-X, and blocking with 10 % goat serum in PBS. TUNEL reaction mixture was added to chamber wells for 1 h. Cell survival counts were performed and subsequently, Hsp27 (1:250) primary antibody was added and incubated overnight at 4°C, followed by washing and detection with Cy-2 labeled secondary antibody. Cell survival is the percentage of live cells compared to total cell numbers. For siRNA experiments, control conditions indicate wells that had been transfected with negative control siRNA.

4.2.6 Statistical Analysis

Data were analyzed for significance using one-way ANOVA, followed by post-hoc testing with Tukey's test for multiple comparisons. Data were expressed as percentages relative to control values. All experiments were replicated a minimum of three times. Data are expressed as the mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$

4.3 Results

4.3.1 Expression of Hsp27 is developmentally regulated

Whole cryosections of adult and neonatal postnatal day 2 (P2) rat dorsal root ganglia were probed with a primary antibody to Hsp27 and immunoreactivity was detected with Cy-2 secondary antibodies (Figures 4.1A and B). Immunoreactivity in adult sections was robust (Figure 4.1A), and present in most cells in all areas of the ganglion. The majority of labelled cells were of the medium to large sized category and there was no difference in expression of Hsp27 among ganglia of different spinal areas (cervical, thoracic, lumbar or sacral – data not shown). Conversely, neonatal DRG sections showed minimal Hsp27 staining (Figure 4.1B) and these results were consistent throughout all spinal levels. As seen in Figure 4.1C, cultures of adult neurons show intense staining, and Hsp27 can also be seen extending into neuritic processes. Growth cones and contact points show staining for Hsp27, which suggests a role for this chaperone in the maintenance of the cytoskeleton and in cell adhesion. On the other hand, the presence of Hsp27 in neonatal cultures is not as extensive, although it is seen in

some neurites (Figure 4.1D). This corresponds to the low constitutive levels present in whole ganglion seen in Figure 4.1B.

To further demonstrate the differences in Hsp27 expression between adult and neonatal neurons, cell counts and western blotting were performed to quantitate expression and protein levels. Approximately 80 % of neurons in adult sections showed staining, whereas only 20 % of neonatal neurons were immunoreactive (Figure 4.2A). Western blotting experiments of whole DRG lysates showed similar results as cell counts; adult levels were taken as 100 % Hsp27 expression and neonatal levels were expressed in comparison to adults (Figure 4.2B). Adult and neonatal DRG primary neuronal cultures also showed differential staining for Hsp27. The number of cells expressing Hsp27 *in vitro* was counted 24 h, 48 h and 72 h after plating for both adult and neonatal cultures and expressed as a percentage of total cells present (Figure 4.2C). The presence or absence of NGF in the cultures had no effect on the percentage of adult cells expressing Hsp27, as 80 % of cells were immunopositive for Hsp27. The percentage of neonatal neurons positive for Hsp27 was significantly less (~25 %) than that in adult neurons and remained at this level for the duration of the experiment. Western blotting data also supported these results, although neonatal Hsp27 expression from cell culture lysates was slightly higher than that from whole DRGs (Figure 4.2D). This data suggests that dissociation may cause an increased expression of Hsp27 in some neonatal cells but does not result in an overall increase in numbers of cells expressing Hsp27.

4.3.2 NGF withdrawal in neonatal cultures causes significant apoptotic cell death and no change in Hsp27 expression.

Neonatal neurons were plated in medium containing NGF, and for NGF withdrawal experiments, NGF was washed out of culture medium over a period of 3-4 h. Figure 4.3A shows the survival of cells over a 48 h period after NGF withdrawal. Cells maintained in the presence of NGF displayed a stable level of survival, after the initial loss due to dissociation. However, as expected, NGF withdrawal resulted in increased cell death over the duration of the experiment – with 70 % loss by 48 h compared to cells maintained in medium supplemented with NGF (taken as 100 % survival). We have previously shown that adult neuronal survival is not affected by NGF withdrawal, and for up to two weeks in culture adult neurons remain viable in the presence of NGF or anti-NGF (Dodge *et al.*, 2002). Figure 4.3B presents Western blotting results showing Hsp27 expression after NGF withdrawal from neonatal neuronal cultures. There is no significant difference in expression in the absence or presence of NGF or over a 48 h time period, as shown in the graph which quantitates Hsp27 expression and also the representative Western blot probed with anti-Hsp27 antibody. This indicates that Hsp27 is not regulated by NGF in these cells.

4.3.3 Exposure of adult and neonatal neurons to heat stress.

Heat shock proteins can be induced in response to various cellular stresses including a sublethal heat shock. We investigated whether a heat stress could induce expression of Hsp27 in our cultured neurons and if this stress could also result in activation of Akt, a signalling intermediate known to be important for cell survival in a

variety of cell types. Cultures of adult and neonatal neurons were exposed to a heat stress of 45°C for 15 min, and samples were collected at recovery periods of 5 min, 1 h, 3 h, 6 h and 24 h after the stress. Equivalent amounts of protein (30 ug) were electrophoresed on SDS-polyacrylamide gels and probed with an antibody to pHsp27^{S15}, Hsp27or pAkt ^{S473} (Figure 4.4). Results show that as early as 1 h after heat shock Hsp27 becomes phosphorylated in both adult (Figure 4.4A) and neonatal neurons (Figure 4.4B). Total levels of Hsp27 were significantly elevated ($p< 0.05$) by 3 h after heat shock and continued to rise over the experimental period ($p< 0.01$). Furthermore, increased Akt activation (phosphorylation of Akt) was observed in both adult (Figure 4.4C) and neonate (Figure 4.4D) cultures after heat shock and this returned to control levels by 3 h of recovery. These results show that even though neonatal neurons have low levels of Hsp27 expression, they can be induced to increase Hsp27 expression after a heat stress and that both the neonatal and adult cultures respond to the acute stress in a similar way – inducing Hsp27 expression after a lag time and an initial activation of Akt, which could potentially play a role in promoting neuronal survival as we have previously shown for PC12 cells (Mearow *et al.*, 2002).

4.3.4 Endogenous Hsp27 induction protects neonatal neurons from NGF withdrawal-induced cell death.

Cells were plated in the presence of NGF, and 3 h prior to NGF withdrawal were heat shocked for 15 min at 45°C. Following NGF withdrawal, survival was assessed at 24 h and 48 h by propidium iodide staining (Figures 4.5A and 4.5B). As seen previously,

there was no significant difference in cellular survival between cells maintained in NGF up to 48 h after the beginning of the experiment. When cells were heat shocked, there was a significant decrease in survival to 65 % 48 h afterwards (Figure 4.5B). Our observations suggest that while adult neurons are quite resistant to a variety of cell stresses, neonatal neurons tend to be more susceptible to certain stresses, such that there is usually an initial loss of a proportion of the neurons. This could be indicative of different populations of neurons, although we have not investigated this in any detail. Cultures that contained no NGF showed significant decreases in cell survival at 24 h (Figure 4.5A), and more dramatically at 48 h (Figure 4.5B) after withdrawal. However, when heat shock was given 3 h before NGF withdrawal, the induction of Hsp27 expression significantly improved cellular survival and delayed neuronal apoptosis. 24 h after withdrawal, there is a significantly higher percentage of cellular survival (85 % as opposed to 55 % in the absence of NGF) (Figure 4.5A). Cell death 48 h after heat shock without NGF in the culture medium was also decreased (60 % survival as opposed to 35 % in the absence of NGF) (Figure 4.5B). Figure 4.5C shows levels of Hsp27 expression by Western blotting, and Figures 4.5D and 4.5E present these results quantitatively. In all conditions where heat shock was given, Hsp27 levels are significantly higher than without heat shock, regardless of whether NGF was present or withdrawn. These data indicate that the induction of Hsp27 prior to NGF withdrawal contributes to increased cellular survival in the face of NGF withdrawal.

4.3.5 Hsp27 siRNA inhibits the rescue of neonatal neurons from NGF-withdrawal by heat shock.

To further investigate the contribution of Hsp27 to cell survival, we used a siRNA approach to decrease Hsp27 expression. Neonatal neurons were transfected with either a scrambled negative control siRNA or a cocktail of 2 Hsp27 siRNAs and plated in medium supplemented with NGF. Transfection resulted in approximately 45-50 % efficiency (data not shown). siRNA was introduced into cultures and left for 24 h before beginning the experiment. Cells were heat shocked and/or subsequently underwent NGF withdrawal. Survival was assayed by TUNEL labelling and results are shown in Figure 4.6A (scrambled siRNA) and Figure 4.6B (Hsp27 siRNA). At 24 h and 48 h after NGF withdrawal there was a significant decrease in survival. Heat shock given prior to NGF withdrawal provides some rescue of neurons from apoptosis. However, as seen in Figure 4.6A and 4.6B, neonatal neurons transfected with Hsp27 siRNA were not rescued from NGF withdrawal induced apoptosis, indicating that induction of Hsp27 via heat stress is sufficient to rescue neonatal neurons from apoptosis after NGF withdrawal. Western blotting data of this experiment (Figure 4.6C) shows that siRNA effectively inhibited the expression of Hsp27, both constitutively (first 4 lanes) and after heat stress (last 4 lanes). These results also show that siRNA used in these experiments is specific for Hsp27, as no change in another heat shock protein, Hsp70, was detected. Figure 4.6D shows TUNEL labelling results of neonatal cells 48 h after NGF withdrawal. Nuclear staining shows cells that are undergoing apoptosis (small arrows), whereas large arrows indicate cells lacking TUNEL labelling. Hsp27 immunostaining is shown in Figure 4.6E, indicating

that cells undergoing apoptosis have no Hsp27 expression, and surviving cells still show robust Hsp27 expression.

4.3.6 Effect of decreasing Hsp27 expression on adult DRG neuronal survival.

Due to the high expression levels of Hsp27 in adult neurons and their independence on NGF for survival, we sought to determine the effect of downregulation of Hsp27 expression. To decrease endogenous levels, adult neurons were transfected with either negative control siRNA (scrambled) or 2 different Hsp27 siRNAs as noted above. Figure 4.7A shows Western blotting experiments of cultures of adult neurons that were transfected with siRNA. siRNA was introduced into cultures and left for 24 h before giving a heat stress. Protein from control (corresponding to 48 h after plating) and heat shocked cultures were then collected after a 24 h recovery period. siRNA substantially decreased expression of Hsp27 in control conditions and also inhibited the induction of Hsp27 24 h after heat shock at the time when Hsp27 expression would have peaked. Immunocytochemical studies for Hsp27 also showed that many cells in culture either showed a decreased intensity in immunofluorescence or no detectable levels of Hsp27 when compared to other cells (Figure 4.7B, arrowheads show cells with moderate Hsp27 expression, arrows show cells lacking Hsp27). Cultures transfected with scrambled siRNA showed no difference in Hsp27 expression and were identical to non-transfected controls.

The effect of Hsp27 siRNA on cell survival was also determined. TUNEL positive cells were counted and values were compared to control conditions (cells transfected with scrambled negative control siRNA only). Figure 4.7C shows that

decreases in cell survival were seen in siRNA conditions beginning 24 h after transfection (< 20 % cell death) and survival continued to decrease up to 72 h afterwards (up to about 30 % cell death). However, as Figure 4.7B shows, not all cells that displayed lack of Hsp27 staining appeared to be dying.

4.4 Discussion

The results of this study show that the small heat shock protein Hsp27 is expressed differentially between neonatal DRG neurons and adult DRG neurons both *in vivo* and in culture, and that this expression in adult neurons may be a contributing factor to their NGF-independent survival. Both adult cultures and whole DRG cryosections were constitutively strongly immunoreactive for Hsp27, whereas expression levels in neonatal cultures were low in comparison. When cultures were subjected to sublethal heat stress, activation of Akt occurred for a brief period of time immediately after the stress and endogenous Hsp27 expression was significantly induced in both adult and neonatal cultures. Hsp27 siRNA served to significantly decrease endogenous Hsp27 levels in adult neuronal cultures and also caused some cell death over time. Induction of endogenous Hsp27 in neonatal neurons was able to protect these cells from NGF withdrawal-dependent cell death. Furthermore, when neonatal neurons were transfected with Hsp27 siRNA to suppress induction of Hsp27, the apparent Hsp27-dependent rescue was not observed. Our results demonstrate the importance of Hsp27 in survival of DRG neuronal cultures and are similar to those reported by others using exogenous overexpression of Hsp27 by herpes simplex viral-based vectors (HSV) (Lewis *et al.*, 1999; Wagstaff *et al.*, 1999; Benn *et al.*, 2002).

It is well established that neurotrophic factors, such as NGF, are important in the development and survival of DRG neurons, although these neurons later lose their dependence on NGF for their survival. The mechanisms by which these neurons acquire their independence of neurotrophins for survival has not been fully elucidated, although neurotrophin signal transduction intermediates, such as the PI3-K pathway, have been implicated in their survival (Borasio *et al.*, 1993; Klinz and Heumann, 1995; Dodge *et al.*, 2002; Edström and Ekström, 2003). While there are likely a number of factors contributing to the survival of mature DRG neurons, BDNF has been implicated as being important *in vitro* (Acheson *et al.*, 1995). However, addition of anti-BDNF or of BDNF in our cultures does not effect survival, indicating that these neurons are not depending on autocrine production of BDNF for survival (Tucker and Mearow, unpublished observations).

During development, many neurons undergo apoptotic cell death, and much of this cell death seems to resemble damage due to stress. Stressors can often result in the induction or upregulation of heat shock proteins. As we, and others (Costigan *et al.*, 1998; Lewis *et al.*, 1999) have shown, Hsp27 expression in neonatal sensory neurons is quite low, and it is possible that their low levels might contribute to cell death during development.

In addition, post-translational modifications, such as phosphorylation of Hsps, are dependent upon intracellular signalling cascades that are activated in response to various stressors (Gaestel, 2002). In response to heat stress, which in our model was 45°C for 15 min, we observed phosphorylation of Hsp27 after a 1 h recovery and a significant

induction of total Hsp27 that occurred within a 3 h recovery period in both adult and neonate cultures. Hsp27 expression remained elevated above control levels up to 24 h afterwards. Our previous studies in PC12 cells have shown that after a similar heat shock Hsp27 levels were increased at 3 h afterwards; in that report we noted that both induction of endogenous Hsp27 and overexpression of exogenous Hsp27 could protect PC12 cells from NGF withdrawal-induced cell death, likely by way of its interactions with Akt and inhibition of caspase activation (Mearow *et al.*, 2002). Whether phosphorylation of Hsp27 is necessary for DRG neuronal survival may depend upon which sites are assessed. Prior work noted that a triple phosphorylation mutant of human Hsp27 (which is phosphorylated on S15, S78 and S82) was unable to protect adult rat DRG neurons after injury (Benn *et al.*, 2002). Our observations have been that there is some amount of constitutive phosphorylation that can be increased by heat shock. Our preliminary results with a series of phosphorylation mutants of rodent Hsp27 (which is phosphorylated on only S15 and S82) suggest that phosphorylation does not play a key role in survival (Williams and Mearow, in preparation), although it does appear to be important in neurite initiation and growth (Williams *et al.*, 2005).

A number of studies have investigated the relationships between Hsp27 and Akt (also known as PKB), and reported that Akt forms a complex with Hsp27 (Konishi *et al.*, 1997; Murashov *et al.*, 2001; Mearow *et al.*, 2002; Rane *et al.*, 2003). These findings were important because they linked Hsp27, a molecular chaperone, to anti-apoptotic machinery of the cell. The activation of Akt after stress or insult can promote survival, via a series of signal transduction events that act to inhibit apoptosis, such as phosphorylation and inactivation of Bad (Datta *et al.*, 1997). Our results in these

experiments have similarly shown that a sublethal heat stress activates Akt immediately, and this stress response may be serving to inhibit apoptosis by interacting with other intermediate proteins. It has also been shown that Hsp27 displays anti-apoptotic ability by inhibiting caspases and cytochrome c release (Mehlen *et al.*, 1996; Garrido *et al.*, 1999; Charette *et al.*, 2000; Samali *et al.*, 2001; Paul *et al.*, 2002).

Hsp27 has been reported to be important for neuron survival in both the CNS and PNS (Lewis *et al.*, 1999; Wagstaff *et al.*, 1999; Benn *et al.*, 2002; Kalwy *et al.*, 2003; Zourlidou *et al.*, 2004; Patel *et al.*, 2005). In the PNS, *in vivo* studies of sciatic nerve injury found that only DRG neurons expressing Hsp27, as detected by immunocytochemistry, survived after P0 axotomy (Lewis *et al.*, 1999). Viral overexpression of Hsp27 inhibited a proportion of the cell death normally observed after NGF-withdrawal in both sympathetic and DRG neonatal neurons (Wagstaff *et al.*, 1999) and protected neonatal motor neurons from injury-induced death, while downregulation of Hsp27 resulted in death of motor neurons and a proportion of sensory neurons (Benn *et al.*, 2002). In our experiments, we have seen that induced expression of Hsp27 by heat stress was sufficient to inhibit apoptotic cell death of neonatal neurons after NGF withdrawal. We have also seen this protection in PC12 cells, which, once differentiated by NGF, are dependent upon it for survival (Mearow *et al.*, 2002). The mechanism involved has not been fully elucidated, but based on results noting the interactions of Hsp27 with Akt and inhibition of pro-apoptotic machinery, it is likely that Hsp27 is acting through these same mechanisms to inhibit apoptosis in our system.

With regard to the effect of siRNA on downregulation of Hsp27 and neuronal survival, Hsp27 antisense delivery using an HSV amplicon vector was previously

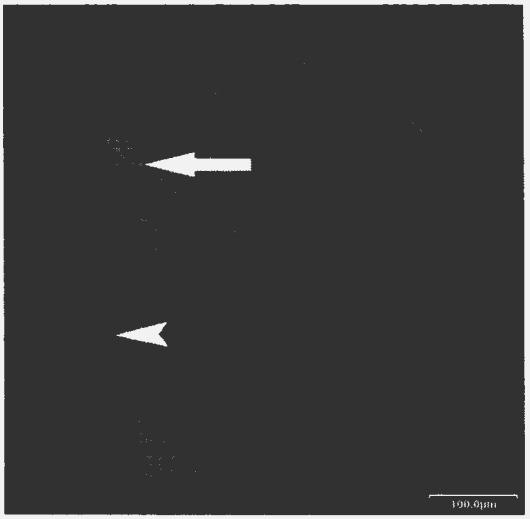
employed to examine the role of Hsp27 in motor and sensory neuronal survival (Benn *et al.*, 2002). However, the results in that report with respect to Hsp27 levels were not quantitated at the level of protein expression, although it was noted that there appeared to be a complete downregulation of Hsp27 as detected by ICC at 12 h after plating. In our experiments, siRNA was shown to downregulate Hsp27 expression before and after heat stress in both neonatal and adult neurons. However, we found that although ICC might indicate lack of Hsp27 expression on a per cell basis, the Western analyses demonstrated that there was no complete downregulation, which is perhaps not surprising for a constitutively expressed protein with a half life of >12 h (Landry *et al.*, 1991) and only 45-50% transfection efficiency was seen. Indeed the siRNA treatment was most effective at blocking the induced upregulation of Hsp27, similar to results reported for CHO cells (Hargis *et al.*, 2004). Downregulation of Hsp27 also resulted in less adult neuronal cell death (<20 % death at 24 h) compared to that previously reported (>75 % at 24 h) (Benn *et al.*, 2002). It is possible that the viral infection approach may be more effective in decreasing Hsp27 compared to siRNA, although our quantitation of the levels clearly show a very efficient decrease in both endogenous and induced Hsp27 protein levels. While basal expression levels of Hsp27 in neonatal neurons are lower than in adult neurons (both in terms of total Hsp27 expression and the percentage of neurons expressing Hsp27), expression was still decreased by the siRNA treatment and served to inhibit the rescue of neurons by heat shock prior to NGF withdrawal. Interestingly, the siRNA treatment did not induce cell death in the neonatal neurons maintained in NGF. Our data would suggest that while Hsp27 does play a role in contributing to the neurotrophin-independence of adult DRG neurons, it is not the only important factor.

Although Hsp27 is a critical component of a cell's anti-apoptotic machinery and plays a role in proper protein folding and transport, the exact mechanisms by which Hsp27 acts to increase neuronal cell survival remain unclear. It is likely that complexes with Akt and other important signalling intermediates may contribute to its mode of action, but more investigations into the differences in signalling between adult neurons and developing neurons are required to understand how Hsp27 contributes to NGF independence of adult DRG neurons. While there are differences in developing and mature DRG neuronal constitutive expression of Hsp27 that might contribute to the acquisition of NGF-independence, further investigation is required to understand underlying mechanisms.

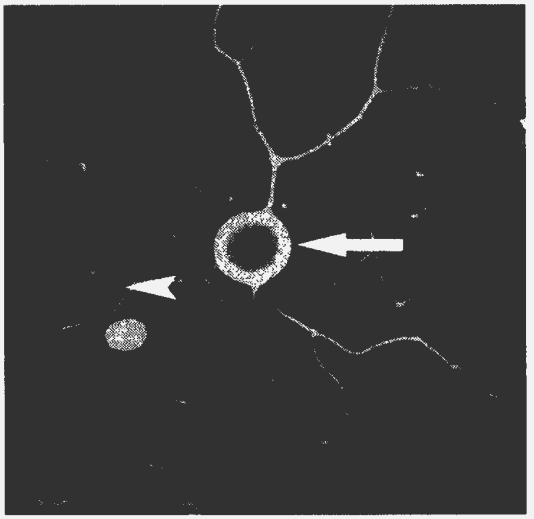
4.5 Figures

Figure 4.1: Expression of Hsp27 in adult and neonatal DRG cryosections and primary cultures. (A,B) DRGs were extracted from adult (A) and neonatal (B) rats, frozen in liquid nitrogen and sectioned into 8 μm (adult) and 16 μm (neonate) thick sections. Cell counts of sections from cervical, thoracic, lumbar and sacral regions together for expression levels of Hsp27 show that there are a significantly greater number of cells in adult DRGs expressing Hsp27. (C,D) Neuronal cultures were also prepared and immunostained with an antibody to Hsp27. (C) Adult neuronal cultures show robust, intense staining for Hsp27 in both the cell body and the surrounding neurites. (D) Neonatal neurons show faint or no staining for Hsp27. Arrowheads indicate cells lacking Hsp27 expression, whereas arrows indicate cells immunopositive for Hsp27. Scale bar for A and B is 100 μM, scale bar for C and D is 50 μm.

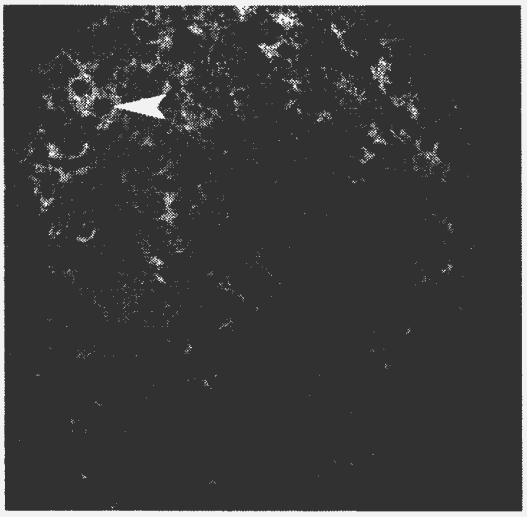
A



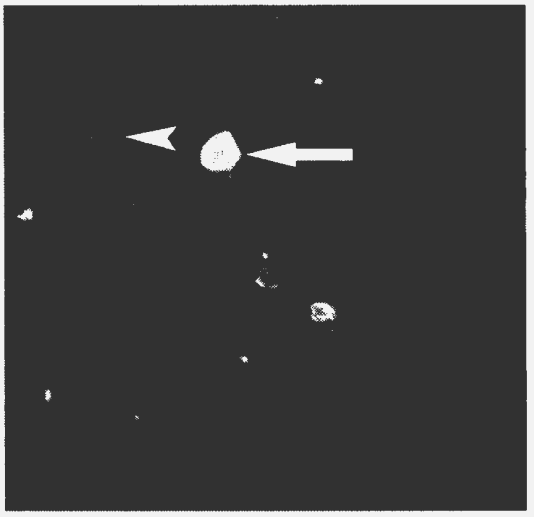
C



B



D



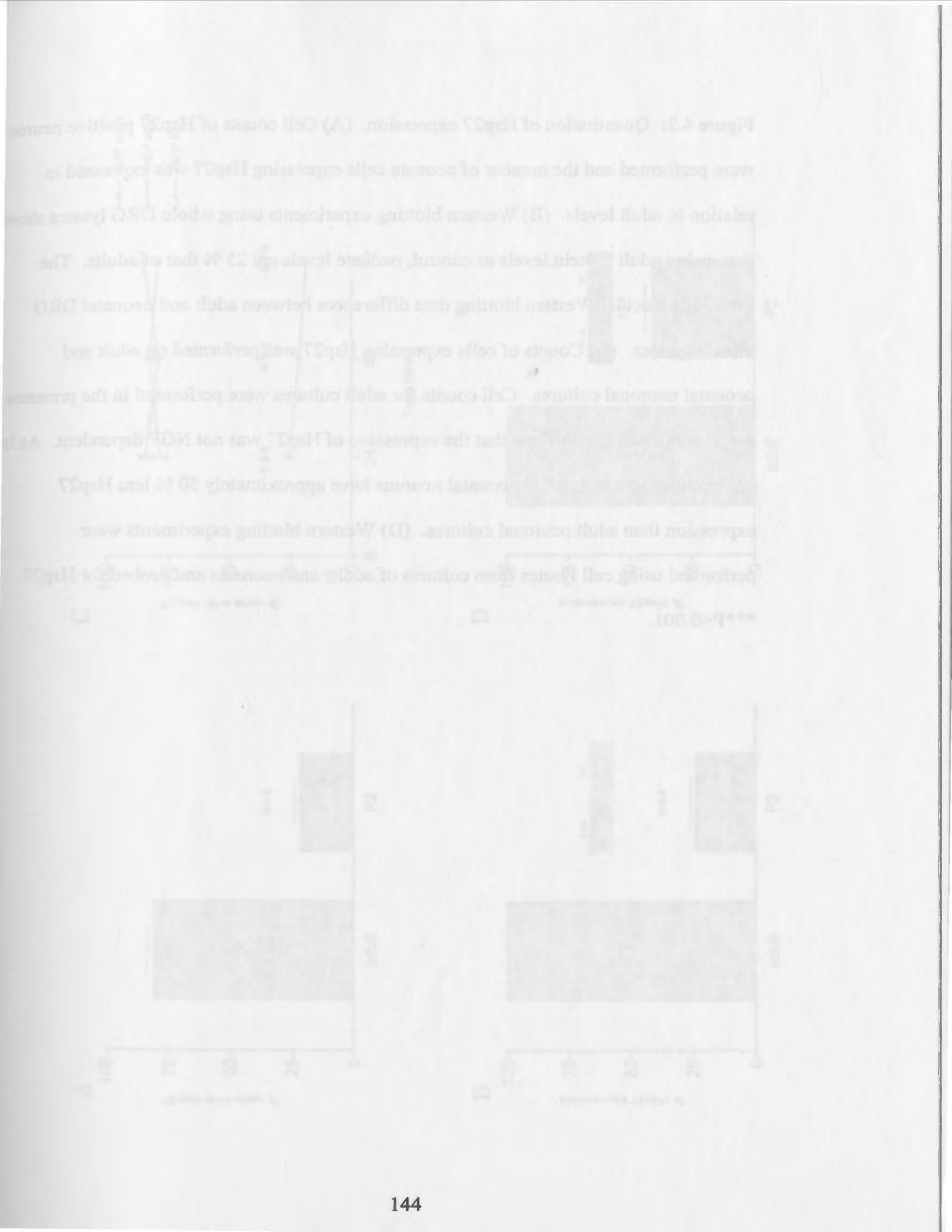


Figure 4.2: Quantitation of Hsp27 expression. (A) Cell counts of Hsp27 positive neurons were performed and the number of neonate cells expressing Hsp27 was expressed in relation to adult levels. (B) Western blotting experiments using whole DRG lysates show that, using adult protein levels as control, neonate levels are 25 % that of adults. The inset shows actual Western blotting data differences between adult and neonatal DRG protein lysates. (C) Counts of cells expressing Hsp27 was performed on adult and neonatal neuronal cultures. Cell counts for adult cultures were performed in the presence and absence of NGF to show that the expression of Hsp27 was not NGF dependent. As in cryosections, cultures of P2 neonatal neurons have approximately 50 % less Hsp27 expression than adult neuronal cultures. (D) Western blotting experiments were performed using cell lysates from cultures of adults and neonates and probed for Hsp27. ***$P<0.001$.

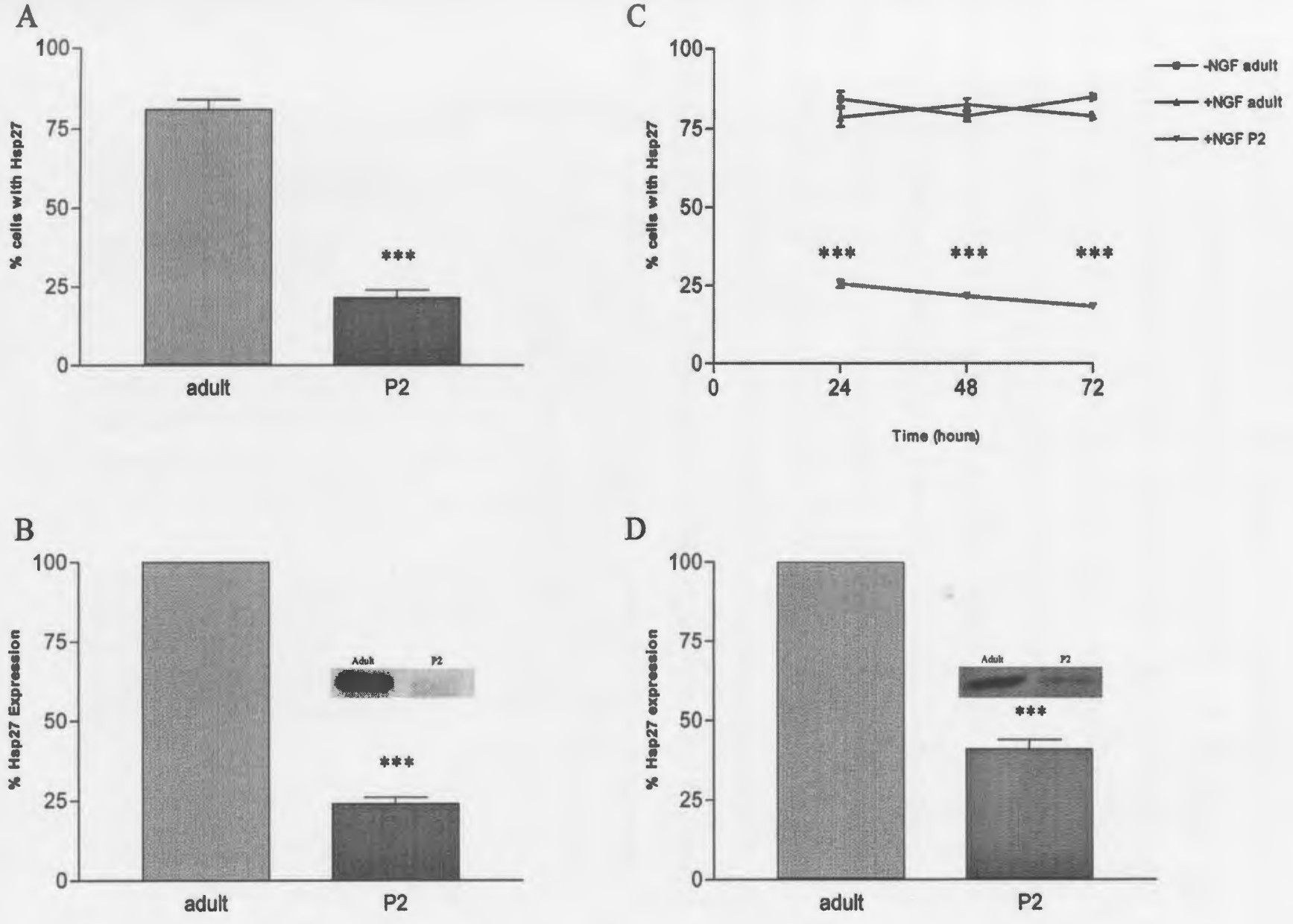
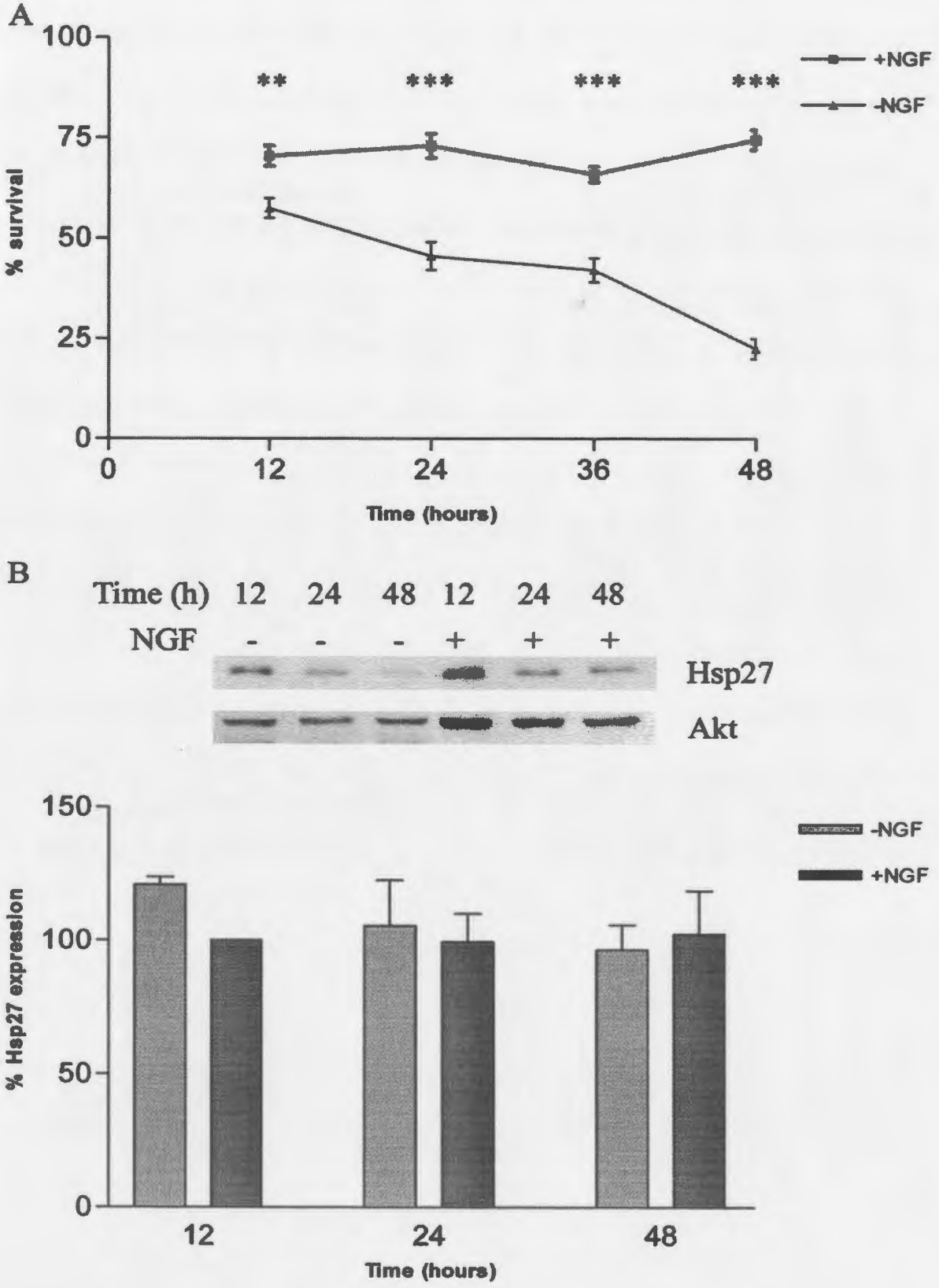

A
100
75
50
25
0
% cells with Hsp27
***
adult
P2
C
100
75
50
25
0
% cells with Hsp27
-NGF adult
+NGF adult
+NGF P2
***
***
***
0
24
48
72
Time (hours)
B
100
75
50
25
0
% Hsp27 Expression
Adult
P2
***
adult
P2
D
100
75
50
25
0
% Hsp27 expression
Adult
P2
***
adult
P2

Figure 4.3: Neonatal DRG neurons undergo apoptosis in the absence of NGF. (A) Neonatal cultures were plated on postnatal day 2 in the presence of 50 ng/mL NGF and left for 3-4 d to allow for mitotic inhibitors to eliminate non-neuronal populations. NGF was subsequently washed out of the cultures over a period of 3-4 h and survival was assayed at 12 h, 24 h, 36 h and 48 h afterwards using propidium iodide (PI) staining. PI positive nuclei were counted in pre-determined quadrants of each well (4 wells per condition, experiments were repeated 3 times) and cells that underwent NGF withdrawal were expressed as a percentage of cells maintained in the presence of NGF. (B) Western blotting experiments showing expression of Hsp27 after NGF withdrawal or maintenance of NGF in P2 neonatal cultures. Cells were cultured as in panel (A). Control condition was 12 h in the presence of NGF. No significant differences were found in Hsp27 expression between the two conditions up to 48 h after withdrawal. Inset for (B) is a representative blot of the graphed results. **$P<0.01$. ***$P<0.001$.


A
100
75
50
25
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% survival
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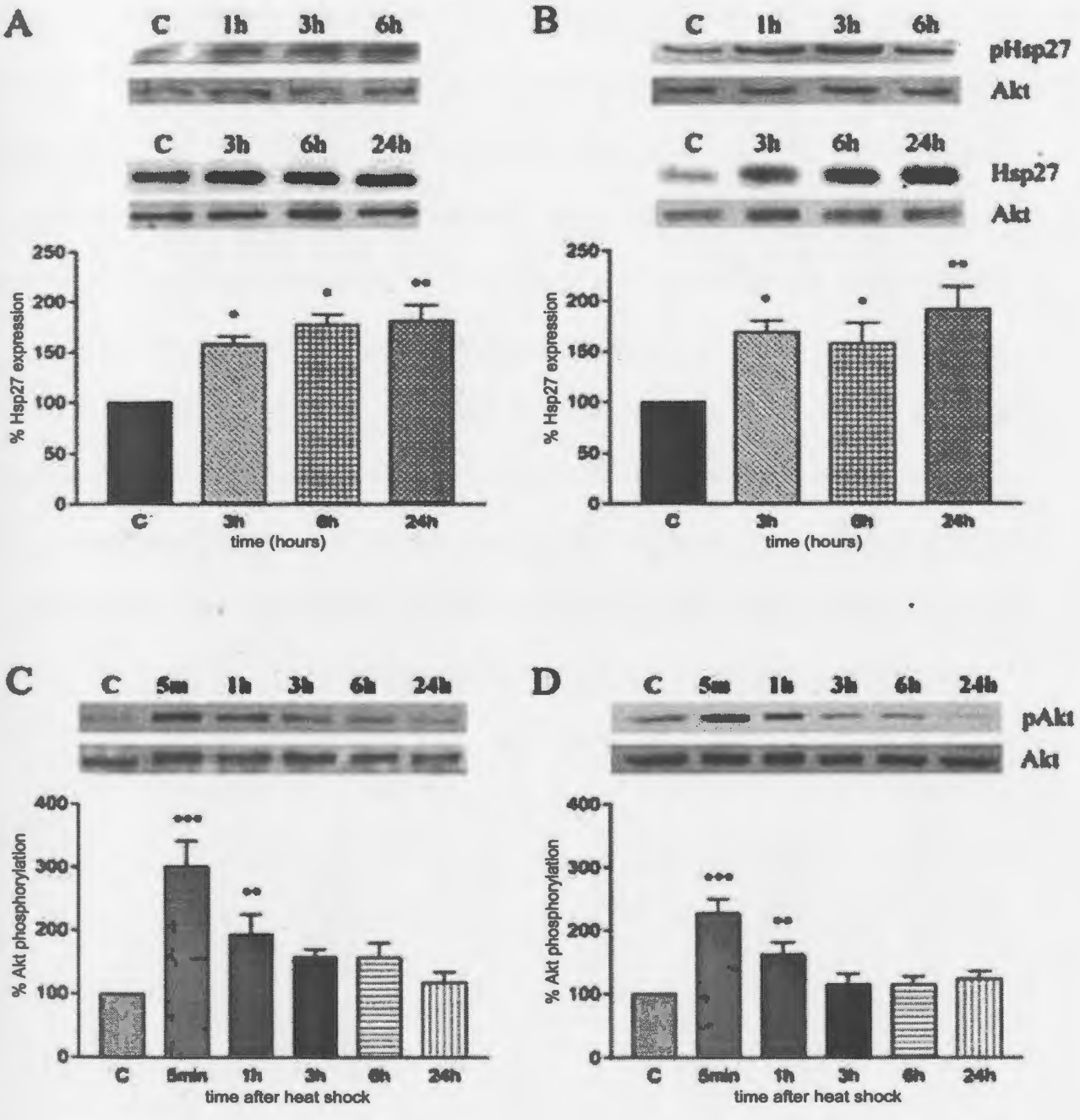
***
***
***
+NGF
-NGF
0
12
24
36
48
Time (hours)
B
Time (h) 12 24 48 12 24 48
NGF - - - + + +
Hsp27
Akt
150
100
50
0
% Hsp27 expression
-NGF
+NGF
12
24
48
Time (hours)

Figure 4.4: Heat stress of adult and neonatal cultures results in induced expression and phosphorylation of Hsp27, and activation of Akt. All cultures were heat shocked at 45°C for 15 min by placing culture dishes directly in a pre-heated water bath, and cultures were subsequently allowed to recover in the incubator at 37°C for varying time periods afterwards. Samples were collected, electrophoresed on a 10 % polyacrylamide gels and transferred to nitrocellulose. (A, B) Protein was collected after 1 h, 3 h, 6 h or 24 h recovery from heat shock and blots were probed with a primary antibody specific for pHsp27 or Hsp27; Akt was used as a loading control. Results show phosphorylation of Hsp27 1 h after heat shock and significant induction of total Hsp27 after 3 h, 6 h, and 24 h recovery from heat shock in adult (A) and neonatal (B) cultures. (C) Protein was collected at 5 min, 1 h, 3 h, 6 h and 24 h after heat shock and blots were probed with a primary antibody to pAkt and Akt. Strong activation of Akt in adult cultures occurred 5 min after heat shock. (D) Activation of Akt also occurred in neonatal cultures and was significantly increased after a 5 min recovery from heat shock. *$P<0.05$, **$P<0.01$, ***$P<0.001$


A
C
1h
3h
6h
C
3h
6h
24h
% Hsp27 expression
250
200
150
100
50
0
C
3h
6h
24h
time (hours)
B
C
1h
3h
6h
pHsp27
Akt
C
3h
6h
24h
Hsp27
Akt
% Hsp27 expression
250
200
150
100
50
0
C
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6h
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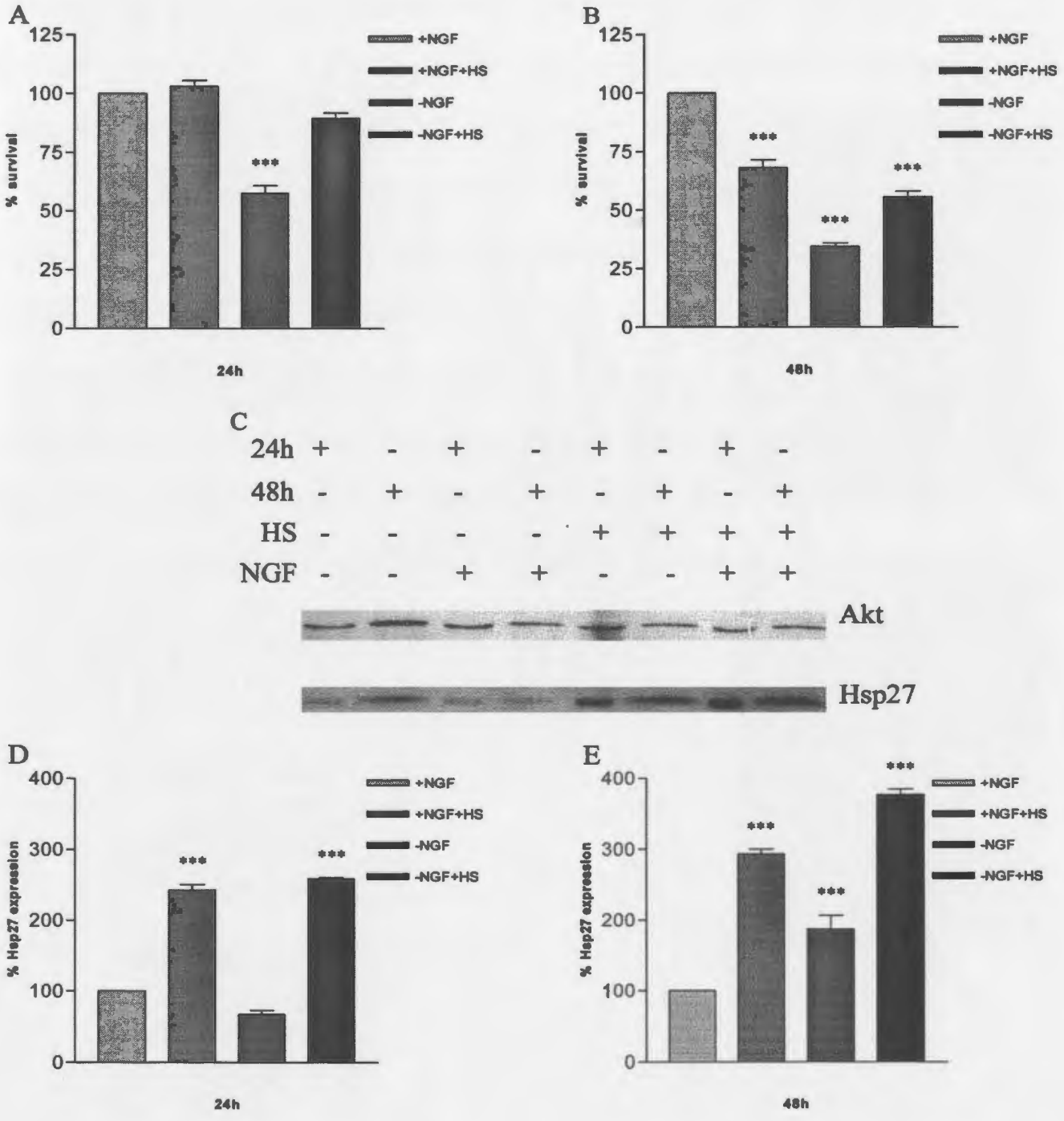
time (hours)
C
C
5m
1h
3h
6h
24h
% Akt phosphorylation
400
300
200
100
0
C
5min
1h
3h
6h
24h
time after heat shock
D
C
5m
1h
3h
6h
24h
pAkt
Akt
% Akt phosphorylation
400
300
200
100
0
C
5min
1h
3h
6h
24h
time after heat shock

Figure 4.5: Rescue of neonatal neurons from NGF withdrawal by heat shock. Neonatal cultures were plated and left in the presence of anti-mitotics for 3-4 d to minimize contamination of cultures by non-neuronal cells. (A, B) Cells were either maintained in the presence of NGF (50 ng/mL) for the entire experiment or underwent NGF withdrawal. Some cells were subjected to heat shock 3 h prior to NGF withdrawal. Waiting to wash NGF out of cultures until after cells had recovered from heat shock for 3 h was to ensure that during the withdrawal there was already an induction of Hsp27 occurring as seen in Figure 4.4. Cell survival was then assessed by PI staining at either 24 h (A) or 48 h (B) after NGF withdrawal. (C) Representative Western blot showing levels of Hsp27 expression with or without heat shock +/- NGF. (D, E) In –NGF and +NGF conditions after heat shock, Hsp27 expression was significantly increased compared to cultures not given a heat shock. Control conditions were taken as 100 % Hsp27 expression, and the cultures were maintained in the presence of NGF at 24 h and 48h (no heat shock). ***$P<0.001$


A
125
100
75
50
25
0
% survival
***
+NGF
+NGF+HS
-NGF
-NGF+HS
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B
125
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75
50
25
0
% survival
***
***
***
+NGF
+NGF+HS
-NGF
-NGF+HS
48h
C
24h + - + - + - + -
48h - + - + - + - +
HS - - - - + + + +
NGF - - + + - - + +
Akt
Hsp27
D
400
300
200
100
0
% Hsp27 expression
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+NGF
+NGF+HS
-NGF
-NGF+HS
24h
E
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% Hsp27 expression
***
***
***
+NGF
+NGF+HS
-NGF
-NGF+HS
48h

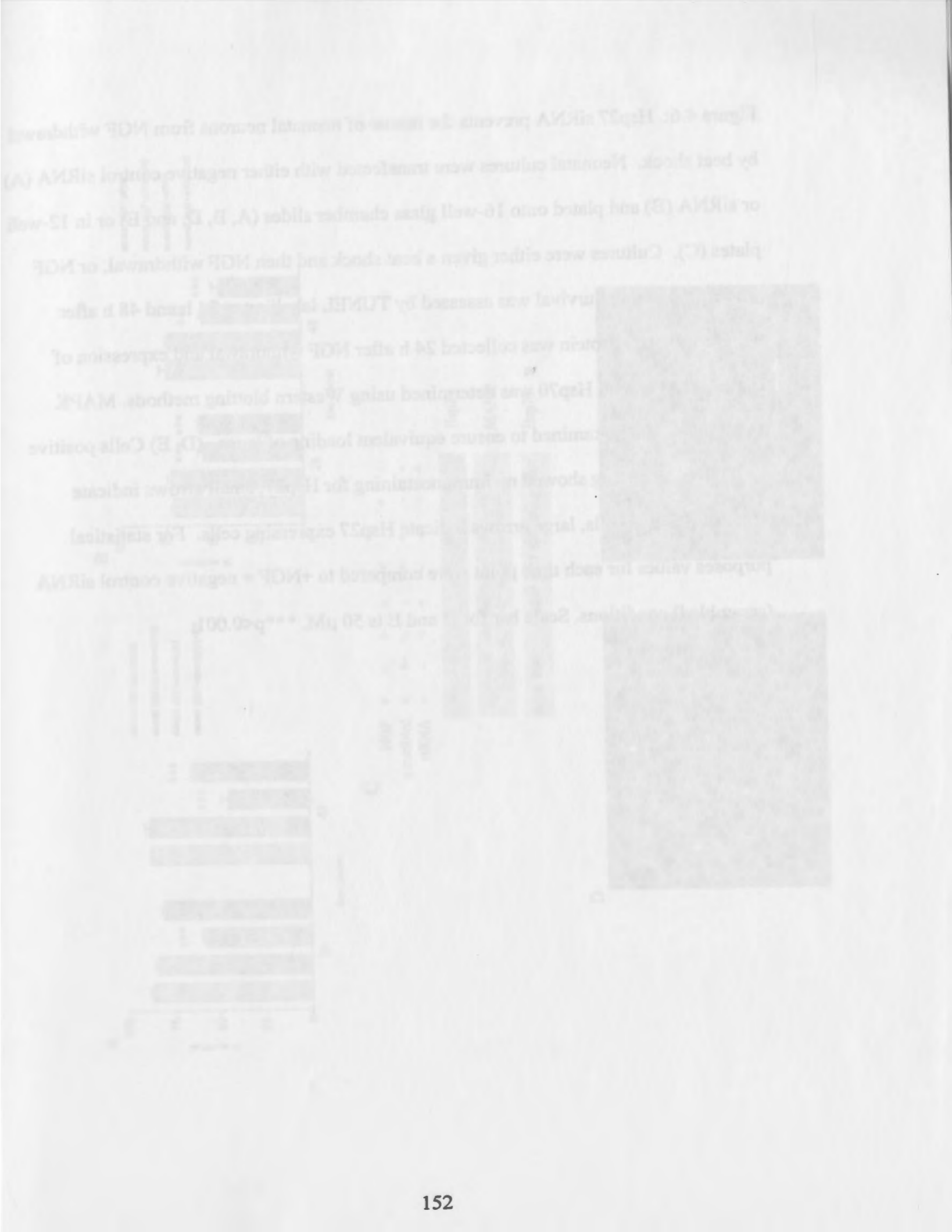


Figure 4.6: Hsp27 siRNA prevents the rescue of neonatal neurons from NGF withdrawal by heat shock. Neonatal cultures were transfected with either negative control siRNA (A) or siRNA (B) and plated onto 16-well glass chamber slides (A, B, D, and E) or in 12-well plates (C). Cultures were either given a heat shock and then NGF withdrawal, or NGF withdrawal alone. Survival was assessed by TUNEL labelling at 24 h and 48 h after withdrawal. (C) Protein was collected 24 h after NGF withdrawal and expression of Hsp27, MAPK, and Hsp70 was determined using Western blotting methods. MAPK 44/42 levels were examined to ensure equivalent loading of lanes. (D, E) Cells positive for TUNEL labelling showed no immunostaining for Hsp27; small arrows indicate TUNEL positive cells, large arrows indicate Hsp27 expressing cells. For statistical purposes values for each time point were compared to +NGF + negative control siRNA (scrambled) conditions. Scale bar for D and E is 50 μM. ***$p<0.001$.

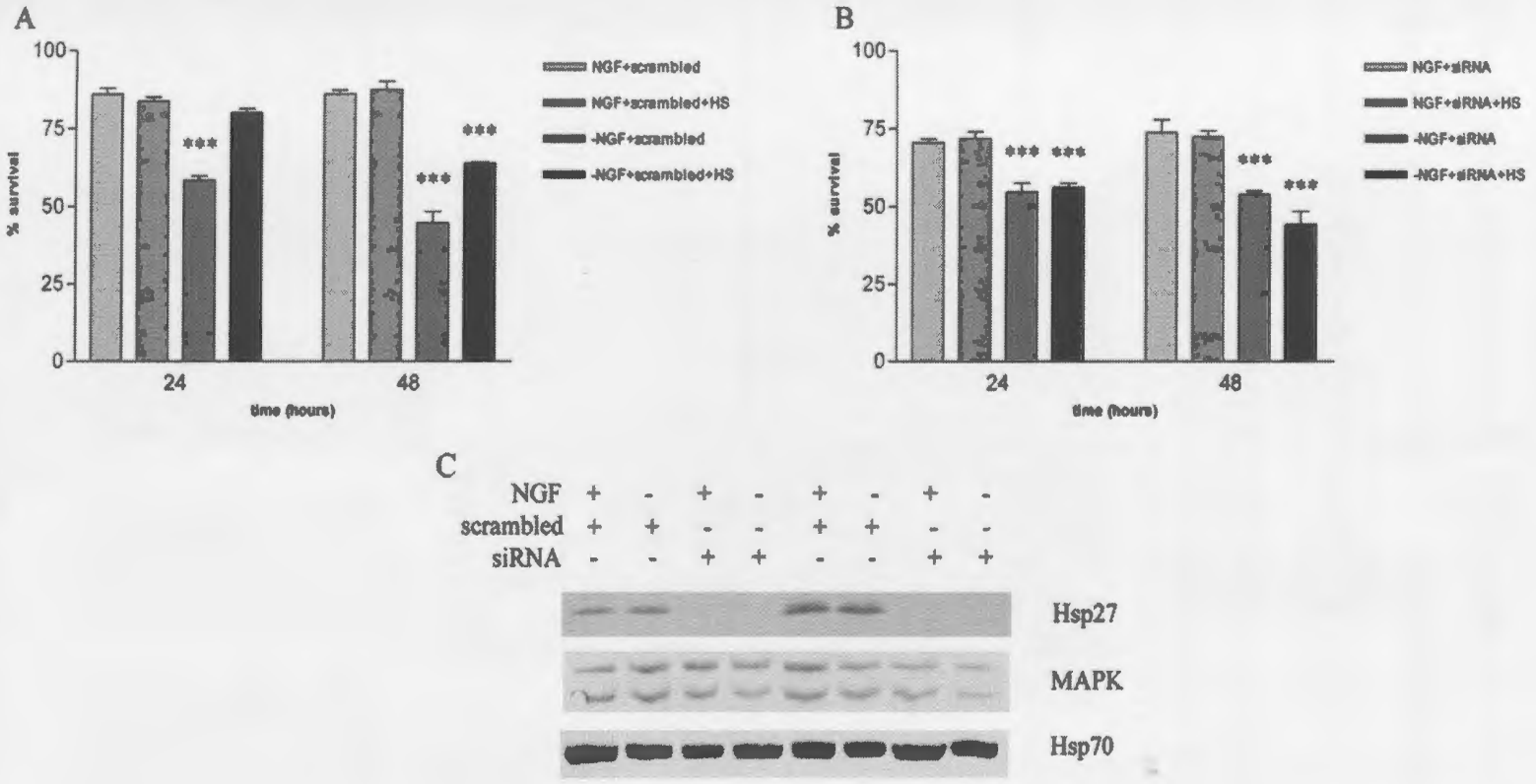
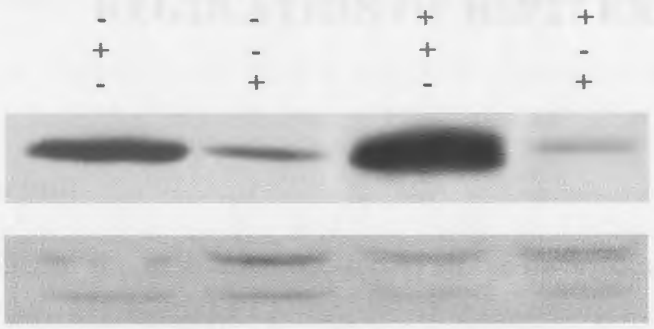
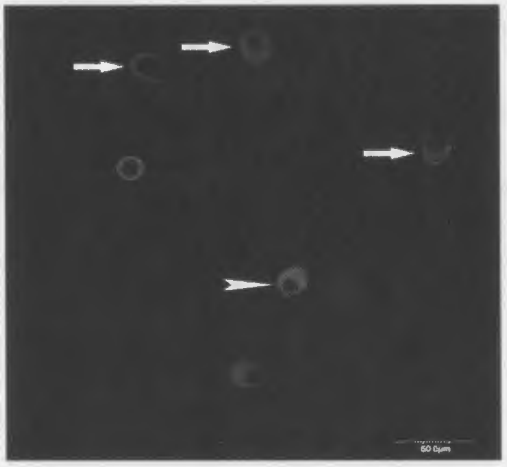

A
100
75
50
25
0
% survival
***
***
***
24
48
time (hours)
NGF+scrambled
NGF+scrambled+HS
-NGF+scrambled
-NGF+scrambled+HS
B
100
75
50
25
0
% survival
*** ***
***
***
24
48
time (hours)
NGF+siRNA
NGF+siRNA+HS
-NGF+siRNA
-NGF+siRNA+HS
C
NGF + - + - + - + -
scrambled + + - - + + - -
siRNA - - + + - - + +
Hsp27
MAPK
Hsp70
D
E

Figure 4.7: Hsp27 siRNA downregulates constitutive and induced expression of Hsp27 in adult neurons. (A) Adult neuronal cultures were plated after being nucleofected with either negative control siRNA (scrambled) or Hsp27 siRNA. Cultures were heat shocked and expression of Hsp27 was evaluated by Western blotting 24 h afterwards. MAPK 44/42 levels were examined to ensure equivalent loading of lanes. (B) Immunofluorescent staining of Hsp27 in the presence of siRNA at 24 h after transfection. Arrows indicate cells lacking Hsp27, arrowheads point to Hsp27 immunopositive cell. (C) Adult cultures were plated after nucleofection with either negative control siRNA or siRNA and survival was assessed at 24 h, 48 h and 72 h afterwards by TUNEL labelling. **$p<0.01$, ***$p<0.001$

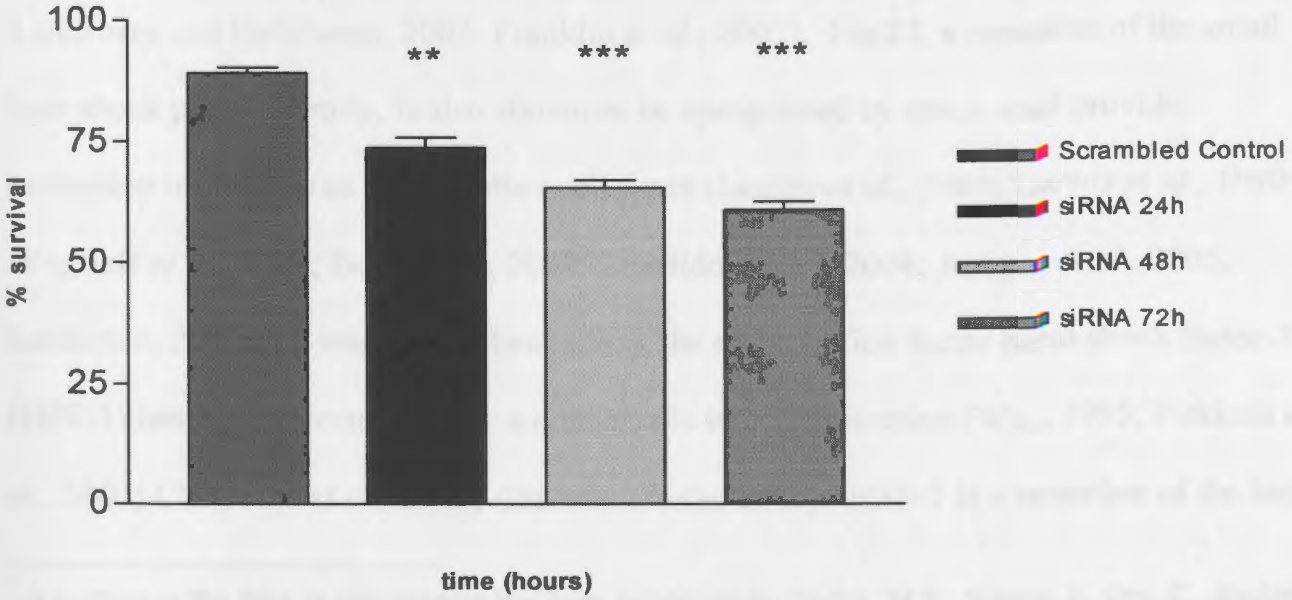
A


HS - - + +
scrambled + - + -
siRNA - + - +
Hsp27
MAPK

B



C


100
75
50
25
0
% survival
**
***
***
time (hours)
Scrambled Control
siRNA 24h
siRNA 48h
siRNA 72h

*CHAPTER 5

REGULATION OF HSP27 EXPRESSION

5.1 Introduction

Studies of the cellular response to stress have focused on induction of heat shock proteins (Hsps) as one of the most common occurrences among virtually all organisms. Stress responses result in changes in gene expression, with resultant changes in protein expression. The induction of these proteins via stress responses are one of a number of changes in protein expression and activation that serves to prevent apoptosis due to detrimental stressors, such as heat shock, oxidative stress and UV, and to repair cellular damage.

Hsp70 and Hsp90 have both been shown to be inducible with stress in the nervous system and to provide protection against insults (Ohtsuka and Suzuki, 2000; Richter-Landsberg and Goldbaum, 2003; Franklin *et al.*, 2005). Hsp27, a member of the small heat shock protein family, is also shown to be upregulated by stress and provide protection in neurons as well as other cell types (Landry *et al.*, 1989; Lewis *et al.*, 1999; Wagstaff *et al.*, 1999; Benn *et al.*, 2002; Zourlidou *et al.*, 2004; Arrigo *et al.*, 2005; Latchman, 2005). In response to heat stress, the transcription factor heat shock factor-1 (HSF-1) has been reported to play a crucial role in cell protection (Wu, 1995; Pirkkala *et al.*, 2001; Christians *et al.*, 2002; Ahn and Thiele, 2003). HSF-1 is a member of the heat

* **A portion of the data in this chapter has been published in:** Dodge, M.E., Wang, J., Guy, C., Rankin, S., Rahimtula, M., Mearow, K.M. (2006) Stress-induced heat shock protein 27 expression and its role in dorsal root ganglion neuronal survival. Brain Research Molecular Brain Research 1068: 34-48.

shock factor family of which there are four heat shock factors (HSF-1, HSF-2, HSF-3, HSF-4); all vary in their purpose and function, and as a family are conserved among many species. To date, only HSF-1 and HSF-3 have been linked to the heat shock response – HSF-1 being involved in mild heat stress and HSF-3 being involved in severe heat stress, although only expressed in avian cells. HSF-2 has been shown to be involved in development and differentiation, and the full function of HSF-4 is yet to be determined. The fact that there are different HSFs indicates that they are functionally and physiologically important in many organisms.

HSF-1 is important for Hsp gene expression, and its activation leads to conformational change from a monomeric form to an active, DNA-binding trimeric form, and this has been shown to be a specific response to heat stress (Mathew *et al.*, 2001). This trimeric form translocates from the cytoplasm to the cell nucleus where it binds via its DNA binding domain, an amino-terminal helix-turn-helix domain (Pirkkala *et al.*, 2001), to an nGAAn motif on the heat shock element (HSE) portion of the Hsp promoter and activates Hsp gene expression (Sarge *et al.*, 1993; Wu, 1995; Tonkiss and Calderwood, 2005). Neurons have relatively low levels of HSF compared to glial cells which has been suggested to result in a relatively compromised heat shock response (Tonkiss and Calderwood, 2005).

In addition, phosphorylation may also occur after HSF-1 has become transcriptionally active and results in increases in its activity. HSF-1 itself has been reported to be under regulation by candidates such as Hsp90, as HSF-1 activation has been shown to occur following the reduction of Hsp90 (Zou *et al.*, 1998). In addition, phosphorylation of HSF-1 on S303 and S307 also serve to negatively regulate HSF-1 and

renders it transcriptionally incompetent in the absence of stress (Chu *et al.*, 1996; Knauf *et al.*, 1996; Kline and Morimoto, 1997), and this phosphorylation has been suggested to occur via GSK-3 (Chu *et al.*, 1996; Chu *et al.*, 1998). Phosphorylation of S230, on the other hand, has been shown to occur after heat stress and to be important for transcriptional activity (Holmberg *et al.*, 2001).

Other factors have been implicated in regulation of Hsp27 expression, and many studies have focused on the estrogen receptor, ERβ. ERβ has the ability to bind ligand and promoter regions of genes, and as a result has been the target of prevention and treatment strategies for cancer (Osborne *et al.*, 2001). However, ER also interacts within other signalling pathways, such as cell survival, stress-activated and cell proliferation pathways, as well as induction of Hsp27. The Hsp27 promotor contains an estrogen response element (ERE) (Knowlton and Sun, 2001; Voss *et al.*, 2003) and treatment of MCF-7 cells with 17 β-estradiol has been shown to induce Hsp27 mRNA expression (Porter *et al.*, 1996). Additionally, in the ischemic brain, as well as in cardiac myocytes, 17 β-estradiol was also shown to induce Hsp27 expression (Lu *et al.*, 2002; Hamilton *et al.*, 2004).

The role of NF-κB, a transcription factor well known for its role in inflammatory and immune responses, is relatively unknown in DRG neurons. Heat stress is known to cause inhibition of NF-κB in both lung epithelium and A549 cells (Wong *et al.*, 1997a, 1997b), and is implicated as playing a role in apoptosis. However, in neurons, studies suggest that NF-κB is playing the opposite role – as a mediator of cell survival, as its inhibition via SN50 has caused neuronal cell death (Maggirwar *et al.*, 1998; Yu *et al.*,

1999; Fernyhough *et al.*, 2005), and this may be through upregulation of anti-apoptotic genes such as Bcl-x (Ma and Bisby, 1998).

In our efforts to understand how Hsp27 expression might be regulated in neurons in order to promote neuronal survival, we have correlated expression and activation of the transcription factor, HSF-1, with the physiological heat stress and the subsequent upregulation of Hsp27 in both neonatal and adult DRG neurons, as well as in PC12 cells. Additionally, alternate regulators of Hsp27 expression were examined for their role in Hsp27 induction.

5.2 Materials and Methods

5.2.1 Cell Culture

Primary DRG neurons were dissected from young adult (4-5 wks old) or postnatal day 2 (P2) Sprague-Dawley rats. Animals were anaesthetized with Somnotol and sacrificed by decapitation. The usage of animals was approved by the Institutional Animal Care Committee, St. John's, NL, Canada. DRG neurons from young adults were cultured as described previously (Dodge *et al.*, 2002). In brief, ganglia were extracted from all spinal cord levels and incubated in 0.25 % collagenase type II (Invitrogen/Life Technologies, Burlington, Canada) for 45 min at 37°C, and then incubated with 0.25 % trypsin (Invitrogen) for 20 min at 37°C. The ganglia were dissociated by a series of manual triturations using polished Pasteur pipettes. The cell suspension was centrifuged at 1000 rpm and the resulting cell pellet was suspended in serum-free Neurobasal medium (Invitrogen) supplemented with B27 additives and antimitotics (10 μM cytosine

arabinoside, Sigma, St. Louis, MO, USA). P2 neurons were cultured similarly with the following changes: DRGs were not incubated with collagenase but were incubated with trypsin for 45 min at 37° C, followed by incubation in DNAse A (10 mg/mL) for 5 min at room temperature. Dissociation was also by manual trituration and suspension of cells was in serum-free Neurobasal medium supplemented with B27 additives, N_2 and antimitotics. Cells were plated at a density of 5000-7000 cells per well of a 12-well plate for protein analysis. Cells to be plated on culture slides were centrifuged on a 30 % Percoll (Amersham Biosciences, Quebec) gradient and plated at a density of 200-300 cells per well. Medium was supplemented with 50 ng/mL NGF (Cedarlane Labs, Hornsby, Canada) where appropriate.

5.2.2 Cell Culture Treatments

For heat shock treatment, culture plates were immersed in a 45°C water bath for 15 min. Cells were allowed to recover for varying intervals after heat shock and sampled for protein analysis or fixed for immunocytochemistry. For experiments testing the effects of estrogen on Hsp27 expression, phenol red-free culture media was supplemented with 10 nM or 50 nM 17 β-estradiol for 24 h prior to protein collection (Berthois *et al.*, 1986).

5.2.3 Western Blotting

Culture medium was aspirated and cells were removed with ice-cold Tris-buffered saline (TBS) with sodium vanadate (200 mM). Cell pellets were suspended in protein lysis buffer consisting of 10 % glycerol, 1 % Nonidet P-40, sodium vanadate (200 mM),

sodium fluoride (200 mM), and one protease inhibitor cocktail tablet (Roche Scientific, Laval, QC). Cell lysates were then centrifuged at 10,000 rpm for 10 min. For experiments involving subcellular fractionation, protein was extracted using the Subcellular Proteome Extraction Kit (Calbiochem, La Jolla, CA). Protein concentrations were determined using the BCA protein assay (Pierce Chemicals, Rockford, IL, USA). Equivalent amounts of protein (30 ug) were electrophoresed on 10 % SDS-polyacrylamide gels. Following transfer to nitrocellulose, blots were stained with Ponceau Red to assess the equivalency of protein loading. Following washing with TBS, the blots were blocked in 3 % milk-TBST (Tris-buffered saline and Tween 20 at 0.2 % v/v) and probed with polyclonal antibodies to pHsp27 (1:800, Affinity Bioreagents, CA), Hsp70 (1:10,000, Stressgen, Victoria, B.C), Hsp27 (1:2000, Stressgen), MAPK (1:1000, Santa Cruz Biotechnologies, California), pAkt (1:1000) and Akt (1:1000, Cell Signalling, Mississauga, Canada) ERβ (1:100, Affinity Bioreagents) and a monoclonal antibody to HSF-1 (1:1000) (Stressgen).

5.2.4 Immunocytochemistry

Adult and neonatal neurons were plated on 16-well glass chamber slides (Nunc, VWR Canlab, Mississauga, ON, Canada). The medium was aspirated and cells were fixed with 4 % formaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1 % Triton-X and blocked with 10 % goat serum in PBS. Subsequently, cells were incubated with a polyclonal primary antibody to Hsp27 (1:250, Stressgen), pHsp27ser15 (1:100, Affinity BioReagents) or HSF-1 (1:100, Stressgen) overnight at 4°C. Secondary

antibodies labelled with Cy2 or Cy5 (1:100, Jackson Immunoresearch Laboratories, West Grove, PA, USA) and conventional fluorescence or scanning laser confocal microscopy was used to visualize the immunolabeled cells.

5.2.5 Electromobility Shift Assay (EMSA)

Oligonucleotides complementary to the heat shock element (HSE, 5' CTA GAA GCT TCT AGA AGC TTC TAG 3' and 5' CTA GAA GCT TCT AGA AGC TTC TAG 3', Integrated DNA Technologies) or NF-κB consensus sequence (5'AGT TGA GGG GAC TTT CCC AGG 3' and 5' GCC TGG GAA AGT CCC CTC AAC T 3') were annealed by incubating 10 pmol of each probe in a 10 μL reaction containing 20 mM Tris HCl (pH 8.4) and 50 mM KCl. Tubes were then heated to 80°C for 5 min, then slowly cooled to room temperature. Half of these mixtures were end-labelled with [γ-^{32}P] dATP by incubating annealed oligonucleotides with 5X forward reaction buffer and 10U T4 polynucleotide kinase (Invitrogen). For preparation of cold competition probes, 100 pmol of oligonucleotide was used for annealing in a 10 μL reaction and 10 pmol (1 μL), which is approximately 100 fold excess of the hot probe, was used for each reaction.

Protein was collected using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology Inc, Rockford, IL, USA). An equal amount of protein (20 ug) from each sample was used in 25 μL binding reactions, which consisted of 1 ug poly dI-dC, 2X binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA, 10% glycerol and 10 mg/mL BSA), labelled probe, and for competition and supershift assays, with either unlabeled probe or 2 μL HSF-1 antibody (Affinity Bioreagents).

Protein lysate volumes were made to equal 5 μL by addition of buffer C (20 mM Hepes pH 7.8, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.5 mM PMSF). To determine specificity of DNA binding, unlabeled competitor DNA was added to the binding reactions and allowed to incubate for 10 min at room temperature. Following this incubation, labelled probe was added and samples were further incubated for 20 min at room temperature. Samples were then electrophoresed on a 5 % nondenaturing polyacrylamide gel in 0.5X TBE for 1.5 h at 120 V and visualized by autoradiography.

5.2.6 Real Time RT-PCR

RNA was isolated using Trizol reagent (Invitrogen) as per the manufacturer's instructions and was subsequently treated with DNase (Ambion, Austin, TX) to remove traces of contaminating DNA. RNA was reverse transcribed to cDNA using MMLV reverse transcriptase (Invitrogen) prior to use as template for real-time PCR amplification using the following PCR primer pairs: HSF-1 plus 5'AAGCCTGAGAGGGATGACAC; HSF-1 minus 5'TTCGACTGCACCAGTGAGAT; Hsp27 plus 5' AAGGAAGGCGTGGTGGAGAT; Hsp27 minus 5' CCTGGAGGGAGCGTGTATTTC; GADPH plus 5' CCATCACCATCTTCCAGGAG; GADPH minus 5' CCTGCTTCACCACCTTCTTG. PCR amplification was performed using the Roche LightCycler (Laval, QC) and quantified using SYBR green I. Hsp27 and HSF-1 mRNA expression was subsequently normalized using the housekeeping gene GADPH.

5.2.7 Statistical Analysis

Data were analyzed for significance using one-way ANOVA, followed by post-hoc testing with Tukey's test for multiple comparisons. Data were expressed as percentages relative to control values. All experiments were replicated a minimum of three times *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.3 Results

5.3.1 Transcriptional regulation of Hsp27

Hsp27 expression can be regulated by the transcription factor, Heat Shock Factor 1 (HSF-1), among other factors. We examined expression and activation of HSF-1 in both neonatal and adult neuronal cultures to determine whether Hsp27 is under transcriptional regulation by HSF-1 in DRG neurons. Adult and neonatal neurons were plated in 16-well chamber culture slides and immunostained (1 h after HS) for Hsp27 and HSF-1. Figure 5.1A shows expression of Hsp27 in adult neurons, while Figure 5.1D shows Hsp27 expression in neonatal neurons. As previously noted (Chapter 4, Figure 4.1), staining in adult neurons is abundant and intense, extending from the cell body into neurites as well, whereas very few neonatal neurons show expression of Hsp27. Interestingly, HSF-1 was located in both adult (Figure 5.1B) and neonatal cells (Figure 5.1E), and following heat shock was observed to translocate to the nucleus in the adult neurons (Figure 5.1B - arrow points to nuclear localization), while nuclear translocation was not clearly observed in the neonatal neurons using immunocytochemical methods (Figure 5.1E). Although levels of Hsp27 in adult and neonatal neurons differ, HSF-1

expression and translocation does appear to occur in both after heat stress. Figures 5.1C and 5.1F show composites of Hsp27 and HSF-1 immunofluorescence.

Using a subcellular fractionation protocol, we examined localization of HSF-1 before and after heat shock. HSF-1 in both adult and neonatal neurons becomes active and translocates to the nucleus (activity is indicated by a slight increase in protein size and a corresponding upwards shift in the band from 85 to 95 kDa). Figure 5.1G presents a representative Western blot of HSF-1 expression in adult and neonatal neurons. Note the primarily cytoplasmic localization in control lysates compared to the primarily nuclear localization after heat shock. These results indicate that HSF-1 is constitutively present in both adult and neonatal neurons, albeit at relatively low levels compared to PC12 or HeLa cells (data not shown) and can be activated after heat stress in both adult and neonatal neurons. However, these results do not explain the differences in constitutive expression of Hsp27 between the adult and neonatal neurons. It may be that other factors play a role in this expression.

To further determine the relationship between HSF-1 translocation and increased expression of Hsp27 after heat shock, a gel-shift assay was performed to analyze the activation of HSF-1 by examining its DNA binding activity at the HSE element of the Hsp27 promoter. In Figure 5.2A, PC12 nuclear protein lysates were incubated with labelled probe specific for HSE of the Hsp27 promoter. All probe not bound to protein in the binding reaction moves much quicker during electrophoresis, as it is not retarded by protein. This free probe can be seen at the bottom of the gel. The band observed in lane 1 corresponds to a protein-probe complex due to the binding of probe to HSF-1 in the protein sample. Lane 2 demonstrates a gel-shift, which is a band further retarded in

movement due to the binding of HSF-1 antibody to the protein-probe complex. Lane 3 contains free probe only, located at the bottom of the gel. These results will be further discussed in Figure 5.5. Our results for DRG neurons, in Figure 5.2B show that as early as 30 min after heat shock (lane 2) and likewise at 1 h afterwards (lane 3), HSF-1 acquires DNA binding activity. The specificity of the observed bands was determined by performing a cold competition with unlabelled probe (lane 4 – HSE oligonucleotides; lane 5- NFkB oligonucleotides). Specificity was additionally determined by a supershift gel mobility shift assay with the HSF-1 antibody (lane 6). Note that the neurons contain relatively little HSF-1 as compared to PC12 cells (lane 7 – HSE binding in PC12 cell lysates).

To determine not only if HSF-1 acquired DNA binding activity after heat shock, but to correlate this with HSF-1 mRNA expression, we carried out real time RT-PCR analysis of both HSF-1 (Figure 5.3A) and Hsp27 (Figure 5.3B) mRNA expression over the time course of 1 h to 6 h after heat shock. Our results show no changes of HSF-1 mRNA expression over this time frame, although there was a significant increase in Hsp27 mRNA expression by 6 h after heat shock. Likewise, Hsp27 protein expression is also significantly increased by this time (see Chapter 4, Figure 4.4).

NF-κB is another transcription factor that has been reported to play a role in the regulation of adult DRG neuronal survival (Fernyhough *et al.*, 2005). To determine the possible role of NF-κB in regulation of the heat shock response in DRG neurons, DNA-binding assays were performed using sequences specific to the NF-κB consensus sequence. Figure 5.4A shows that activation of NF-κB DNA-binding activity occurs as

early as 30 min after heat shock (lane 3) and continued at 1 h after heat shock (lane 4-6). Specificity was determined by cold competition with unlabeled NF-κB oligonucleotide (lane 2 and 5) or unlabeled HSE oligonucleotide (lane 6). However, we were unable to determine whether p50, p65 or both components were responsible for this activation. This was likely due to the particular antibody reagents we had available for use.

To determine other possible factors that may play a part in regulation of Hsp27 expression in DRG neurons, the effect of estrogen on Hsp27 expression was determined. Our results show that treatment of cultures with either 10 nM or 50 nM 17 β-estradiol for 24 h caused a resultant induction of Hsp27 expression (Figure 5.4B). However, no effects on estrogen receptor (ERβ) expression were observed after heat shock (data not shown).

As we had previously determined that PC12 cells also showed induction of Hsp27 after heat shock (Chapter 3, Figure 3.2), possible regulation of this expression by HSF-1 and/or NF-κB was determined in PC12 cells. Gel-shift assays were performed to determine the DNA-binding activity of HSF-1 at the HSE of the Hsp27 promoter (Figure 5.5A). DNA-binding activity was observed 1 h after heat shock (lanes 2-5) and this band was successfully competed out with unlabeled cold HSE oligonucleotide (lane3), but not with unlabeled cold NF-κB oligonucleotide (lane 4). Specificity was additionally determined by a supershift gel mobility shift assay with the HSF-1 antibody (lane 5). Figure 5.5B shows NF-κB DNA-binding activity before (lane 1) and 1 h after heat shock (lanes 2-4). Specificity was determined by competition with unlabeled cold NF-κB oligonucleotide (lane 3) or with cold unlabeled HSE oligonucleotide (lane 4). Both bands

appear to be specific for NF-κB DNA-binding activity, although we were unable to determine whether p50, p65 or both components were responsible for this activation.

5.4 Discussion

The results of this study show that when cultures were subjected to sublethal heat stress, HSF-1 translocated from the cytoplasm to the nucleus, and also acquired DNA-binding ability. In addition, the NF-κB consensus sequence also showed DNA-binding activity after heat stress.

Hsp27 expression and induction by heat stress is regulated at the transcriptional level by the transcription factor HSF-1. HSF-1 is rapidly activated by heat shock, acquires DNA binding activity once activated and can be measured by a shift in protein band size during western blotting (Wu, 1995; Cotto *et al.*, 1997; Holmberg *et al.*, 2000; Tonkiss and Calderwood, 2005). Under stressful cellular conditions such as heat shock, proteins are altered and may become denatured or misfolded. Although it is not understood how these changes in proteins trigger the activation of HSF-1, its activation and translocation from the cytoplasm to the nucleus results in induction of heat shock proteins, which serve to refold proteins and repair damage by stress (Wu, 1995; Tonkiss and Calderwood, 2005). Our results show that a sublethal heat stress does indeed activate HSF-1, causing its translocation from the cytoplasmic to the nuclear protein fraction. These results suggested that it is involved in the heat shock response and induction of Hsp27. We also examined the DNA binding activity of HSF-1, as well as mRNA levels of both HSF-1 and Hsp27 after heat shock. Following heat shock, we observed an early

increase in the DNA binding activity of HSF-1 to the HSE of Hsp27. Our observations of activation and translocation of HSF-1 to the nucleus within 1 h of recovery, acquisition of DNA binding ability, and the resultant increase in both Hsp27 mRNA and protein support the view that Hsp27 is regulated by constitutive levels of HSF-1. Although a number of studies have studied HSF-1 activation and its role in Hsp27 induction (Neininger and Gaestel, 1998; Trinklein *et al.*, 2004; Yan *et al.*, 2005), there is relatively little known about HSF-1 and regulation of Hsp expression in neurons. CNS neuronal levels of HSF-1 are low compared to glial cells and this has been suggested to be a factor in the relatively compromised ability of neurons to upregulate heat shock proteins (Tonkiss and Calderwood, 2005). To our knowledge, this is the first report of HSF-1 activation and correlation with Hsp27 induction in PNS neurons.

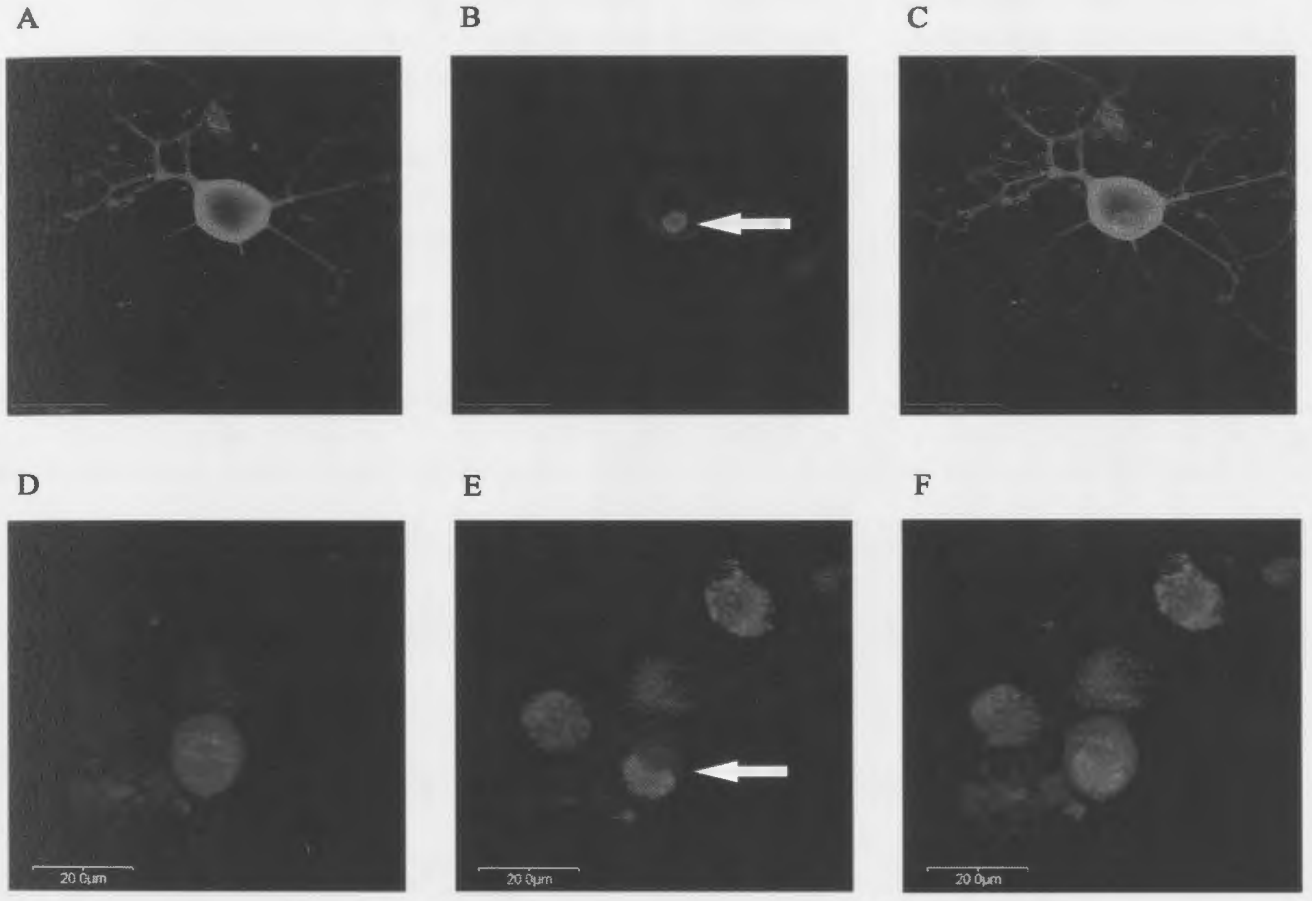
Reports of a possible estrogen response element located in the Hsp27 gene (Knowlton and Sun, 2001; Voss *et al.*, 2003) had also raised the question of possible regulation of Hsp27 by multiple factors. The level of HSF-1 in DRG neurons is low compared to other cell types, such as Hela cells (data not shown), and investigation of estrogen as a possible regulator of the heat shock stress response led to the conclusion that estrogen causes induction of Hsp27 in our cultures. An earlier study had shown that estradiol induces HSF-1, resulting in Hsp27 induction in the absence of stress (Lu *et al.*, 2002), and further study will be necessary to determine the mechanism by which Hsp27 becomes induced by estradiol in DRG neurons.

Recently, NF-κB was reported to play an important role in adult DRG neuronal survival after axotomy (Fernyhough *et al.*, 2005) . NF-κB activation results from various

stresses or injury, and can promote survival or apoptosis depending upon the cellular context and/or the stimulus (Bhakar *et al.*, 2002; Pizzi *et al.*, 2002; Zhu *et al.*, 2004; Kratsovnik *et al.*, 2005). Our results show that not only is HSF-1 likely involved in induction of Hsp27 after heat shock, but that NF-κB also acquires DNA binding activity following heat stress. The relationship between heat shock and NF-kB is complex, with reports indicating both positive (Rossi *et al.*, 1998; Maroni *et al.*, 2003) and negative effects of heat stress on NF-kB activation (DeMeester *et al.*, 2001; Chen *et al.*, 2004). Although we have not investigated the role that NF-κB plays directly in Hsp27 induction, further investigation is needed to determine the role of NF-κB after a heat stress and if it is possibly serving to inhibit apoptosis through other signalling intermediates.

5.5 Figures

Figure 5.1: HSF-1 becomes activated after heat shock in adult and neonatal neuronal cultures. Adult and neonatal DRGs were dissociated and plated on 16-well chamber glass slides. Cultures were heat shocked, fixed 1 h afterwards and incubated with either Hsp27 or HSF-1 primary antibody. (A, D) Expression of Hsp27 after a 1 h recovery from heat shock in adult (A) and neonatal (D) cultures. (B, E) show expression of HSF-1 and its translocation from the cytoplasm to the nucleus after heat shock in adult (B) and to the perinuclear region in neonatal (E) cultures. (C) is a merged image of (A) and (B), and (F) is the merged image of (D) and (E). (G) Representative western blot of HSF-1 expression in adult and neonatal neurons. Before and 1 h after heat shock, protein from adult and neonatal cultures was extracted with the Subcellular Proteome Extraction Kit to yield proteins from different cellular compartments: cytoplasm, membrane, nucleus and cytoskeleton. Activation of HSF-1 was observed in both adult and neonatal cultures, as evidenced by its translocation to the nucleus and by the shift in protein band size in western blotting experiments (arrows point to cells with nuclear immunostaining for HSF-1).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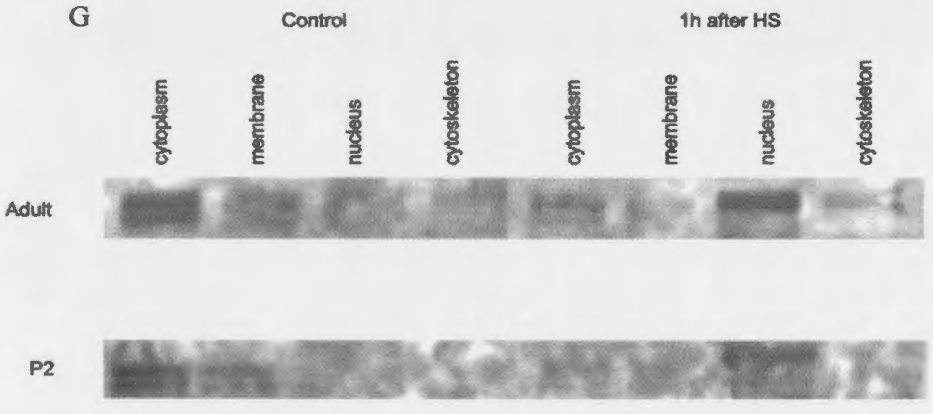
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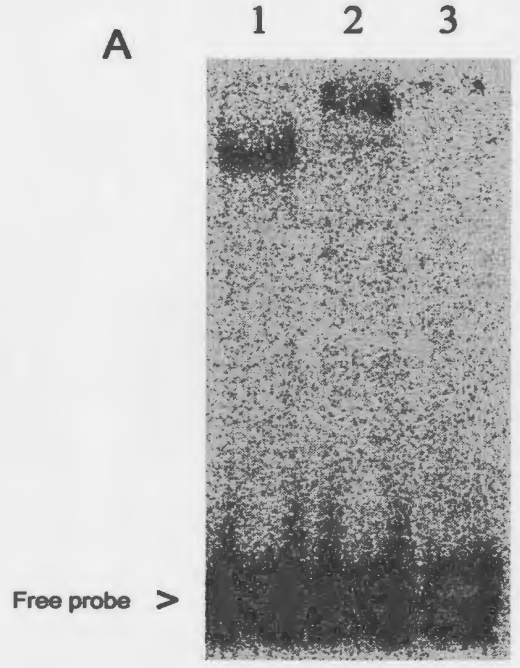
Figure 5.2: HSF-1 acquires DNA-binding activity. **(A).** PC12 cell protein lysates were incubated with radioactive labelled oligonucleotide specific for the HSE of the Hsp27 promoter. Lane 1 and 2 show bands specific for protein-probe complexes and protein-probe-HSF-1 antibody complexes, respectively. Also demonstrated is the mobility of free probe, located at the bottom of the gel. (B). Adult neuronal cultures were heat shocked at 45°C for 15 min and nuclear protein was then collected at 30 min and 1 h afterwards. HSF-binding activity was examined by native gel-shift assay before heat shock (lane 1), 30 min after heat shock (lane 2), and 1 h after heat shock (lanes 3-6). Bands of HSE-protein complexes were visualized by autoradiography. Specificity was determined by competition with a unlabeled HSE oligonucleotides (5' cta gaa gct tct aga agc ttc tag 3' and 5' cta gaa gct tct aga agc ttc tag 3') (lane 4), unlabeled NF-κB consensus sequence oligonucleotides (5' agt tga ggg gac ttt ccc agg c 3' and 5'gcc tgg gaa agt ccc ctc aac t 3') (lane 5), and by pre- incubation with an HSF-1 antibody (lane 6). HSE binding activity of nuclear protein from PC12 cells collected 1 h after heat shock is shown in lane 7. Lane 8 contains free probe only. Lower arrowhead – binding to HSE element; Upper arrowhead – band shift with anti-HSF in lane 6.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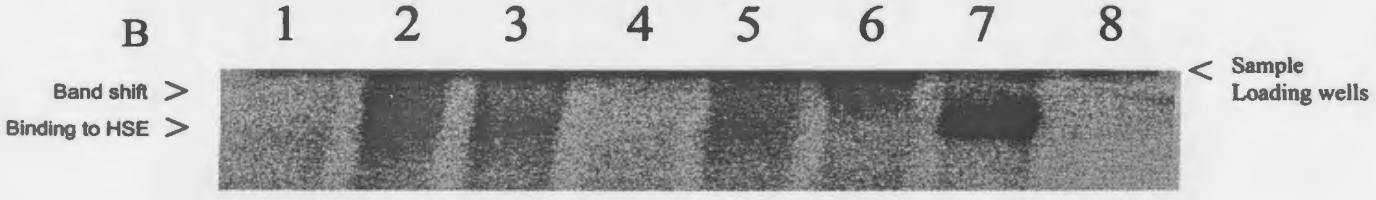
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Figure 5.3: mRNA expression of HSF-1 and Hsp27. (A) HSF-1 and Hsp27 (B) mRNA expression before and 1 h, 3 h and 6 h after heat shock, as determined by real time RT-PCR quantification of SYBR green I. Data is expressed as mean ± SEM. **$p < 0.01$

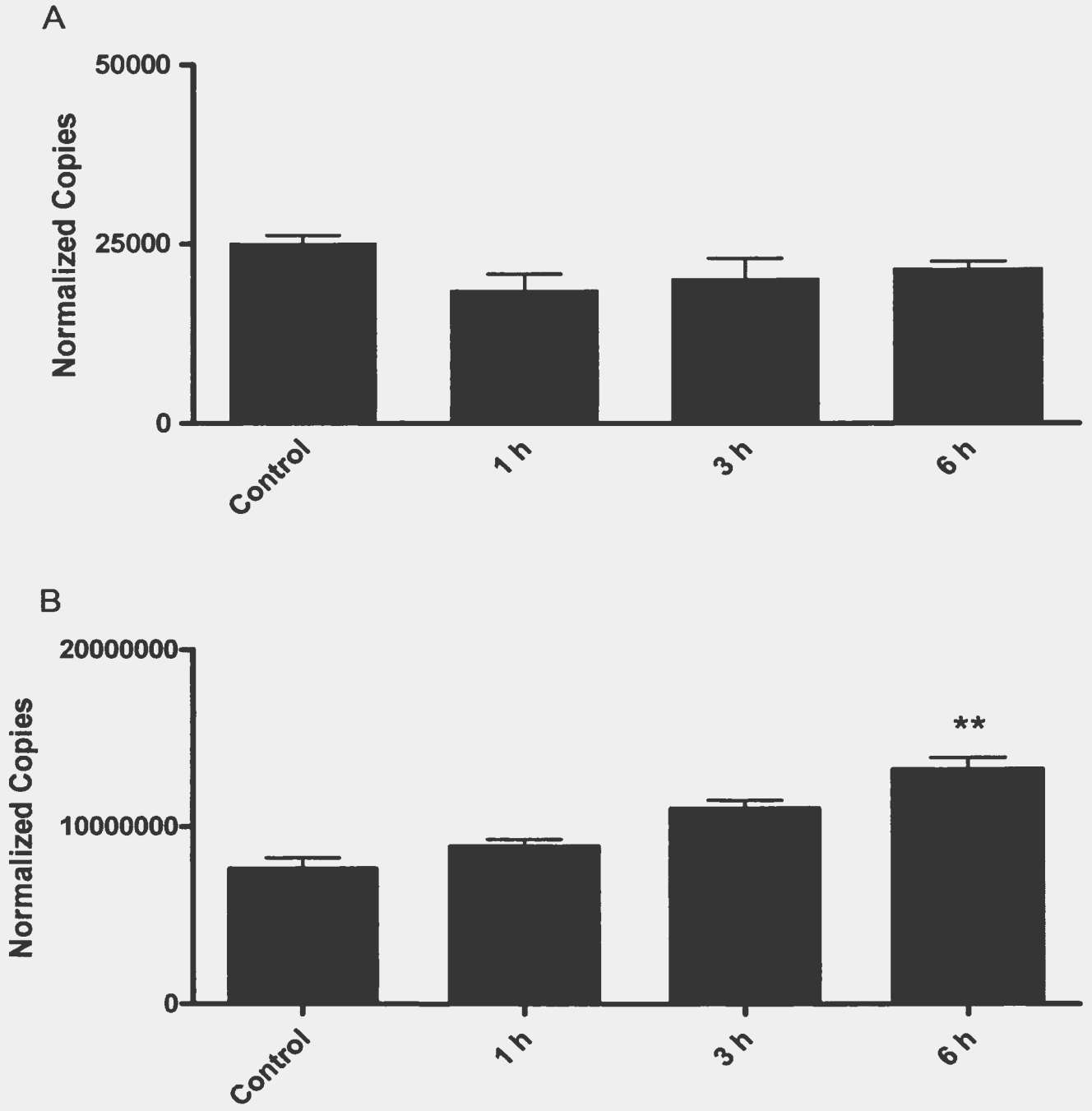
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Figure 5.4: Alternate regulators of Hsp27. (A) NF-κB DNA-binding was determined by gel-shift assay before heat shock (lanes 1 and 2), 30 min after heat shock (lane 3) and 1 h after heat shock (lanes 4-6). Specificity was determined by competition with unlabeled NF-κB oligonucleotide (5' agt tga ggg gac ttt ccc agg c 3' and 5'gcc tgg gaa agt ccc ctc aac t 3') (lanes 2 and 5) or unlabeled HSE oligonucleotides (5' cta gaa gct tct aga agc ttc tag 3' and 5' cta gaa gct tct aga agc ttc tag 3') (lane 6). Lane 7 contains free probe only. (B) Response of Hsp27 to 17-β estradiol was determined by incubation of adult neuronal cultures in media containing 17-β estradiol for 24 h.

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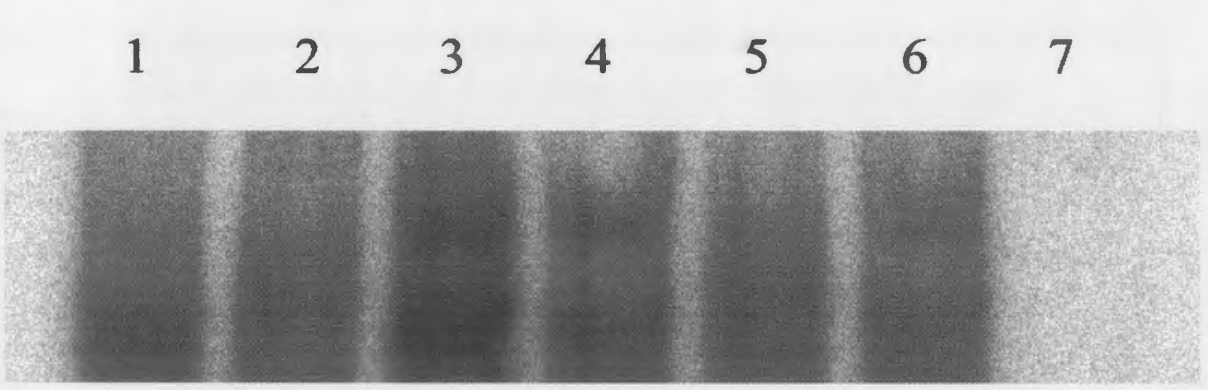

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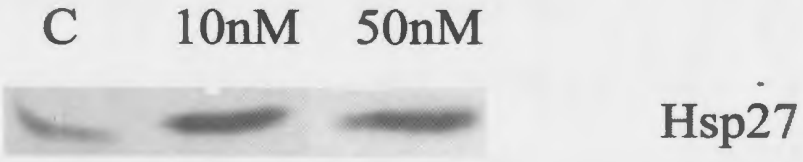

Figure 5.5: **(A)** Possible regulators of Hsp27 in PC12 cells. (A) PC12 cell cultures were heat shocked at 45°C for 15 min and nuclear protein was then collected at 30 min and 1 h afterwards. HSF-1 binding activity was examined by native gel-shift assay before heat shock (lane 1) and 1 h after heat shock (lanes 2-5). Bands of HSE-protein complexes were visualized by autoradiography. Specificity was determined by competition with unlabeled HSE oligonucleotides (5' cta gaa gct tct aga agc ttc tag 3' and 5' cta gaa gct tct aga agc ttc tag 3') (lane 3), unlabeled NF-κB consensus sequence oligonucleotides (5' agt tga ggg gac ttt ccc agg c 3' and 5'gcc tgg gaa agt ccc ctc aac t 3') (lane 4), and by pre-incubation with an HSF-1 antibody (lane 5). Lane 6 contains free probe only. Lower arrowhead – binding to HSE element; Upper arrowhead – band shift with anti-HSF in lane 6. (B) NF-κB DNA-binding was determined by gel-shift assay before heat shock(lane 1) and 30 min after heat shock (lane 2-4). Specificity was determined by competition with unlabeled NF-κB oligonucleotides (5' agt tga ggg gac ttt ccc agg c 3' and 5'gcc tgg gaa agt ccc ctc aac t 3') (lanes 3) or unlabeled HSE oligonucleotides (5' cta gaa gct tct aga agc ttc tag 3' and 5' cta gaa gct tct aga agc ttc tag 3') (lane 4). Lane 5 contains free probe only.

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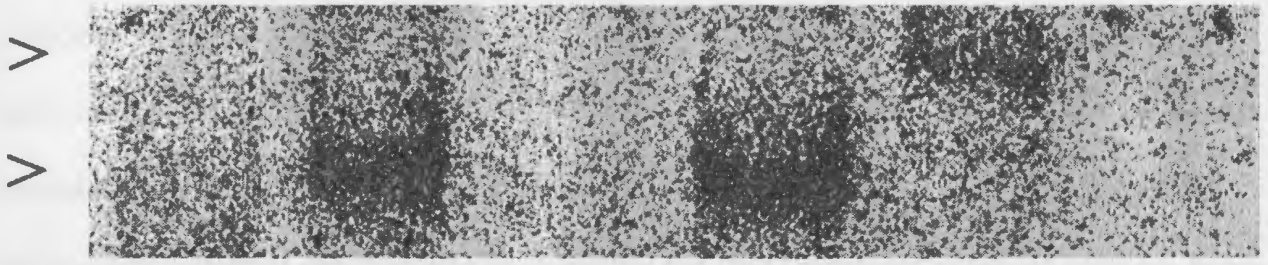

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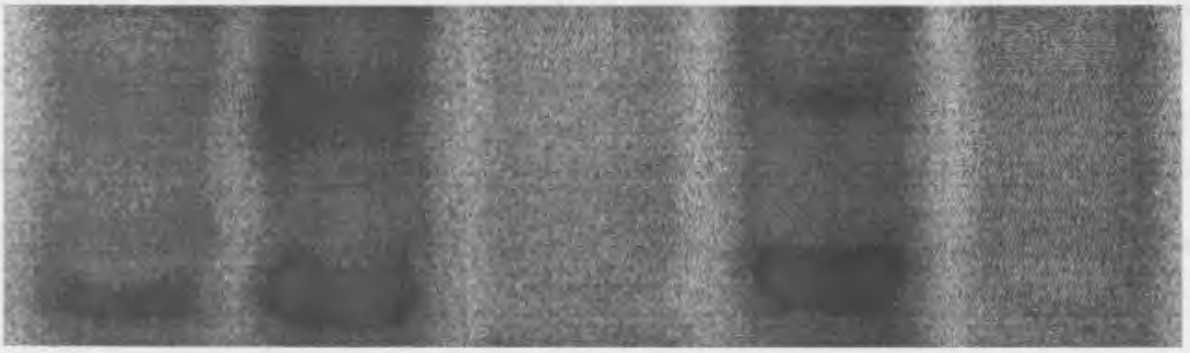

CHAPTER 6

DISCUSSION

This thesis involved an investigation of the signal transduction mechanisms in DRG neurons potentially necessary for their survival during neurotrophin-independence in adulthood, emphasizing the importance of Hsp27 as an anti-apoptotic factor in response to stressful stimuli. Testing of my hypotheses in some instances required preliminary experimentation using PC12 cell lines for the study of neurotrophin signal transduction mechanisms. The ultimate goal of this thesis, however, has been to understand potential mechanisms responsible for DRG neuronal survival with the hope that my results will further the understanding of how PNS neurons respond to stress and injury.

Multiple signal transduction pathways and intermediates have been identified within the DRG and attempts have been made to determine their relative importance with regards to survival. This thesis has focused on cellular survival, as in order for PNS regeneration to occur, it is essential that there must first be a healthy cellular environment in which to promote growth. Although select intermediates have been proposed to be key players, there are still unknown variables in signalling pathways that remain to be elucidated. Neurotrophin-dependent developing DRG neurons have been one model used to determine signalling intermediates important for neuronal survival. It is as important to determine signal transduction events in the neurotrophin-independent adult neurons, as mature neurons are most commonly involved in neurological injury or disease.

Our initial studies involved confirming adult DRG neurotrophin-independence, and our results supported previous reports where adult sensory neurons survive *in vitro* in the absence of exogenous NGF and neutralization of neurotrophin does not result in cell death (Lindsay, 1988; Mendell, 1999). Subsequently, possible factors that contribute towards neurotrophin-independent survival of adult DRG neurons were investigated, with concentration on previously identified pathways and intermediates that have been proven to be relevant, such as the PI-3K, MAPK, and PKC pathways.

Previous studies in our laboratory have determined that the PI-3K/Akt pathway is important for neonatal neuronal survival in DRG neurons, and as shown in Chapter 2 this pathway is also important to survival of adult DRG neurons, although to a lesser extent. Involvement of PLC in neurotrophin-dependent differentiation and survival does not carry over into adulthood, although in our studies it was determined that the catalytic site of PKC does contribute to neurotrophin-independent survival, since protein inhibitors specific for this site effected a significant decrease in survival.

Concurrent studies involving PC12 cells resulted in shifting focus to Hsp27 and its role in cellular survival and interactions with signalling intermediates we previously determined to be important for neuronal survival. Constitutive expression of Hsps, specifically Hsp70 and Hsp27, vary in different body regions and tissues and are highly inducible in response to stress. Chapter 3 examined the protective role of Hsp27 during stress in Hsp27-overexpressing PC12 cells as well as NGF-withdrawal in differentiated PC12 cells. These studies are relevant as we showed that Hsp27 is important in protecting NGF-differentiated cells from apoptosis due to NGF-withdrawal, heat and oxidative stress. Use of experimental methods such as viral Hsp overexpressing vectors

to elucidate their function in neurons have also been effective (Benn *et al.*, 2002; Kalwy *et al.*, 2003), although in our studies we have primarily used the physiological stimulus of heat shock to stimulate a stress response. During stressful stimuli in our experiments, phosphorylation of Hsp27 and activation of Akt and p38MAPK occurs. Complexes involving Hsp27, Akt and p38MAPK resulted after heat stress and these complexes have been observed in other cell types as well, such as neutrophils (Rane *et al.*, 2003). These findings lend further evidence to the importance of Hsp27 and its ability to interact with multiple signalling intermediates in pathways involved in cellular survival.

Hsps are also developmentally regulated (Morimoto *et al.*, 1992). We found that Hsp27 is differentially expressed in DRG neurons with low levels of expression in postnatal neurons compared to adult neurons, and subsequently investigated Hsp27 as a contributor to NGF-independent survival of adult DRG neurons. At the time of these studies, there were few hypotheses regarding the role of Hsp27 in DRG neuronal survival, although since the commencement of my studies there have been other reports demonstrating its importance in prevention of apoptosis (Costigan *et al.*, 1998; Lewis *et al.*, 1999; Wagstaff *et al.*, 1999). Our studies of differentiated PC12 cells (Chapter 3) showed that Hsp27 can prevent apoptosis due to NGF-withdrawal, and we applied the same technique to determine whether Hsp27 could prevent apoptosis during NGF-withdrawal of postnatal DRG neurons. That it was possible to rescue these neurons lends support to my second hypothesis, which is that low levels of Hsp27 in neonatal neurons account in part for their dependence on NGF for survival and it is the abundance of Hsp27 in adult DRG neurons that imparts, at least partially, their NGF-independence.

We suggest that induction of Hsp27 after heat stress is likely to be part of an element of cell preservation and protection, as this induction occurs in multiple cell types throughout the body. *In vivo* studies investigating the effect of sciatic nerve axotomy on neuronal survival showed that surviving neurons are strongly immunoreactive for Hsp27, suggesting a protective function for Hsp27 in neuronal survival after injury (Lewis *et al.*, 1999). Induction of Hsps has been observed after peripheral and central nerve injury in rat models. These observations have implications for not only injury but also for neurodegenerative disease. Consideration of studies documenting induction of Hsp expression provides an explanation for induction of Hsps as a pre-conditioning method to protect against further stress (Currie *et al.*, 2000; Li *et al.*, 2003; Valentim *et al.*, 2003; Dhodda *et al.*, 2004).

As we determined Hsp27 to be significant in promoting survival in DRG neurons, we investigated the mechanism of Hsp27 induction. A number of techniques were employed to determine if HSF-1 was also involved in Hsp27 induction in DRG neurons, as it is in a number of other cell types. Unfortunately, many tools available for use, such as antibodies, were human specific and thus were difficult to use with our rat primary neuronal cell cultures. In addition, another problem was the relatively low levels of HSF-1 present in neurons compared to other cell types such as Hela or PC12 cells. However, we obtained results for our western blotting, ICC and EMSA experiments involving DRG neurons, and found that heat stress resulted in nuclear translocation of HSF-1 as well as induction of its DNA-binding ability to the HSE found in the Hsp27 promoter.

Additionally, our investigations of alternate regulators of Hsp27 induction in adult DRG neurons led us to the conclusion that NF-κB may play a role in the heat shock

response, as its DNA-binding activity increased following heat shock. There is a region in the Hsp27 promoter similar to the NF-κB consensus sequence with only a 1 bp difference, and further experiments will be required to determine whether NF-κB binds directly to the Hsp27 promoter and induces expression. Likewise, increases in NF-κB DNA-binding activity was observed in PC12 cells after heat shock and further studies will be needed to further determine if it directly influences Hsp27 induction.

Determination of signal transduction pathways important in adult DRG neurotrophin-independent survival is important as it allows for understanding of the workings within the adult PNS and identifies possible targets and mechanisms for interventions after injury or onset of disease. The result of stressful stimuli on body tissues and organs is complex and one that is multi-cellular in nature. It is the nature of the stimuli that will dictate cellular behaviour and the survival outcome of cells within these tissues and organs. The ability of Hsps to act both as chaperones and as intermediates in anti-apoptotic signalling may be necessary for neuroprotection to occur. Hsp induction can be time specific, stimulus specific, and cell specific, and it must be considered that cells require Hsps for normal functioning and become part of an essential protection and repair mechanism in these cells after stress. In fact, mutations of Hsp genes are implicated in diseases of hereditary motor neuropathy, as this results in axonal loss and reduced neuronal survival (Evgrafov *et al.*, 2004). Constitutive Hsp27 is involved in stabilizing the actin cytoskeleton, as we have demonstrated in our laboratory (Williams *et al.*, 2005). Cellular survival promoting effects of Hsp27 induction after stress have been extensively examined in this thesis, which lends strong evidence to

support the importance of Hsps in the PNS. The most effective therapies involving heat shock proteins, however, will integrate knowledge about interactions of Hsps with one another, with other signalling intermediates so that in the nervous system Hsps may contribute to the preservation of irreplaceable neurons.

6.1 Future Directions

Future studies investigating neurotrophin-independent survival of the DRG and stress responses should include further examination of key elements discussed in this thesis. The role Hsp27 plays in signal transduction pathways involved in the stress response and survival, interaction with protein intermediates, and its regulation in these cells must be examined in further detail.

The studies in Chapter 2 demonstrated the importance of the PI-3K and PKC pathways in adult DRG neuronal survival. Subsequent chapters focused on how Hsp27 is a significant factor in this survival as well as in response to stress. There has been much effort invested to learn how neurons respond to different stressors, but much remains to be learned about the role of Hsp27.

First, in adult DRG neurotrophin-independent survival, is Hsp27 solely playing a role in promotion of survival or is it performing multiple simultaneous tasks? Although its proven interaction with anti-apoptotic intermediates leads to the conclusion that Hsp27 is important for cellular survival, Hsp27 may also be functioning to chaperone proteins to prevent misfolding or protect against dephosphorylation of activated signalling intermediates, as we observed in Chapter 3. Hsp27 was able to protect Akt from

dephosphorylation in PC12 cells. There is a need to determine the interaction of Hsp27 with signalling intermediates in these pathways and, as discussed previously, some interactions have been identified.

Secondly, are there co-chaperones that aid Hsp27 in its functions? For example, do Hsp27 and Hsp70 interact with each other and/or complement each other in response to stress and protein misfolding/unfolding? Hsps are known for their role as chaperones, especially the Hsp70 family, as able to sequester denatured/unfolded proteins and/or facilitate their proper refolding. Possible interactions of Hsp27 with other Hsps and its functions as a chaperone in cellular stress and apoptosis remain to be determined, especially in DRG neurons.

Third, Hsp27 is known to interact with cytoskeletal elements, especially in the stabilization of actin monomers. The role of Hsp27 as a pro-survival factor in addition to its interaction with the cytoskeleton indicates its importance, because Hsp27 is recognized as a protein involved in multiple signalling pathways and leads to the conclusion that Hsp27 is crucial in many cellular functions.

The present study clearly demonstrates the importance of Hsp27 in survival of DRG neurons and how a physiological stress can induce expression of this protein and enhance cellular survival. Initially, Chapter 2 investigated signal transduction pathway intermediates in an attempt to identify factors crucial for survival of the adult DRG. We used protein inhibitors to determine effects on survival, and this method has become more useful, as more inhibitors are available for use than there were at the time of my initial studies. In addition to protein inhibitors, function-blocking antibodies specific for target portions of intermediates are now available. Studying protein interactions, however, can

be difficult, as abundance of the target protein in a sample may be a problem, and different methods of sampling can alter how proteins will interact.

Our observations led us to investigate the regulation of Hsp27 in these neurons, especially because HSF-1 was found in significantly smaller quantities compared to other cell types. Through the use of promoter-bashing techniques it will be possible to determine exactly what genes and regions of the Hsp27 promoter are important in its induction. Promoter-bashing is a technique that requires transfection of target DNA sequences into cells and due to the recent availability of systems, such as Amaxa, to increase efficiency of transfection of primary neuronal cultures, we now have the ability to perform many experiments using primary cultures, in preference to using cell lines. The postulation that other factors may be, at least in part, responsible for Hsp27 induction may be investigated using techniques such as microarray and ChIP. These techniques can identify genes involved in the response to stresses such as oxidative stress, heat, UV and ischemia.

As outlined in my thesis, Hsp27 is important for neurotrophin-independent survival of adult DRG neurons in response to physiological heat stress. However, Hsps, including Hsp27 are expressed in other cell types as well. It is necessary to determine why only certain neuronal cell types express or upregulate Hsp27 and some do not. Beyond the difficulty of working with post-mitotic DRG neuronal cultures, additional difficulties may also arise. For certain techniques, finding reagents that are rat-specific is also difficult and, therefore, may involve custom production of reagents or first using cell lines for initial experiments.

It seems as if we have only scratched the surface of the role of Hsp27 and other Hsp families in mammalian cells, as its ubiquitous expression and scope of action appears to be quite broad and across many cell types and even species. It is hoped that the *in vitro* experiments presented in this thesis can provide a basis for therapeutic and treatment paradigms for the PNS.

REFERENCES

Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 374:450-453.

Ahn SG, Thiele DJ (2003) Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. Genes and Development 17:516-528.

Akbar MT, Wells DJ, Latchman DS, de Belleroche J (2001) Heat shock protein 27.shows a distinctive widespread spatial and temporal pattern of induction in CNS glial and neuronal cells compared to heat shock protein 70 and caspase 3 following kainate administration. Brain Res Mol Brain Res 93:148-163.

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha. Curr Biol 7:261-269.

Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR, Miller FD (1998) P53 Is Essential for Developmental Neuron Death as Regulated by the TrkA and p75 Neurotrophin Receptors. J Cell Biol 143:1691-1703.

Amin J, Ananthan J, Voellmy R (1988) Key features of heat shock regulatory elements. Mol Cell Biol 8:3761-3769.

Andjelkovic M, Sudian HS, Meier R, Frech M, Alessi DR, Hemmings BA (1998) Nerve growth factor promotes activation of the alpha, beta and gamma isoforms of protein kinase B in PC12 pheochromocytoma cells. Eur J Biochem 251:195-200.

Aoki M, Tamatani M, Taniguchi M, Yamaguchi A, Bando Y, Kasai K, Miyoshi Y, Nakamura Y, Vitek MP, Tohyama M (2001) Hypothermic treatment restores glucose regulated protein 78 (GRP78) expression in ischemic brain. Molecular Brain Research 95:117-128.

Arrigo AP (2000) sHsp as novel regulators of programmed cell death and tumorigenicity. Pathol Biol 48:280-288.

Arrigo AP (2001) Hsp27: novel regulator of intracellular redox state. IUBMB Life 52:303-307.

Arrigo AP, Suhan JP, Welch WJ (1988) Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein. Mol Cell Biol 8:5059-5071.

Arrigo AP, Firdaus WJ, Mellier G, Moulin M, Paul C, Diaz-latoud C, Kretz-remy C (2005) Cytotoxic effects induced by oxidative stress in cultured mammalian cells and protection provided by Hsp27 expression. Methods 35:126-138.

Ashcroft M, Stephens RM, Hallberg B, Downward J, Kaplan DR (1999) The selective and inducible activation of endogenous PI 3-kinase in PC12 cells results in efficient NGF-mediated survival but defective neurite outgrowth. Oncogene 18:4586-4597.

Baler R, Dahl G, Voellmy R (1993) Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. Mol Cell Biol 13:2486-2496.

Barger SW, Horster D, Furukawa K, Goodman Y, Krieglstein J, Mattson MP (1995) Tumor necrosis factors alpha and beta protect neurons against amyloid beta-peptide toxicity: evidence for involvement of a kappa B-binding factor and attenuation of peroxide and Ca2+ accumulation. Proc Natl Acad Sci USA 92:9328-9332.

Barker PA (1998) p75NTR: A study in contrasts. Cell Death Differ 5:346-356.

Battiani F (2001) Protein kinase C isoforms as therapeutic targets in nervous system disease states. Pharmacol Res 44:353-361.

Bechtold DA, Brown IR (2000) Heat shock proteins Hsp27 and Hsp32 localize to synaptic sites in the rat cerebellum following hyperthermia. Molecular Brain Research 75:309-320.

Beere HM (2001) Stressed to death: regulation of apoptotic signaling pathways by the heat shock proteins. Sci STKE 93:RE1.

Beere HM (2004) "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. J Cell Sci 117:2641-2651.

Benn SC, Perrelet D, Kato AC, Scholz J, Decosterd I, Mannion RJ, Bakowska JC, Woolf CJ (2002) Hsp27 upregulation and phosphorylation is required for injured sensory and motor neuron survival. Neuron 36:45-56.

Benndorf R, Hayess K, Ryazantsev S, Wieske M, Behlke J, Lutsch G (1994) Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. J Biol Chem 269:20780-20784.

Bennett DL, Averill S, Clary DO, Priestley JV, McMagon SB (1996) Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. Eur J Neurosci 8:2204-2208.

Berg D, Youdim MB, Riederer P (2004) Redox Imbalance. Cell Tissue Res 318:201-213.

Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal F (1991) Neurotrophin-5: a novel neurotrophin that activates Trk and TrkB. Neuron 8:947-956.

Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496.

Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, Barker PA (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. J Neurosci 22:8466-8475.

Bibel M, Hoppe E, Barde YA (1999) Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J 18:616-622.

Bijur GN, Jope RS (2000) Opposing Actions of Phosphatidylinositol 3-Kinase and Glycogen Synthase Kinase-3 in the Regulation of HSF-1 Activity. Journal of Neurochemistry 75:2401.

Bitar KN (2002) HSP27 phosphorylation and interaction with actin-myosin in smooth muscle contraction. AJP - Gastrointestinal and Liver Physiology 282:G894-903.

Boellmann F, Guettouche T, Guo Y, Fenna M, Mnayer L, Voellmy R (2004) DAXX interacts with heat shock factor 1 during stress activation and enhances its transcriptional activity. PNAS 101:4100-4105.

Borasio GD, Markus A, Wittinghofer A, Barde YA, Heumann R (1993) Involvement of ras p21 in neurotrophin-induced response of sensory, but not sympathetic neurons. J Cell Biol 121:665-672.

Bova MP, McHaourab HS, Han Y, Fung BK (2000) Subunit exchange of small heat shock proteins. Analysis of oligomer formation of alphaA-crystallin and Hsp27 by fluorescence resonance energy transfer and site-directed truncations. J Biol Chem 275:1035-1042.

Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E, Garrido C (2000) Hsp27 negatively regulates cell death by interacting with cytochrome c. Nat Cell Biol 2:645-652.

Burgering BM, Coffer PJ (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 376:599-602.

Carter BD, Dechant G, Frade JM, Kaltschmidt C, Barde YA (1996) Neurotrophins and their p75 receptor. Cold Spring Harb Symp Quant Biol 61:407-415.

Casaccia-Bonnefil P, Kong H, Chao MV (1998) Neurotrophins: the biological paradox of survival factors eliciting apoptosis. Cell Death Differ 5:343-345.

Chalecka-Franaszek E, Chuang D (1999) Lithium activates the serine-threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. Proc Natl Acad Sci 96:8745-8750.

Chao MV, Hempstead BL (1995) p75 and Trk: a two-receptor system. Trends Neurosci 18:321-326.

Charette SJ, Landry J (2000) The Interaction of HSP27 with Daxx Identifies a Potential Regulatory Role of HSP27 in Fas-Induced Apoptosis. Ann NY Acad Sci 926:126-131.

Charette SJ, Lambert H, Landry J (2001) A Kinase-independent Function of Ask1 in Caspase-independent Cell Death. Journal of Biological Chemistry 276:36071.

Charette SJ, Lavoie JN, Lambert H, Landry J (2000) Inhibition of Daxx-Mediated Apoptosis by Heat Shock Protein 27. Mol Cell Biol 20:7602-7612.

Chen H, Hewison M, Hu B, Sharma M, Sun Z, Adams JS (2004) An Hsp27-related, Dominant-negative-acting Intracellular Estradiol-binding Protein. Journal of Biological Chemistry 279:29944-29951.

Chen YR, Tan TH (2000) The c-Jun-N-terminal kinase pathway and apoptotic signalling. Int J Oncol 16:651-662.

Cheng B, Christakos S, Mattson MP (1994) Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. Neuron 12:139-153.

Christians ES, Yan LJ, Benjamin IJ (2002) Heat shock factor 1 and heat shock proteins: Critical partners in protection against acute cell injury. Crit Care Med 30:S43.

Chu B, Soncin F, Price BD, Stevenson MA, Calderwood SK (1996) Sequential Phosphorylation by Mitogen-activated Protein Kinase and Glycogen Synthase Kinase 3 Represses Transcriptional Activation by Heat Shock Factor-1. J Biol Chem 271:30847-30857.

Chu B, Zhong R, Soncin F, Stevenson MA, Calderwood SK (1998) Transcriptional Activity of Heat Shock Factor 1 at 37 degrees C Is Repressed through Phosphorylation on Two Distinct Serine Residues by Glycogen Synthase Kinase 3 alpha and Protein Kinases C alpha and C zeta. J Biol Chem 273:18640-18646.

Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW (1997) Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappaB control. Proc Natl Acad Sci USA 94:10057-10062.

Concannon C, Orrenius S, Samali A (2001) Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c. Gene Expr 9:195-201.

Cooper LF, Uoshima K, Guo Z (2000) Transcriptional regulation involving the intronic heat shock element of the rat hsp27 gene. Biochim Biophys Acta 1490:348-354.

Costigan M, Mannion RJ, Kendall G, Lewis SE, Campagna JA, Coggeshall RE, Meridith-Middleton J, Tate S, Woolf CJ (1998) Heat Shock Protein 27: Developmental Regulation and Expression after Peripheral Nerve Injury. J Neurosci 18:5891-5900.

Cotto J, Fox S, Morimoto R (1997) HSF1 granules: a novel stress-induced nuclear compartment of human cells. J Cell Sci 110:2925-2934.

Cotto JJ, Kline M, Morimoto RI (1996) Activation of Heat Shock Factor 1 DNA Binding Precedes Stress-induced Serine Phosphorylation. J Biol Chem 271:3355-3358.

Coughlin M, Collins M (1985) Nerve growth factor-independent development of embryonic mouse sympathetic neurons in dissociated cell culture. Dev Biol 110:392-401.

Creedon DJ, Johnson EM, Jr., Lawrence JC, Jr. (1996) Mitogen-activated Protein Kinase-independent Pathways Mediate the Effects of Nerve Growth Factor and cAMP on Neuronal Survival. J Biol Chem 271:20713-20718.

Crossthwaite AJ, Hasan S, Williams RJ (2002) Hydrogen peroxide-mediated phosphorylation of ERK1/2, Akt/PKB and JNK in cortical neurones: dependence on Ca and PI3-kinase. J Neurochem 80:24-35.

Crowder RJ, Freeman RS (1998) Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 18:2933-2943.

Crowder RJ, Freeman RS (2000) Glycogen Synthase Kinase-3beta Activity Is Critical for Neuronal Death Caused by Inhibiting Phosphatidylinositol 3-Kinase or Akt but Not for Death Caused by Nerve Growth Factor Withdrawal. J Biol Chem 275:34266-34271.

Currie RW, Ellison JA, White RF, Feuerstein GZ, Wang X, Barone FC (2000) Benign focal ischemic preconditioning induces neuronal Hsp70 and prolonged astrogliosis with expression of Hsp27. Brain Res 863:169-181.

da Silva JS, Dotti CG (2002) Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. Nat Rev Neurosci 3:694-704.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231-241.

Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. Cell 103:239-252.

Deacon E, Pongracz J, Griffiths G, Lord J (1997) Isoenzymes of protein kinase C: differential involvement in apoptosis and pathogenesis. J Clin Pathol 50:124-131.

Deckwerth TL, Elliott JL, Knudson CM, Johnson JEM, Snider WD, Korsmeyer SJ (1996) BAX Is Required for Neuronal Death after Trophic Factor Deprivation and during Development. Neuron 17:401.

Dehmelt L, Smart FM, Ozer RS, Halpain S (2003) The role of microtubule-associated protein 2c in the reorganization of microtubules and lamellipodia during neurite initiation. J Neurosci 23:9479-9490.

DeMeester SL, Buchman TG, Cobb JP (2001) The heat shock paradox: does NF-kappaB determine cell fate? FASEB J 15:270-274.

Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karim M, Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76:1025-1037.

Detera-Wadleigh S (2001) Lithium-related genetics of bipolar disorder. Ann Med 33:272-285.

Dhodda VK, Sailor KA, Bowen KK, Vemuganti R (2004) Putative endogenous mediators of preconditioning-induced ischemic tolerance in rat brain identified by genomic and proteomic analysis. J Neurochem 89:73-89.

Diamond J, Foerster A, Holmes M, Coughlin M (1992) Sensory nerves in adult rats regenerate and restore sensory function to the skin independently of endogenous NGF. J Neurosci 12:1467-1476.

Dodge ME, Rahimtula M, Mearow KM (2002) Factors contributing to neurotrophin-independent survival of adult sensory neurons. Brain Res 953:144-156.

Dodge ME, Wang J, Guy C, Rankin S, Rahimtula M, Mearow KM (2006) Stress-induced heat shock protein 27 expression and its role in dorsal root ganglion neuronal survival. Brain Research 1068:34-48.

Dorion S, Landry J (2002) Activation of the mitogen-activated protein kinase pathways by heat shock. Cell Stress Chap 7:200-206.

Dragunow M, Xu R, Walton M, Woodgate A-M, Lawlor P, MacGibbon GA, Young D, Gibbons H, Lipski J, Muravlev A (2000) c-Jun promotes neurite outgrowth and survival in PC12 cells. Molecular Brain Research 83:20.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of Neuronal Survival by the Serine-Threonine Protein Kinase Akt. Science 275:661-665.

Dunn DK, Whelan RDH, Hill B, King RJB (1993) Relationship of HSP27 and oestrogen receptor in hormone sensitive and insensitive cell lines. J Steroid Biochem Mol Biol 46:469-479.

Dwyer D, Liu Y, Miao S, Bradley R (1996) Neuronal differentiation in PC12 cells is accompanied by diminished inducibility of Hsp70 and Hsp60 in response to heat and ethanol. Neurochem Res 21:659-666.

Edström A, Ekström P (2003) Role of phosphatidylinositol 3-kinase in neuronal survival and axonal outgrowth of adult mouse dorsal root ganglia explants. J Neurosci Res 74:726-735.

Ehrnsperger M, Graber S, Gaestel M, Buchner J (1997) Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. EMBO J 16:221-229.

Eichler ME, Rich KM (1989) Death of sensory ganglion neurons after acute withdrawal of nerve growth factor in dissociated cell cultures. Brain Res 482:340-346.

Encinas M, Iglesias M, Llecha N, Comella JX (1999) Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. J Neurochem 73:1409-1421.

Ernfors P (2001) Local and target-derived actions of neurotrophins during peripheral nervous system development. Cell Mol Life Sci 58:1036-1044.

Ernfors P, Rosario CM, Merlio JP, Grant G, Aldskogius H, Persson H (1993) Expression of mRNAs for neurotrophin receptors in the dorsal root ganglion and spinal cord during development and following peripheral or central axotomy. Brain Res Mol Brain Res 17:217-226.

Evgrafov OV, Mersiyanova I, Irobi J, Van Den Bosch L, Dierick I, Leung CL, Schagina O, Verpoorten N, Van Impe K, Fedotov V, Dadali E, Auer-Grumbach M, Windpassinger C, Wagner K, Mitrovic Z, Hilton-Jones D, Talbot K, Martin JJ, Vasserman N, Tverskaya S, Polyakov A, Liem RK, Gettemans J, Robberecht W, De Jonghe P, Timmerman V (2004) Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. Nat Genet 36:602-606.

Feng W, Webb P, Nguyen P, Liu X, Li J, Karin M, Kushner PJ (2001) Potentiation of estrogen receptor activation function 1 (AF-1) by Src/JNK through a serine 118-independent pathway. Mol Endocrinol 15:32-45.

Fernyhough P, Smith DR, Schapansky J, VanDer Ploeg R, Gardiner NJ, Tweed CW, Kontos A, Freeman L, Purves-Tyson TD, Glazner GW (2005) Activation of nuclear factor-kB via endogenous tumor necrosis factor alpha regulates survival of axotomized adult sensory neurons. J Neurosci 25:1682-1690.

Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359:1-16.

Franklin TB, Krueger-Naug AM, Clarke DB, Arrigo AP, Currie RW (2005) The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. Int J Hyperthermia 21:379-392.

Gabai VL, Sherman MY (2002) Invited review: Interplay between molecular chaperones and signaling pathways in survival of heat shock. J Appl Physiol 92:1743-1748.

Gaestel M (2002) sHsp-phosphorylation: enzymes, signaling pathways and functional implications. Prog Mol Subcell Biol 28:151-169.

Gaestel M, Gotthardt R, Muller T (1993) Structure and organisation of a murine gene encoding small heat-shock protein Hsp25. Gene 128:279-283.

Ganea E (2001) Chaperone-like activity of alpha-crystallin and other small heat shock proteins. Curr Prot Pep Sci 2:205-225.

Garnier P, Ying W, Swanson RA (2003) Ischemic Preconditioning by Caspase Cleavage of Poly(ADP-Ribose) Polymerase-1. Journal of Neuroscience 23:7967-7973.

Garrido C, Gurbuxani S, Ravagnan L, Kroemer G (2001) Heat Shock Proteins: Endogenous Modulators of Apoptotic Cell Death. Biochemical and Biophysical Research Communications 286:433.

Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP, Solary E (1999) HSP27 inhibits cytochrome c-dependent activation of procaspase-9. Faseb J 13:2061-2070.

Gerthoffer WT, Gunst SJ (2001) Signal Transduction in Smooth Muscle: Invited Review: Focal adhesion and small heat shock proteins in the regulation of actin remodeling and contractility in smooth muscle. J App Physiol 91:963-972.

Geum D, Son GH, Kim K (2002) Phosphorylation-dependent cellular localization and thermoprotective role of heat shock protein 25 in hippocampal progenitor cells. J Biol Chem 277:19913-19921.

Goodson ML, Sarge KD (1995) Heat-inducible DNA binding of purified heat shock transcription factor 1. J Biol Chem 270:2447-2450.

Green DR (2005) Apoptotic pathways: Ten minutes to dead. Cell 121:671-674.

Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3[beta] in cellular signaling. Progress in Neurobiology 65:391-426.

Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, Landry J (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci 110:357-368.

Gubina E, Rinaudo M, Szallasi Z, Blumberg P, Mufson R (1998) Overexpression of protein kinase C isoform epsilon but not delta in human interleukin-3-dependent cells suppresses apoptosis and induces Bcl-2 expression. Blood 91:823-829.

Gusev NB, Bogatcheva NV, Marston SB (2002) Structure and properties of small heat shock proteins (sHsp) and their interaction with cytoskeleton proteins. Biochem (Moscow) 67:613-623.

Guzhova I, Kislyakova K, Moskaliova O, Fridlanskaya I, Tytell M, Cheetham M, Margulis B (2001) In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. Brain Research 914:66-73.

Hamilton KL, Gupta S, Knowlton AA (2004) Estrogen and regulation of heat shock protein expression in female cardiomyocytes: cross-talk with NFkB signaling. J Mol Cell Cardiol 36:577-584.

Hanstein B, Djahansouzi S, Dall P, Beckmann MW, Bender HG (2004) Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. Eur J Endocrinol 150:243-255.

Harding TC, Xue L, Bienemann A, Haywood D, Dickens M, Tolkovsky AM, Uney JB (2001) Inhibition of JNK by Overexpression of the JNK Binding Domain of JIP-1 Prevents Apoptosis in Sympathetic Neurons. J Biol Chem 276:4531-4534.

Hargis MT, Storck CW, Wickstrom E, Yakubov LA, Leeper DB, Coss RA (2004) Hsp27 anti-sense oligonucleotides sensitize the microtubular cytoskeleton of Chinese hamster ovary cells grown at low pH to 42 degrees C-induced reorganization. Int J Hyperthermia 20:491-502.

Hatayama T, Takahashi H, Yamagishi N (1997) Reduced induction of HSP70 in PC12 cells during neuronal differentiation. J Biochem (Tokyo) 122:904-910.

Hatayama T, Yamagishi N, Minobe E, Sakai K (2001) Role of hsp105 in protection against stress-induced apoptosis in neuronal PC12 cells. Biochem Biophys Res Comm 288:528-534.

Hightower LE (1991) Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 66:191-197.

Hohn A, Leibrock J, Bailey K, Barde Y-A (1990) Identification and characterization of a novel member of the nerve growth factor family. Nature 344:3339-3341.

Holmberg CI, Illman SA, Kallio M, Mikhailov A, Sistonen L (2000) Formation of nuclear HSF1 granules varies depending on stress stimuli. Cell Stress Chaperones 5:219-228.

Holmberg CI, Hietakangas V, Mikhailov A, Rantanen JO, Kallio M, Meinander A, Hellman J, Morrice N, MacKintosh C, Morimoto RI, Eriksson JE, Sistonen L (2001) Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. EMBO J 20:3800-3810.

Hopkins DA, Plumier JC, Currie RW (1998) Induction of the 27-kDa heat shock protein (Hsp27) in the rat medulla oblongata after vagus nerve injury. Exp Neurol 153:173-183.

Horman S, Fokan D, Mosselmans R, Mairesse N, Galand P (1999) Anti-sense inhibition of small-heat-shock-protein (HSP27) expression in MCF-7 mammary-carcinoma cells induces their spontaneous acquisition of a secretory phenotype. Int J Cancer 82:574-582.

Huang EJ, Reichardt LF (2003) Trk receptors: Roles in neuronal signal transduction. Ann Rev Biochem 72:609-642.

Huot J, Houle F, Spitz DR, Landry J (1996) HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. Cancer Res 56:273-279.

Huot J, Lambert H, Lavoie JN, Guimond A, Houle F, Landry J (1995) Characterization of 45-kDa/54-kDa HSP27 kinase, a stress-sensitive kinase which may activate the phosphorylation-dependent protective function of mammalian 27-kDa heat-shock protein HSP27. Eur J Biochem 227:416-427.

Ip NY, Ibanez SH, Nye SH, McClain J, Jones PF, Gies DR, Belluso L, LeBeau MM, Espinosa R, Squinto SP, Persson H, Yancopoulus GD (1991) Mammalian NT-4: structure, chromosome location, tissue distribution and receptor specificity. Proc Natl Acad Sci USA 89:3060-3064.

Irobi J, Van Impe K, Seeman P, Jordanova A, Dierick I, Verpoorten N, Michalik A, De Vriendt E, Jacobs A, Van Gerwen V, Vennekens K, Mazanec R, Tournev I, Hilton-Jones D, Talbot K, Kremensky I, Van Den Bosch L, Robberecht W, Van Vandekerckhove J, Broeckhoven C, Gettemans J, De Jonghe P, Timmerman V (2004) Hot-spot residue in small heat-shock protein 22 causes distal motor neuropathy. Nat Genet 36:597-601.

Ito H, Nomoto H, Furukawa S (2003) Growth arrest of PC12 cells by nerve growth factor is dependent on the phosphatidylinositol 3-kinase/Akt pathway via p75 neurotrophin receptor. Jf Neurosci Res 72:211-217.

Jakob U, Gaestel M, Engel K, Buchner J (1993) Small heat shock proteins are molecular chaperones. J Biol Chem 268:1517-1520.

Jayachandran M, Miller VM (2002) Ovariectomy upregulates expression of estrogen receptors, NOS, and HSPs in porcine platelets. Am J Physiol Heart Cir Physiol 283:H220-226.

Jin Z, El-Deiry WS (2005) Overview of cell death signalling pathways. Cancer Biol Ther 4:139-163.

Jolly C, Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Nat Cancer Inst 92:1564-1572.

Jolly C, Usson Y, Morimoto RI (1999) Rapid and reversible relocalization of heat shock factor 1 within seconds to nuclear stress granules. Proc Natl Acad Sci USA 96:6769-6774.

Jones DM, Tucker BA, Rahimtula M, Mearow KM (2003) The synergistic effects of NGF and IGF-1 on neurite growth in adult sensory neurons: convergence on the PI 3-kinase signaling pathway. J Neurochem 86:1116-1128.

Kalwy SA, Akbar MT, Coffin RS, de Belleroche J, Latchman DS (2003) Heat shock protein 27 delivered via a herpes simplex virus vector can protect neurons of the

hippocampus against kainic-acid-induced cell loss. Molecular Brain Research 111:91-103.

Kamradt MC, Chen F, Cryns VL (2001) The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. J Biol Chem 276:16059-16063.

Kaplan D, Miller F (1997) Signal transduction by the neurotrophin receptors. Curr Opin Cell Biol 9:213-221.

Kaplan DR, Stephens RM (1994) Neurotrophin signal transduction by the Trk receptor. J Neurobiol 25:1404-1417.

Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10:381-391.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252:554-558.

Karin M, Ben-Neriah Y (2000) Phosphorylation Meets Ubiquitination: The Control of NF-KB Activity. Ann Rev Immunol 18:621-663.

Kashiba H, Noguchi K, Ueda Y, Senba E (1995) Coexpression of trk family members and low-affinity neurotrophin receptors in rat dorsal-root ganglion neurons. Mol Brain Res 30:158-164.

Kato K, Ito H, Kamei K, Iwamoto I (1999) Selective stimulation of Hsp27 and alphaB-crystallin but not Hsp70 expression by p38 MAP kinase activation. Cell Stress Chaperones 4:94-101.

Khosravi-Far R, Esposti MD (2004) Death receptor signals to mitochondria. Cancer Biol Ther 3:1051-1057.

Khursigara G, Bertin J, Yano H, Moffett H, DiStefano PS, Chao MV (2001) A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2. J Neurosci 21:5854-5863.

Kim R (2005) Unknotting the roles of Bcl-2 and Bcl-xL in cell death. Biochem Biophys Res Comm 333:336-343.

Kim R, Emi M, Tanabe K (2005) Caspase-dependent and -independent cell death pathways after DNA damage. Oncol Rep 14:595-599.

Kimpinski K, Mearow K (2001) Neurite growth promotion by nerve growth factor and insulin-like growth factor-1 in cultured adult sensory neurons: Role of phosphoinositide 3-kinase and mitogen activated protein kinase. J Neurosci Res 63:486-499.

Kimpinski K, Campenot RB, Mearow K (1997) Effects of the neurotrophins nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (BDNF) on neurite growth from adult sensory neurons in compartmented cultures. J Neurobiol 33:395-410.

Kimpinski K, Jelinski S, Mearow K (1999) The anti-p75 antibody, MC192, and brain-derived neurotrophic factor inhibit nerve growth factor-dependent neurite growth from adult sensory neurons. Neurosci 93:253-263.

Kitamura Y, Nomura Y (2003) Stress proteins and glial functions: possible therapeutic targets for neurodegenerative disorders. Pharm Therapeutics 97:35-53.

Klein P, Melton D (1996) A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA 93:8455-8459.

Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991a) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Klein R, Nanduri V, Jing SA, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991b) The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66:395-403.

Klesse LJ, Parada LF (1998) p21 Ras and Phosphatidylinositol-3 Kinase Are Required for Survival of Wild-Type and NF1 Mutant Sensory Neurons. J Neurosci 18:10420-10428.

Klesse LJ, Parada LF (1999) Trks: Signal transduction and intracellular pathways. Microsc Res Tech 45:210-216.

Klettner A (2004) The induction of heat shock proteins as a potential strategy to treat neurodegenerative disorders. Drug News Perspect 17:299-306.

Kline MP, Morimoto RI (1997) Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. Mol Cell Biol 17:2107-2115.

Klinz F-J, Heumann R (1995) Time-Resolved Signaling Pathways of Nerve Growth Factor Diverge Downstream of the p140trk Receptor Activation Between Chick Sympathetic and Dorsal Root Ganglion Sensory Neurons. J Neurochem 65:1046-1053.

Knauf U, Newton EM, Kyriakis J, Kingston RE (1996) Repression of human heat shock factor 1 activity at control temperature by phosphorylation. Genes Dev 10:2782-2793.

Knowlton AA, Sun L (2001) Heat-shock factor-1, steroid hormones, and regulation of heat-shock protein expression in the heart. Am J Physiol Heart Circ Physiol 280:H455-464.

Kohn G, Wong HR, Bshesh K, Zhao B, Vasi N, Denenberg A, Morris C, Stark J, Shanley TP (2002) Heat shock inhibits tnf-induced ICAM-1 expression in human endothelial cells via I kappa kinase inhibition. Shock 17:91-97.

Konishi H, Matsuzaki H, Tanaka M, Takemura Y, Kuroda S, Ono Y, Kikkawa U (1997) Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27. FEBS Lett 410:493.

Kopito RR (2000) Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 10:524-530.

Koroshetz WJ, Bonventre JV (1994) Heat shock response in the central nervous system. Experientia 50:1085-1091.

Koteiche HA, McHaourab HS (2002) The determinants of the oligomeric structure in Hsp16.5 are encoded in the alpha-crystallin domain. FEBS Lett 519:16-22.

Kratsovnik E, Bromberg Y, Sperling O, Zoref-Shani E (2005) Oxidative Stress Activates Transcription Factor NF-kB-Mediated Protective Signaling in Primary Rat Neuronal Cultures. J Mol Neurosci 26:27-32.

Krueger-Naug AM, Hopkins DA, Armstrong JN, Plumier JC, Currie RW (2000) Hyperthermic induction of the 27-kDa heat shock protein (Hsp27) in neuroglia and neurons of the rat central nervous system. J Comp Neurol 428:495-510.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR (1994) The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369:156-160.

Lamballe F, Klein R, Barbacid M (1991) trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66:967-979.

Lambert H, Charette SJ, Bernier AF, Guimond A, Landry J (1999) HSP27 Multimerization Mediated by Phosphorylation-sensitive Intermolecular Interactions at the Amino Terminus. J Biol Chem 274:9378-9385.

Landry J, Huot J (1995) Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. Biochem Cell Biol 73:703-707.

Landry J, Chretien P, Laszlo A, Lambert H (1991) Phosphorylation of HSP27 during development and decay of thermotolerance in Chinese hamster cells. J Cell Physiol 147:93-101.

Landry J, Chretien P, Lambert H, Hickey E, Weber LA (1989) Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. J Cell Biol 109:7-15.

Landry J, Lambert H, Zhou M, Lavoie JN, Hickey E, Weber LA, Anderson CW (1992) Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. J Biol Chem 267:794-803.

Latchman DS (2005) HSP27 and cell survival in neurones. Int J Hyperthermia 21:393-402.

Lavoie J, Chretien P, Landry J (1990) Sequence of the Chinese hamster small heat shock protein HSP27. Nucleic Acids Res 18:1637.

Lavoie JN, Gingras-Breton G, Tanguay RM, Landry J (1993) Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. J Biol Chem 268:3420-3429.

Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J (1995) Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. Mol Cell Biol 15:505-516.

Lee H, Jiang F, Wang Q, Nicosia SV, Yang J, Su B, Bai W (2000) MEKK1 activation of human estrogen receptor alpha and stimulation of the agonistic activity of 4-hydroxytamoxifen in endometrial and ovarian cancer cells. Mol Endocrinol 14:1882-1896.

Lee JE, Yenari MA, Sun GH, Xu L, Emond MR, Cheng D, Steinberg GK, Giffard RG (2001a) Differential neuroprotection from human heat shock protein 70 overexpression in in vitro and in vivo models of ischemia and ischemia-like conditions. Exp Neurol 170:129-139.

Lee KH, Hwang YH, Lee CT, Kim YW, Han SK, Shim YS, Yoo CG (2004) The heat-shock-induced suppression of the IkB/NF-kB cascade is due to inactivation of upstream regulators of IkBalpha through insolubilization. Exp Cell Res 299:49-56.

Lee MW, Park SC, Chae HS, Bach JH, Lee HJ, Lee SH, Kang YK, Kim KY, Lee WB, Kim SS (2001b) The protective role of HSP90 against 3-hydroxykynurenine-induced neuronal apoptosis. Biochem Biophys Res Commun 284:261-267.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Theonen H, Barde Y-A (1989) Molecular cloning and expression of BDNF. Nature 341:149-152.

Lentz SI, Knudson CM, Korsmeyer SJ, Snider WD (1999) Neurotrophins Support the Development of Diverse Sensory Axon Morphologies. J Neurosci 19:1038-1048.

Levi-Montalcini R, Booker B (1960) Destruction of sympathetic ganglia in mammals by an antiserum to a nerve growth protein. Proc Natl Acad Sci USA 46:384-391.

Lewin GR (1996) Neurotrophins and the specification of neuronal phenotype. Philos Trans R Soc Lon B Biol Sci 351:405-411.

Lewis SE, Mannion RJ, White FA, Coggeshall RE, Beggs S, Costigan M, Martin JL, Dillmann WH, Woolf CJ (1999) A Role for HSP27 in Sensory Neuron Survival. J Neurosci 19:8945-8953.

Li L, Lorenzo P, Bogi K, Blumberg P, Yuspa S (1999) Protein kinase Cd targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. Mol Cell Biol 19:8547-8558.

Li X, Jope R (1995) Selective inhibition of the expression of signal transduction proteins by lithium in nerve growth factor-differentiated PC12 cells. J Neurochem 65:2500-2508.

Li Y, Roth S, Laser M, Ma JX, Crosson CE (2003) Retinal preconditioning and the induction of heat-shock protein 27. 44:1299-1304.

Lindner RA, Carver JA, Ehrnsperger M, Buchner J, Esposito G, Behlke J, Lutsch G, Kotlyarov A, Gaestel M (2000) Mouse Hsp25, a small shock protein. The role of its C-terminal extension in oligomerization and chaperone action. Eur J Biochem 267:1923-1932.

Lindquist S (1986) The heat-shock response. Annu Rev Biochem 55:1151-1191.

Lindsay RM (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J Neurosci 8:2394-2405.

Locke M, Noble EG, Tanguay RM, Feild MR, Ianuzzo SE, Ianuzzo CD (1995) Activation of heat-shock transcription factor in rat heart after heat shock and exercise. Am J Physiol 268:C1387-1394.

Lu A, Ran RQ, Clark J, Reilly M, Nee A, Sharp FR (2002) 17-beta-estradiol induces heat shock proteins in brain arteries and potentiates ischemic heat shock protein induction in glia and neurons. J Cereb Blood Flow Metab 22:183-195.

Ma W, Bisby MA (1998) Increased activation of nuclear factor kappa B in rat lumbar dorsal root ganglion neurons following partial sciatic nerve injuries. Brain Res 797:243-254.

Maggirwar SB, Sarmiere PD, Dewhurst S, Freeman RS (1998) Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. J Neurosci 18:10356-10365.

Maher P (2001) How Protein Kinase C Activation Protects Nerve Cells from Oxidative Stress-Induced Cell Death. J Neurosci 21:2929-2938.

Mailhos C, Howard MK, Latchman DS (1993) Heat shock protects neuronal cells from programmed cell death by apoptosis. Neurosci 55:621-627.

Mailhos C, Howard MK, Latchman DS (1994) Heat shock proteins hsp90 and hsp70 protect neuronal cells from thermal stress but not from programmed cell death. J Neurochem 63:1787-1795.

Maisonpierre PC, Beluscio L, Squinto S, Ip NY, Furth M, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446-1451.

Majdan M, Walsh GS, Aloyz R, Miller FD (2001) TrkA mediates developmental sympathetic neuron survival in vivo by silencing an ongoing p75NTR-mediated death signal. J Cell Biol 155:1275-1285.

Malhotra VW, H.R. (2002) Interactions between the heat shock response and the nuclear factor-kappaB signaling pathway. Crit Care Med 30:S89-S95.

Manji H, Lenox R (2000) Signaling: cellular insights into the pathophysiology of bipolar disorder. Biol Psychiatry 48:518-530.

Maroni P, Bendinelli P, Tiberio L, Rovetta F, Piccoletti R, Schiaffonati L (2003) In vivo heat-shock response in the brain: signalling pathway and transcription factor activation. Mol Brain Res 119:90-99.

Martin MB, Franke TF, Stoica GE, Chambon P, Katzenellenbogen BS, Stoica BA, McLemore MS, Olivo SE, Stoica A (2000) A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. Endocrinol 141:4503-4511.

Martinou I, Missotten M, Fernandez PA, Sadoul R, Martinou JC (1998) Bax and Bak proteins require caspase activity to trigger apoptosis in sympathetic neurons. Neuroreport 9:15-19.

Mathew A, Mathur SK, Jolly C, Fox SG, Kim S, Morimoto RI (2001) Stress-Specific Activation and Repression of Heat Shock Factors 1 and 2. Mol Cell Biol 21:7163.

Mazzoni IE, Said FA, Aloyz R, Miller FD, Kaplan D (1999) Ras Regulates Sympathetic Neuron Survival by Suppressing the p53-Mediated Cell Death Pathway. J Neurosci 19:9716-9727.

McMahon SB, Armanini MP, Ling LH, Phillips HS (1994) Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. Neuron 12:1161-1171.

Mearow K, Mazzoni I, Boudreau M, Marsh HN, Miller F, Kaplan D (1998) Signaling pathways required for embryonic and neonatal DRG neuron survival, Soc Neurosci Abstr.:1293.

Mearow KM, Dodge ME, Rahimtula M, Yegappan C (2002) Stress-mediated signaling in PC12 cells - the role of the small heat shock protein, Hsp27, and Akt in protecting cells from heat stress and nerve growth factor withdrawal. J Neurochem 83:452-462.

Mehlen P, Arrigo AP (1994) The serum-induced phosphorylation of mammalian hsp27 correlates with changes in its intracellular localization and levels of oligomerization. Eur J Biochem 221:327-334.

Mehlen P, Schulze-Osthoff K, Arrigo AP (1996) Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. J Biol Chem 271:16510-16514.

Meier M, King GL, Clermont A, Perez A, Hayashi M, Feener EP (2001) Angiotensin AT(1) receptor stimulates heat shock protein 27 phosphorylation in vitro and in vivo. Hypertension 38:1260-1265.

Memberg SP, Hall AK (1995) Proliferation, differentiation, and survival of rat sensory neuron precursors in vitro require specific trophic factors. Mol Cell Neurosci 6:323-335.

Mendell LM (1999) Neurotrophin action on sensory neurons in adults: an extension of the neurotrophic hypothesis. Pain Suppl 6:S127-132.

Merck KB, Groenen PJ, Voorter CE, de Haard-Hoekman WA, Horwitz J, Bloemendal H, de Jong WW (1993) Structural and functional similarities of bovine alpha-crystallin and mouse small heat-shock protein. A family of chaperones. J Biol Chem 268:1046-1052.

Michael GJ, Averill S, Nitkunan A, Rattray M, Bennett DLH, Yan Q, Priestley JV (1997) Nerve Growth Factor Treatment Increases Brain-Derived Neurotrophic Factor Selectively in TrkA-Expressing Dorsal Root Ganglion Cells and in Their Central Terminations within the Spinal Cord. J Neurosci 17:8476-8490.

Middleton G, Hamanoue M, Enokido Y, Wyatt S, Pennica D, Jaffray E, Hay RT, Davies AM (2000) Cytokine-induced nuclear factor kappa B activation promotes the survival of developing neurons. J Cell Biol 148:325-332.

Mielke K, Herdegen T (2000) JNK and p38 stresskinases--degenerative effectors of signal-transduction-cascades in the nervous system. Prog Neurobiol 61:45-60.

Miller FD, Kaplan DR (1998) Life and death decisions: a biological role for the p75 neurotrophin receptor. Cell Death Differ 5:343-345.

Miller FD, Kaplan DR (2001) Neurotrophin signalling pathways regulating neuronal apoptosis. Cell Mol Life Sci 58:1045-1053.

Miron T, Wilchek M, Geiger B (1988) Characterization of an inhibitor of actin polymerization in vinculin-rich fraction of turkey gizzard smooth muscle. Eur J Biochem 178:543-553.

Miron T, Vancompernolle K, Vandekerckhove J, Wilchek M, Geiger B (1991) A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J Cell Biol 114:255-261.

Mobley WC, Schenker A, Shooter EM (1976) Characterization and isolation of proteolytically modified nerve growth factor. Biochem 15:5543-5549.

Molliver DC, Snider WD (1997) Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. J Comp Neurol 381:428-438.

Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19:849-861.

Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12:3788.

Morimoto RI, Sarge KD, Abravaya K (1992) Transcriptional regulation of heat shock genes. A paradigm for inducible genomic responses. J Biol Chem 267:21987-21990.

Morimoto RI, Kline MP, Bimston DN, Cotto JJ (1997) The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays Biochem 32:17-29.

Mosser DD, Theodorakis NG, Morimoto RI (1988) Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. Mol Cell Biol 8:4736-4744.

Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B (2000) The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol Cell Biol 20:7146-7159.

Mounier N, Arrigo AP (2002) Actin cytoskeleton and small heat shock proteins: how do they interact? Cell Stress Chap 7:167-176.

Mu X, Silos-Santiago I, Carroll SL, Snider WD (1993) Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. J Neurosci 13:4029-4041.

Murashov AK, Ul H, I, Hill C, Park E, Smith M, Wang X, Wang X, Goldberg DJ, Wolgemuth DJ (2001) Crosstalk between p38, Hsp25 and Akt in spinal motor neurons after sciatic nerve injury. Brain Res Mol Brain Res 93:199-208.

Nakatsue T, Katoh I, Nakamura S, Takahashi Y, Ikawa Y, Yoshinaka Y (1998) Acute infection of Sindbis virus induces phosphorylation and intracellular translocation of small heat shock protein HSP27 and activation of p38 MAP kinase signaling pathway. Biochem Biophys Res Commun 253:59-64.

Neininger A, Gaestel M (1998) Evidence for a hsp25-specific mechanism involved in transcriptional activation by heat shock. Exp Cell Res 242:285-293.

Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B (2000) The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol Cell Biol 20:7146-7159.

Mounier N, Arrigo AP (2002) Actin cytoskeleton and small heat shock proteins: how do they interact? Cell Stress Chap 7:167-176.

Mu X, Silos-Santiago I, Carroll SL, Snider WD (1993) Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. J Neurosci 13:4029-4041.

Murashov AK, Ul H, I, Hill C, Park E, Smith M, Wang X, Wang X, Goldberg DJ, Wolgemuth DJ (2001) Crosstalk between p38, Hsp25 and Akt in spinal motor neurons after sciatic nerve injury. Brain Res Mol Brain Res 93:199-208.

Nakatsue T, Katoh I, Nakamura S, Takahashi Y, Ikawa Y, Yoshinaka Y (1998) Acute infection of Sindbis virus induces phosphorylation and intracellular translocation of small heat shock protein HSP27 and activation of p38 MAP kinase signaling pathway. Biochem Biophys Res Commun 253:59-64.

Neininger A, Gaestel M (1998) Evidence for a hsp25-specific mechanism involved in transcriptional activation by heat shock. Exp Cell Res 242:285-293.

Nobes C, Tolkovsky A (1995) Neutralizing anti-p21ras Fabs suppress rat sympathetic neuron survival induced by NGF, LIF, CNTF and cAMP. Eur J Neurosci 7:344-350.

Nollen EA, Morimoto RI (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. J Cell Sci 115:2809-2816.

Ohtsuka K, Suzuki T (2000) Roles of molecular chaperones in the nervous system. Brain Res Bull 53:141-146.

Orike N, Thrasivoulou C, Wrigley A, Cowen T (2001a) Differential regulation of survival and growth in adult sympathetic neurons: An in vitro study of neurotrophin responsiveness. J Neurobiol 47:295-305.

Orike N, Middleton G, Borthwick E, Buchman V, Cowen T, Davies AM (2001b) Role of PI 3-kinase, Akt and Bcl-2-related proteins in sustaining the survival of neurotrophic factor-independent adult sympathetic neurons. J Cell Biol 154:995.

Osaki M, Oshimura M, Ito H (2004) PI3K-Akt pathway: Its functions and alterations in human cancer. Apoptosis 9:667-676.

Osborne CK, Schiff R, Fuqua SAW, Shou J (2001) Estrogen receptor: Current understanding of its activation and modulation. In: Clin Cancer Res, pp S4338-4342.

Pandey P, Farber R, Nakazawa A, Kumar S, Bharti A, Nalin C, Weichselbaum R, Kufe D, Kharbanda S (2000) Hsp27 functions as a negative regulator of cytochrome c-dependent activation of procaspase-3. Oncogene 19:1975-1981.

Pantano C, Shrivastava P, McElhinney B, Janssen-Heininger Y (2003) Hydrogen peroxide signaling through tumor necrosis factor receptor 1 leads to selective activation of c-jun N-terminal kinase. J Biol Chem 278:44091-44096.

Parekh DB, Ziegler W, Parker PJ (2000) Multiple pathways control protein kinase C phosphorylation. EMBO J 19:496-503.

Park HG, Han SI, Oh SY, Kang HS (2005) Cellular responses to mild heat stress. Cell Mol Life Sci 62:10-23.

Patapoutian A, Reichardt LF (2001) Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 11:272-280.

Patel YJ, Payne Smith MD, de Belleroche J, Latchman DS (2005) Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant-induced cell death in mammalian neuronal cells. Brain Res Mol Brain Res 134:256-274.

Patrone C, Andersson S, Korhonen L, Lindholm D (1999) Estrogen receptor-dependent regulation of sensory neuron survival in developing dorsal root ganglion. Proc Natl Acad Sci USA 96:10905-10910.

Paul C, Manero F, Gonin S, Kretz-Remy C, Virot S, Arrigo AP (2002) Hsp27 as a negative regulator of cytochrome C release. Mol Cell Biol 22:816-834.

Perng MD, Cairns L, van den Ijssel P, Prescott A, Hutcheson AM, Quinlan RA (1999) Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin. J Cell Sci 112:2099-2112.

Phillips HS, Armanini MP (1996) Expression of the trk family of neurotrophin receptors in developing and adult dorsal root ganglion neurons. Philos Trans R Soc Lond B Biol Sci 351:413-416.

Philpott KL, McCarthy MJ, Klippel A, Rubin LL (1997) Activated Phosphatidylinositol 3-Kinase and Akt Kinase Promote Survival of Superior Cervical Neurons. J Cell Biol 139:809-815.

Pichon S, Bryckaert M, Berrou E (2004) Control of actin dynamics by p38 MAP kinase - Hsp27 distribution in the lamellipodium of smooth muscle cells. J Cell Sci 117:2569-2577.

Pirkkala L, Nykanen P, Sistonen L (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J 15:1118-1131.

Pittet JF, Lee H, Pespeni M, O;Mahoney A, Roux J, Welch WJ (2005) Stress-induced inhibition of the Nf-kB signaling pathway results from the insolubilization of the IkB kinase complex following its dissociation from heat shock protein 90. J Immunol 174:384-394.

Pizzi M, Goffi F, Boroni F, Benarese M, Perkins SE, Liou HC, Spano P (2002) Opposing roles for NF-kappa B/Rel factors p65 and c-Rel in the modulation of neuron survival elicited by glutamate and interleukin-1beta. J Biol Chem 277:20717-20723.

Plumier JC, Hopkins DA, Robertson HA, Currie RW (1997) Constitutive expression of the 27-kDa heat shock protein (Hsp27) in sensory and motor neurons of the rat nervous system. J Comp Neurol 384:409-428.

Porter W, Wang F, Wang W, Duan R, Safe S (1996) Role of estrogen receptor/Sp1 complexes in estrogen-induced heat shock protein 27 gene expression. In: Mol Endocrinol, pp 1371 -1378.

Pratt WB, Toft DO (2003) Regulation of Signaling Protein Function and Trafficking by the hsp90/hsp70-Based Chaperone Machinery. Exp Biol Med 228:111-133.

Preville X, Schultz H, Knauf U, Gaestel M, Arrigo AP (1998) Analysis of the role of Hsp25 phosphorylation reveals the importance of the oligomerization state of this small heat shock protein in its protective function against TNFalpha- and hydrogen peroxide-induced cell death. J Cell Biochem 69:436-452.

Quigney DJ, Gorman AM, Samali A (2003) Heat shock protects PC12 cells against MPP+ toxicity. Brain Res 993:133-139.

Rabindran SK, Haroun RI, Clos J, Wisniewski J, Wu C (1993) Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. Science 259:230-234.

Rane MJ, Pan Y, Singh S, Powell DW, Wu R, Cummins T, Chen Q, McLeish KR, Klein JB (2003) Heat Shock Protein 27 Controls Apoptosis by Regulating Akt Activation. J Biol Chem 278:27828-27835.

Renkawek K, Stege GJ, Bosman GJ (1999) Dementia, gliosis and expression of the small heat shock proteins hsp27 and alpha B-crystallin in Parkinson's disease. Neuroreport 10:2273-2276.

Rich KM, Tip HK, Osborne PA, Schmidt RE, Johnson EMJ (1984) Role of nerve growth factor in the adult dorsal root ganglia neuron and its response to injury. J Comp Neurol 230:110-118.

Richter-Landsberg C, Goldbaum O (2003) Stress proteins in neural cells: functional roles in health and disease. Cell Mol Life Sci 60:337-349.

Ritossa F (1962) A new puffing pattern induced by heat shock and DNP in *Drosophila*. Experientia 18:571-573.

Rodriguez-Tebar A, Dechant G, Barde YA (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487-492.

Rodriguez-Tebar A, Dechant G, Gotz R, Barde YA (1992) Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J 11:917-922.

Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M, Arrigo AP, Buchner J, Gaestel M (1999) Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. J Biol Chem 274:18947-18956.

Rossi A, Elia G, Santoro MG (1998) Activation of the heat shock factor 1 by serine protease inhibitors. An effect associated with nuclear factor-kB inhibition. J Biol Chem 273:16446-16452.

Rothwarf DM, Karin M (1999) The NF-kappa B Activation Pathway: A Paradigm in Information Transfer from Membrane to Nucleus. Sci STKE 1999:RE1.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78:1027-1037.

Roux PP, Bhakar AL, Kennedy TE, Barker PA (2001) The p75 Neurotrophin Receptor Activates Akt (Protein Kinase B) through a Phosphatidylinositol 3-Kinase-dependent Pathway. J Biol Chem 276:23097-23104.

Ruit KG, Elliott JL, Osborne PA, Yan Q, Snider WD (1992) Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. Neuron 8:573-587.

Salvesen GS, Dixit VM (1999) Caspase activation: the induced-proximity model. Proc Natl Acad Sci USA 96:10964-10967.

Samali A, Robertson JD, Peterson E, Manero F, van Zeijl L, Paul C, Cotgreave IA, Arrigo AP, Orrenius S (2001) Hsp27 protects mitochondria of thermotolerant cells against apoptotic stimuli. Cell Stress Chap 6:49-58.

Sarge KD, Morimoto RI (1991) Surprising features of transcriptional regulation of heat shock genes. Gene Expr 1:169-173.

Sarge KD, Murphy SP, Morimoto RI (1993) Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol Cell Biol 13:1392-1407.

Sastry PS, Rao KS (2000) Apoptosis and the Nervous System. J Neurochem 74:1-20.

Sato S, Fujita N, Tsuruo T (2000) Modulation of Akt kinase activity by binding to Hsp90. Proc Nat Acad Sci USA 97:10832-10837.

Schafers M, Geis C, Brors D, Yaksh TL, Sommer C (2002) Anterograde transport of tumor necrosis factor-alpha in the intact and injured rat sciatic nerve. J Neurosci 22:536-545.

Schafers M, Geis C, Svensson CI, Luo ZD, Sommer C (2003) Selective increase of tumour necrosis factor-alpha in injured and spared myelinated primary afferents after chronic constrictive injury of rat sciatic nerve. Eur J Neurosci 17:791-804.

Segal RA, Greenberg ME (1996) Intracellular signaling pathways activated by neurotrophic factors. Ann Rev Neurosci 19:463-489.

Shanley TP, Ryan MA, Eaves-Pyles T, Wong HR (2000) Heat shock inhibits phosphorylation of I-kappaBalpha. Shock 14:447-450.

Shaw M, Cohen P, Alessi D (1998) The activation of protein kinase B by H2O2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. Biochem J 336:241-246.

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407:538-541.

Snider WD (1994) Functions of the neurotrophins during nervous system development: What the knockouts are teaching us. Cell 77:627-638.

Snider WD, Silos-Santiago I (1996) Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. Philos Trans R Soc Lon B Biol Sci 351:395-403.

Sohrabji F, Miranda RC, Toran-Allerand CD (1994) Estrogen differentially regulates estrogen and nerve growth factor receptor mRNAs in adult sensory neurons. J Neurosci 14:459-471.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, Nikolics K, Parada LF (1991) The neurotrophic factors BDNF and NT-3 are ligands for the TrkB tyrosine kinase receptor. Cell 65:895-903.

Soti C, Csermely P (2002a) Chaperones come of age. Cell Stress & Chaperones 7:186-190.

Soti C, Csermely P (2002b) Chaperones and aging: role in neurodegeneration and in other civilizational diseases. Neurochem Int 41:383-389.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, DiStefano PS, Yancopoulus GD (1991) trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65:885-893.

Stambolic V, Ruel L, Woodgett J (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr Biol 6:1664-1668.

Stefanis L (2005) Caspase-dependent and -independent neuronal death: two distinct pathways to neuronal injury. Neuroscientist 11:50-62.

Stephens R, Loeb D, Copeland T, Pawson T, Greene L, Kaplan D (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron 12:691-704.

Stokoe D, Engel K, Campbell DG, Cohen P, Gaestel M (1992) Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. FEBS Letters 313:307-313.

Taleghany N, Sarajari S, DonCarlos LL, Gollapudi L, Oblinger MM (1999) Differential expression of estrogen receptor alpha and beta in rat dorsal root ganglion neurons. J Neurosci Res 57:603-615.

Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T, Tohyama M (1999) Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. J Biol Chem 274:8531-8538.

Tanabe M, Nakai A, Kawazoe Y, Nagata K (1997) Different Thresholds in the Responses of Two Heat Shock Transcription Factors, HSF1 and HSF3. J Biol Chem 272:15389-15395.

Tanaka H, Yokota H, Jover T, Cappuccio I, Calderone A, Simionescu M, Bennett MV, Zukin RS (2004) Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. J Neurosci 24:2750-2759.

Tanaka S, Koike T (2001) Activation of protein kinase C delays apoptosis of nerve growth factor-deprived rat sympathetic neurons through a Ca(2+)-influx dependent mechanism. Neurosci Lett 313:9-12.

Taylor JM, Crack PJ (2004) Impact of oxidative stress on neuronal survival. Clin Exp Pharmacol Physiol 31:397-406.

Teng KK, Hempstead BL (2004) Neurotrophins and their receptors: signaling trios in complex biological systems. Cell Mol Life Sci 61:35-48.

Theriault JR, Lambert H, Chavez-Zobel AT, Charest G, Lavigne P, Landry J (2004) Essential role of the NH2-terminal WD/EPF motif in the phosphorylation-activated protective function of mammalian Hsp27. J Biol Chem 279:23463-23471.

Thoenen H (1991) The changing scene of neurotrophic factors. Trends Neurosci 14:165-170.

Toker A (1998) Signaling through protein kinase C. Front Biosci 3:D1134-1147.

Tong JX, Eichler ME, Rich KM (1996) Intracellular calcium levels influence apoptosis in mature sensory neurons after trophic factor deprivation. Exp Neurol 138:45-52.

Tonkiss J, Calderwood SK (2005) Regulation of heat shock gene transcription in neuronal cells. Int J Hyperthermia 21:433-444.

Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi N, Jones SN, Flavell RA, Davis RJ (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 288:870-874.

Trinklein ND, Chen WC, Kingston RE, Myers RM (2004) Transcriptional regulation and binding of heat shock factor 1 and heat shock factor 2 to 32 human heat shock genes during thermal stress and differentiation. Cell Stress Chap 9:21-28.

Troy CM, Friedman JE, Friedman WJ (2002) Mechanisms of p75-mediated death of hippocampal neurons. Role of caspases. J Biol Chem 277:34295-34302.

Tucker BA, Rahimtula M, Mearow KM (2005) Integrin activation and neurotrophin signaling cooperate to enhance neurite outgrowth in sensory neurons. J Comp Neurol 486:267-280.

Uoshima K, Handelman B, Cooper LF (1993) Isolation and Characterization of a Rat HSP 27 Gene. Biochemical and Biophysical Research Communications 197:1388-1395.

Vaillant A, Mazzoni I, Tudan C, Boudreau M, Kaplan D, Miller F (1999) Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol 146:955-966.

Valentim LM, Rodnight R, Geyer AB, Horn AP, Tavares A, Cimarosti H, Netto CA, Salbego CG (2003) Changes in heat shock protein 27 phosphorylation and immunocontent in response to preconditioning to oxygen and glucose deprivation in organotypic hippocampal cultures. Neurosci 118:379-386.

van der Zee C, Rashid K, Le K, Moore K, Stanisz J, Diamond J, Racine R, Fahnestock M (1995) Intraventricular administration of antibodies to nerve growth factor retards kindling and blocks mossy fiber sprouting in adult rats. J Neurosci 15:5316-5323.

Verge VMK, Merlio JP, Grondin J (1992) Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. J Neurosci 12:4011-4022.

Virdee K, Tolkovsky AM (1996) Inhibition of p42 and p44 Mitogen-Activated Protein Kinase Activity by PD98059 Does Not Suppress Nerve Growth Factor-Induced Survival of Sympathetic Neurones. J Neurochem 67:1801-1805.

Vogelbaum MA, Tong JX, Rich KM (1998) Developmental Regulation of Apoptosis in Dorsal Root Ganglion Neurons. J Neurosci 18:8928-8935.

Voss MR, Stallone JN, Li M, Cornelussen RNM, Knuefermann P, Knowlton AA (2003) Gender differences in the expression of heat shock proteins: the effect of estrogen. Am J Physiol Heart Circ Physiol 285:H687-692.

Wagstaff MJ, Collaco-Moraes Y, Smith J, de Belleroche JS, Coffin RS, Latchman DS (1999) Protection of Neuronal Cells from Apoptosis by Hsp27 Delivered with a Herpes Simplex Virus-based Vector. J Biol Chem 274:5061-5069.

Walsh GS, Orike N, Kaplan DR, Miller FD (2004) The invulnerability of adult neurons: a critical role for p73. J Neurosci 24:9638-9647.

Walter S, Buchner J (2002) Moleclar chaperones - cellular machines for protein folding. Angew Chem Int Ed 41:1098-1113.

Welch WJ (1985) Phorbol ester, calcium ionophore, or serum added to quiescent rat embryo fibroblast cells all result in the elevated phosphorylation of two 28,000-dalton mammalian stress proteins. J Biol Chem 260:3058-3062.

Wert M, Palfrey H (2000) Intraventricular administration of antibodies to nerve growth factor retards kindling and blocks mossy fiber sprouting in adult rats. Biochem J 352:175-182.

White FA, Silos-Santiago I, Molliver DC, Nishimura M, Phillips H, Barbacid M, Snider WD (1996) Synchronous Onset of NGF and TrkA Survival Dependence in Developing Dorsal Root Ganglia. J Neurosci 16:4662-4672.

Williams KL, Rahimtula M, Mearow KM (2005) Hsp27 and axonal growth in adult sensory neurons in vitro. BMC Neurosci 6:24.

Wong HR, Ryan M, Wispe JR (1997a) Stress response decreases NF-kappaB nuclear translocation and increases I-kappaBalpha expression in A549 cells. J Clin Invest 99:2423-2428.

Wong HR, Ryan M, Wispe JR (1997b) The heat shock response inhibits inducible nitric oxide synthase gene expression by blocking I kappa-B degradation and NF-kappa B nuclear translocation. Biochem Biophys Res Comm 231:257-263.

Wood JN (1995) Regulation of Nf-kB activity in rat dorsal root ganglia and PC12 cells by tumour necrosis factor and nerve growth factor. Neurosci Lett 192:41-44.

Woodgett J (2001) Judging a protein by more than its name: GSK-3. Sci STKE 100:RE12.

Woolf BB, Green DR (1999) Suicidal Tendencies: Apoptotic Cell Death by Caspase Family Proteinases. J Biol Chem 274:20049-20052.

Wooten MW, Seibenhener ML, Neidigh KB, Vandenplas ML (2000) Mapping of atypical protein kinase C within the nerve growth factor signaling cascade: relationship to differentiation and survival of PC12 cells. Mol Cell Biol 20:4494-4504.

Wright DE, Snider WD (1995) Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. J Comp Neurol 351:329-338.

Wu C (1984) Two protein-binding sites in chromatin implicated in the activation of heat shock genes. Nature 309:229-234.

Wu C (1995) Heat shock transcription factors: structure and regulation. Annu Rev Cell Dev Biol 11:441-469.

Wyttenbach A, Sauvageot O, Carmichael J, Diaz-Latoud C, Arrigo AP, Rubinsztein DC (2002) Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. Hum Mol Genet 11:1137-1151.

Xia W, Voellmy R (1997) Hyperphosphorylation of Heat Shock Transcription Factor 1Is Correlated with Transcriptional Competence and Slow Dissociation of Active Factor Trimers. J Biol Chem 272:4094-4102.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326-1331.

Xiao H, Lis JT (1988) Germline transformation used to define key features of heat-shock response elements. Science 239:1139-1142.

Xiao J, Liu Y (2003) Differential roles of ERK and JNK in early and late stages of neuritogenesis: a study in a novel PC12 model system. J Neurochem 86:1516-1523.

Yan LJ, Rajasekaran NS, Sathyanarayanan S, Benjamin IJ (2005) Mouse HSF1 disruption perturbs redox state and increases mitochondrial oxidative stress in kidney. Antioxid Redox Signal 7:465-471.

Yenari MA (2002) Heat shock proteins and neuroprotection. Adv Exp Med Biol 513:281-299.

Yip HK, Rich KM, Lampe PA, Johnson EMJ (1984) The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. J Neurosci 4:2986-2992.

Yu Z, Zhou D, Bruce-Keller AJ, Kindy MS, Mattson MP (1999) Lack of the p50 subunit of nuclear factor-kappaB increases the vulnerability of hippocampal neurons to excitotoxic injury. J Neurosci 19:8856-8865.

Zhang X, Beuron F, Freemont PS (2002) Machinery of protein folding and unfolding. Curr Opin Struct Biol 12:231-238.

Zhu Y, Culmsee C, Klumpp S, Krieglstein J (2004) Neuroprotection by transforming growth factor-beta1 involves activation of nuclear factor-kappaB through phosphatidylinositol-3-OH kinase/Akt and mitogen-activated protein kinase-extracellular-signal regulated kinase1,2 signaling pathways. Neuroscience 123:897-906.

Zirrgiebel U, Ohga Y, Carter B, Berninger B, Inagaki N, Thoenen H, Lindholm D (1995) Characterization of TrkB receptor-mediated signalling pathways in rat cerebellar granule neurons:involvement of protein kinase C in neuronal survival. J Neurochem 65:2241-2250.

Zou J, Guo Y, Guettouche T, Smith DF, Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell 94:471-480.

Zourlidou A, Payne Smith MD, Latchman DS (2004) HSP27 but not HSP70 has a potent protective effect against alpha-synuclein-induced cell death in mammalian neuronal cells. J Neurochem 88:1439-1448.