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Original

**CELL TYPE-SPECIFIC AND STRESS-DEPENDENT EXPRESSION OF THE  
SMALL HEAT SHOCK PROTEIN HSP27 IN THE ADULT RAT CENTRAL  
NERVOUS SYSTEM**

by

**Anne Marie R. Krueger-Naug**

**Submitted in partial fulfilment of the requirements for the degree of**

**Doctor of Philosophy**

**in Anatomy and Neurobiology**

**at**

**Dalhousie University**

**Halifax, Nova Scotia**

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Cell Type-Specific and Stress-Dependent Expression of the Small Heat Shock Protein Hsp27 in the Adult Rat Central Nervous System" by Anne Marie R. Krueger-Naug in partial fulfilment of the requirements of the degree of Doctor of Philosophy.

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**so much depends  
upon**

**a red wheel  
barrow**

**glazed with rain  
water**

**beside the white  
chickens**

**William Carlos Williams**

**From Collected Poems: 1909-1939, Volume 1**

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*'Hogwarts, Hogwarts, Hoggy Warty Hogwarts  
Teach us something please,  
Whether we be old and bald  
Or young with scabby knees,  
Our heads could do with filling  
With some interesting stuff,  
For now they're bare and full of air  
Dead flies and bits of fluff,  
So teach us things worth knowing,  
Bring back what we've forgot,  
Just do your best, we'll do the rest,  
And learn until our brains all rot.'*

J.K. Rowling  
from *Harry Potter and the Philosopher's Stone*  
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**to Jason and to baby Anna**

**All the diamonds in this world**

**that mean anything to me**

**Bruce Cockburn  
Circles in the Stream © 1997**

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## **ABSTRACT**

Heat shock proteins are a diverse group of evolutionarily conserved proteins that are found in all organisms. The small heat shock protein Hsp27 is constitutively expressed in many neurons of the brainstem and spinal cord, is strongly induced in glial cells in response to ischemia, seizures, or spreading depression, and is selectively induced in peripheral neurons after axotomy. This thesis examined the cell-type specific and stress-dependent expression of Hsp27 in the rat central nervous system following hyperthermic treatment and following transection of the optic nerve.

The expression of Hsp27 was examined in brains of adult rats from 1.5 hours to 6 days after brief hyperthermic stress (core body temperature of 42°C for 15 min). Twenty-four hours following hyperthermia, Western blot analysis showed that Hsp27 was elevated in the cerebral cortex, hippocampus, cerebellum and brainstem. Immunohistochemistry for Hsp27 revealed a time-dependent, but transient, increase in the level of Hsp27 immunoreactivity in several types of neuroglia and in specific neuronal populations. The pattern of neuronal Hsp27 immunoreactivity suggests that some of the activated cells are involved in physiological responses related to body fluid homeostasis and temperature regulation.

Hsp27 has recently been shown to play a role in sensory neuron survival following peripheral nerve axotomy (Lewis et al., 1999). To investigate the role of Hsp27 in injured central nervous system sensory neurons, the induction and cell-specific expression of Hsp27 was studied in rat retinal ganglion cells from 1-28 days after optic nerve transection. The results of this work demonstrated that transection of the optic nerve induced the expression of Hsp27 in three distinct regions of the rat visual system:

**sensory retinal ganglion cells in the eye, glial cells of the optic tract, and astrocytes in the optic layer of the superior colliculus. Hsp27 may be associated with enhanced survival of a subset of retinal ganglion cells, providing evidence of a protective role for Hsp27 in central nervous system neuronal injury.**

**In addition, the administration of the neurotrophin BDNF at the time of optic nerve transection was shown to delay retinal ganglion cell death and also to suppress retinal ganglion cell expression of Hsp27. These results indicate that exogenously applied protective molecules may interfere with other endogenous cell survival pathways important for the long-term survival of some neurons following injury.**

## LIST OF ABBREVIATIONS

<b>%</b>	<b>percent</b>
<b>μl</b>	<b>microliter</b>
<b>μm</b>	<b>micrometer</b>
<b>°C</b>	<b>degrees Celsius</b>
<b>Aq</b>	<b>aqueduct</b>
<b>AP</b>	<b>area postrema</b>
<b>Ask-1</b>	<b>apoptosis signal regulated kinase-1</b>
<b>AT<sub>1</sub></b>	<b>angiotensin II receptor 1</b>
<b>BDNF</b>	<b>brain derived neurotrophic factor</b>
<b>CA3c</b>	<b>(cornu ammonis) pyramidal cell layer sector 3c</b>
<b>cc</b>	<b>central canal</b>
<b>CC</b>	<b>corpus callosum</b>
<b>caspase</b>	<b>cysteine proteases with aspartate specificity</b>
<b><i>c-fos</i></b>	<b>fos gene</b>
<b>CNS</b>	<b>central nervous system</b>
<b>Cor</b>	<b>cortex</b>
<b>CPu</b>	<b>caudate putamen</b>
<b>CSD</b>	<b>cortical spreading depression</b>
<b>DAB</b>	<b>diaminobenzidine</b>
<b>DAG</b>	<b>diaminoglycerol</b>
<b>Daxx</b>	<b>death associated protein</b>

<b>DD</b>	<b>death domain</b>
<b>DMV</b>	<b>dorsal motor nucleus of the vagus nerve</b>
<b>DpWh</b>	<b>deep white layer of the superior colliculus</b>
<b>DRG</b>	<b>dorsal root ganglion</b>
<b>ERK</b>	<b>extracellular signal-regulated kinase (MAPK)</b>
<b>FADD</b>	<b>Fas associated death domain</b>
<b>Fas-L</b>	<b>Fas ligand</b>
<b>FG</b>	<b>Fluorogold</b>
<b>fl</b>	<b>fiber layer</b>
<i><b>fos</b></i>	<b>fos gene</b>
<b>FOS</b>	<b>fos protein</b>
<b>G</b>	<b>granule cell layer (cerebellum)</b>
<b>GC</b>	<b>granule cell layer</b>
<b>GCL</b>	<b>ganglion cell layer</b>
<b>GFAP</b>	<b>glial fibrillary acidic protein</b>
<b>Gr</b>	<b>gracile nucleus and tract</b>
<b>GTP</b>	<b>guanosine triphosphate</b>
<b>Hil</b>	<b>hilus of the dentate gyrus</b>
<b>HSE</b>	<b>heat shock element</b>
<b>HSF1</b>	<b>heat shock protein factor 1</b>
<i><b>hsp</b></i>	<b>heat shock protein gene</b>
<b>Hsp(s)</b>	<b>heat shock protein(s)</b>
<b>Hsp25</b>	<b>heat shock protein 25 (mouse)</b>

<b>Hsp27</b>	<b>heat shock protein 27 (rat)</b>
<i><b>hsp27</b></i>	<b>heat shock protein 27 gene</b>
<b>i.p.</b>	<b>intraperitoneally</b>
<b>IgG</b>	<b>immunoglobulin G</b>
<b>IL-IR1</b>	<b>interleukin-1 receptor 1</b>
<b>ILM</b>	<b>inner limiting membrane</b>
<b>InG</b>	<b>intermediate gray layer of the superior colliculus</b>
<b>INL</b>	<b>inner nuclear layer</b>
<b>InW</b>	<b>intermediate white layer of the superior colliculus</b>
<b>IPL</b>	<b>inner plexiform layer</b>
<b>IR</b>	<b>immunoreactivity</b>
<b>KCl</b>	<b>potassium chloride</b>
<b>kDa</b>	<b>kiloDaltons</b>
<b>kg</b>	<b>kilogram</b>
<b>Lmol</b>	<b>lacunosum moleculaire</b>
<b>LS</b>	<b>lateral septum</b>
<b>LV</b>	<b>lateral ventricle</b>
<b>M</b>	<b>molar</b>
<b>MAP3K</b>	<b>mitogen activated protein kinase kinase kinase</b>
<b>MAPK</b>	<b>mitogen activated protein kinase</b>
<b>MAPKAP-2</b>	<b>mitogen activated protein kinase activated protein kinase-2</b>
<b>MAPKK</b>	<b>mitogen activated protein kinase kinase</b>
<b>mg</b>	<b>milligram</b>

<b>MKK3</b>	<b>mitogen activated protein kinase kinase 3</b>
<b>MKK6</b>	<b>mitogen activated protein kinase kinase 3</b>
<b>mM</b>	<b>millimolar</b>
<b>mRNA</b>	<b>messenger ribonucleic acid</b>
<b>n</b>	<b>number</b>
<b>NGF</b>	<b>nerve growth factor</b>
<b>NT 4/5</b>	<b>neurotrophin 4/5</b>
<b>NTS</b>	<b>nucleus of the tractus solitarius</b>
<b>OLM</b>	<b>outer limiting membrane</b>
<b>ONL</b>	<b>outer nuclear membrane</b>
<b>Op</b>	<b>optic layer of the superior colliculus</b>
<b>OPL</b>	<b>outer plexiform layer</b>
<b>Or</b>	<b>stratum oriens</b>
<b>ORF</b>	<b>open reading frame</b>
<b>p</b>	<b>postnatal day</b>
<b>PB</b>	<b>phosphate buffer</b>
<b>PBS</b>	<b>phosphate buffered saline</b>
<b>PBS-X</b>	<b>phosphate buffered saline-triton X</b>
<b>PC</b>	<b>Purkinje cell layer</b>
<b>PGP9.5</b>	<b>protein gene product 9.5</b>
<b>PKC</b>	<b>protein kinase C</b>
<b>PLC</b>	<b>phospholipase C</b>
<b>PNS</b>	<b>peripheral nervous system</b>



<b>Py</b>	<b>pyramidal cell layer</b>
<b>Pyk2</b>	<b>proline-rich tyrosine kinase-2</b>
<b>Rad</b>	<b>stratum radiatum</b>
<b>RGC(s)</b>	<b>retinal ganglion cell(s)</b>
<b>RPE</b>	<b>retinal pigment epithelium</b>
<b>RSL</b>	<b>receptor segment layer</b>
<b>S</b>	<b>serine residue</b>
<b>SAPK/JNK</b>	<b>stress activated protein kinase/c-jun NH<sub>2</sub>-terminal kinase</b>
<b>SC</b>	<b>superior colliculus</b>
<b>SDS</b>	<b>sodium dodecyl sulfate</b>
<b>SFO</b>	<b>subfornical organ</b>
<b>sHsp(s)</b>	<b>small heat shock protein(s)</b>
<b>SuG</b>	<b>superficial gray layer of the superior colliculus</b>
<b>TAPA</b>	<b>target for the antiproliferative antibody</b>
<b>TNF<math>\alpha</math></b>	<b>tumor necrosis factor <math>\alpha</math></b>
<b>Trk(s)</b>	<b>tyrosine receptor kinase (s)</b>
<b>TrkB</b>	<b>tyrosine receptor kinase B</b>
<b>V</b>	<b>volt</b>
<b>wm</b>	<b>white matter</b>
<b>Z</b>	<b>zonal layer of the superior colliculus</b>
<b>III</b>	<b>third ventricle</b>
<b>IV</b>	<b>fourth ventricle</b>
<b>V<sub>m</sub></b>	<b>trigeminal motor nucleus</b>

**XII**

**hypoglossal nucleus**

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## **CHAPTER 1:**

### **INTRODUCTION**

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## **The Heat Shock Response and Heat Shock Proteins: Discovery and Classification**

At the molecular level, the heat shock response is defined as the transient inhibition of normal gene transcription and the preferential transcription and translation of a specific set of highly conserved proteins, the heat shock proteins (Hsps) (Lindquist, 1986). The heat shock response was first observed in *Drosophila*, where Ritossa (1962) reported a new set of puffs on salivary gland chromosomes in response to heat. Further investigation revealed that this set of puffs was associated with newly synthesized RNA and protein (Ritossa, 1962; Tissières et al., 1974) and that previously active puffs were absent (reviewed in Ashburner, 1982; Lindquist, 1986). The heat shock response is considered to be a universal phenomenon evident in organisms as diverse as bacteria and rodent, human and soybean (reviewed in Ashburner, 1982; Lindquist, 1986; Morimoto et al., 1990) and is induced by a wide variety of stressors in eukaryotes including environmental stress (heat shock, ultraviolet radiation, inhibitors of energy metabolism), pathophysiological stress (fever and inflammation, ischemia, neuronal injury) and conditions associated with growth and differentiation (reviewed in Morimoto et al., 1994a).

Hsps are classified into 5 main families: the large molecular mass Hsps (ranging in mass from 100-110 kDa), the Hsp90 family (ranging from 83 to 90 kDa), Hsp70 family (ranging from 66-78 kDa), Hsp60 family (found in bacteria, mitochondria and chloroplasts), and a diverse group of small molecular mass Hsps (ranging in size from 15 to 40 kDa) called the Hsp27 family of small Hsps (sHsps) (reviewed in Lindquist, 1988; Morimoto et al., 1994b).

## **Small Heat Shock Protein Family**

sHsps, found in prokaryotic, archaea and eukaryotic organisms, form a family of proteins ranging in molecular mass from 15 to 40 kDa (Lindquist, 1988, de Jong et al., 1993, 1998; Arrigo and Landry, 1994). All sHsps contain a conserved amino acid sequence located toward the carboxy terminus of the protein called the  $\alpha$ -crystallin domain (de Jong et al., 1993; 1998; MacRae, 2000). sHsps are thought to have evolved to cope with the destabilizing effects of stressful conditions on cell integrity (de Jong et al., 1993).

In mammals, the best characterized members of the sHsp family include Hsp27 (Hsp25 in mouse) and the lens proteins  $\alpha$ A and  $\alpha$ B-crystallin, all of which contain the conserved  $\alpha$ -crystallin domain (Kato et al., 2002).  $\alpha$ A and  $\alpha$ B crystallins are major structural proteins in the vertebrate lens (reviewed in de Jong et al., 1993), but have been detected in other tissues (Kato et al., 1991a,b). Early heat shock studies report the induction and expression of a 27-kDa protein in mammalian cells in response to heat treatment (Hickey and Weber, 1982). The genes encoding Hsp27 were later isolated from various mammals including human (Hickey et al., 1986), mouse (Gaestel et al., 1993) and rat (Uoshima et al., 1993).

Both  $\alpha$ B-crystallin and Hsp27 are induced by various forms of stress (reviewed in Welch, 1990, 1992; Head et al., 1994; Ito et al., 1995, 1996, 1997; Bajramovic et al., 2000) and share many functional characteristics including chaperone function (Jakob et al., 1993; Merck et al., 1993), intermediate filament stabilization (Perng et al., 1999) and antiapoptotic properties (Mehlen et al., 1995a,b; 1996a,b). The work presented in this thesis focuses on the inducible expression of Hsp27.

The rat *hsp27* gene is composed of three exons and two introns (Figure 1.1, Uoshima et al., 1993). The promoter region of the *hsp27* gene contains several regulatory elements (CATT and TAATA boxes) and includes a heat shock element (HSE; tandem nGAAn pentamer in opposing orientations). In mammals, the transcriptional activation of *hsps* in response to stressful stimuli is mediated by the binding of heat shock transcription factor (HSF1) to HSE (reviewed in Morimoto et al., 1994a). Cytosolic HSF1 forms a trimer, localizes to the nucleus, acquires DNA binding ability and becomes phosphorylated in order to induce *hsp* gene transcription (Sarge et al., 1993; reviewed in Morimoto et al., 1994a). The presence of a HSE in the promoter region of *hsp27* indicates that, in response to stress, the transcription of *hsp27* is most likely regulated by the binding of HSF1 to HSE.

Hsp27 is a 27 kDa protein (25 kDa in mouse) that consists of 206 amino acid residues (Figure 1.2, Uoshima et al., 1993). The primary structure of Hsp27 contains a highly conserved core  $\alpha$ -crystallin domain extending from amino acids 91 to 188 (Wistow, 1985; deJong 1993; reviewed in MacRae, 2000). The  $\alpha$ -crystallin domain is flanked by an N-terminal domain of 90 amino acids and a flexible C-terminal extension of 18 amino acids (Carver et al., 1995). The secondary structure is predominantly  $\beta$ -pleated sheet (Merck et al., 1993). The tertiary structure of Hsp27 is variable and dependent on specific sequences in the peptide. Hsp27 can form stable dimers through  $\alpha$ -crystallin domain interactions (Lambert et al., 1999; Ehrnsperger et al., 1999; Liu and Welsh, 1999) and sequences in the N-terminal domain are necessary for the stabilization of larger oligomers of up to 800 kDa (Lambert et al., 1999). In the mouse, the flexible C-terminal extension contributes to the stability and solubility of Hsp27 oligomers and

**Figure 1.1: Structure of *hsp27* gene and promoter region.** The promoter region contains several regulatory elements, CATT and TAATA boxes and a heat shock element (HSE). The gene is comprised of 3 exons (E) and a putative HSE is located in the first intron (866-990). The three exons form the open reading frame (ORF) of the *hsp27* mRNA. Sequences encoding the  $\alpha$ -crystallin domain of Hsp27 protein are found in each of the exons. Adapted from Uoshima et al., 1993.



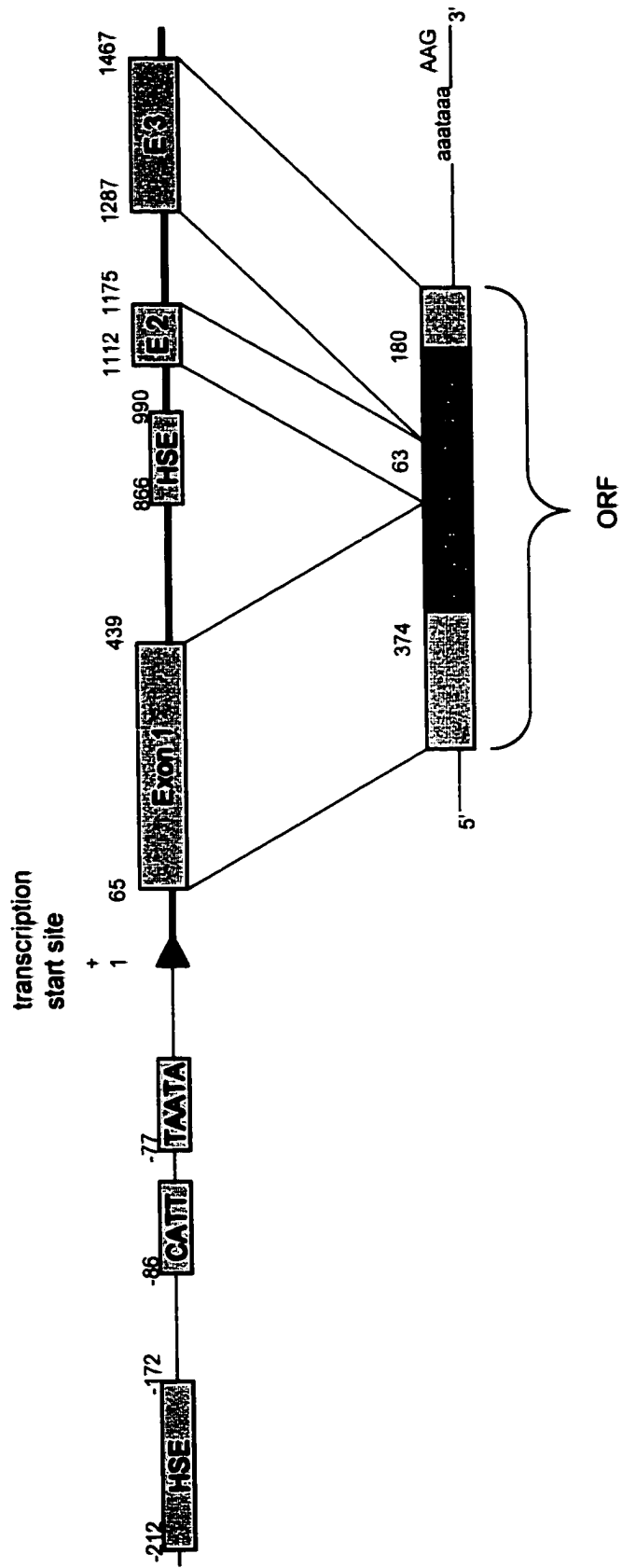


Figure 1.1

Hsp27-substrate complexes (Lindner et al., 2000). The tertiary structure of Hsp27 (monomer, dimer or oligomer) is modulated by phosphorylation. In the rodent, phosphorylation of Hsp27 occurs at two sites in the N-terminal domain of the protein, serine 15 and serine 86 (Figure 1.2, Gaestel et al., 1991). Phosphorylation of serine residues modulates the intramolecular interactions of the larger Hsp27 oligomers, causing the dissociation of the Hsp27 oligomers into dimer and monomeric structures (Kato et al., 1994b; Lambert et al., 1999).

### **Hsp27 Function**

The expression of Hsp27 enhances the survival of mammalian cells when exposed to environmental and physiological stressors (Landry et al., 1989; Rollet et al., 1992; Mehlen et al., 1993; Kampinga et al., 1994; Wissing and Jäättelä, 1996). Specifically, Hsp27 acts as a molecular chaperone (Jakob et al., 1993; Ehrnsperger et al., 1997), inhibits actin and intermediate filament polymerization (Lavoie et al., 1993a,b; Benndorf et al., 1994; Perng et al., 1999; Wieske et al., 2001), reduces oxidative stress related to tumor necrosis factor  $\alpha$  (TNF $\alpha$ )-mediated cell death (Mehlen et al., 1995a,b) and inhibits both receptor-mediated and mitochondrial-mediated apoptosis (Mehlen et al., 1996a,b; Garrido et al., 1999; Bruey et al., 2000; Charette et al., 2000; Pandey et al., 2000; reviewed in Beere et al., 2001; Concannon et al., 2001). Phosphorylation modulates the structure of Hsp27 between monomeric and multimeric forms that are important for the specific functions of the protein (Préville et al., 1998). The unphosphorylated monomer of Hsp27 is required for the inhibition of actin polymerization while phosphorylation

**Figure 1.2:** Sequence and primary structure of Hsp27 protein. **A:** sequence of Hsp27 protein. Asterisks indicate phosphorylation sensitive sites at serine 15 and serine 86. The  $\alpha$ -crystallin domain is underlined. **B:** primary structure of Hsp27 protein. Phosphorylation sensitive serine residues (S) and  $\alpha$ -crystallin domain (box) are indicated.

**A**

\*  
 MTERRVPFSL LRSPSWEPFR DWYPAHSRLF DQAFGVPRFP DEWSQWFSSA  
 \*  
 GWPGYVRPLP AATAEGPAAV TLARPFSTRAL NRQLSSGVSE IRQTADRWRV  
SLDVNHFAPE ELTVKTEGV VEITGKHEER QDEHGYISRC FTRKYTLPPG  
VDPTLVSSSL SPEGTLTVEA PLPKAVTQSA EITIPVTFEA RAQIGGPESE  
 QSGAK

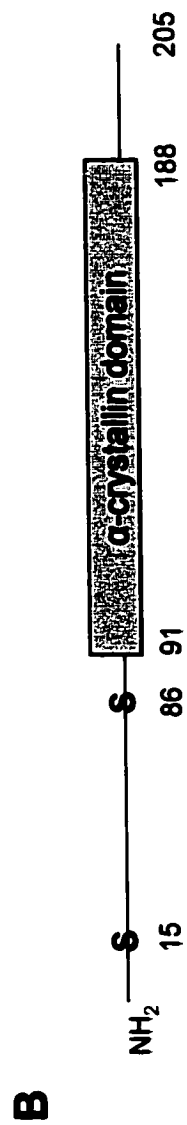


Figure 1.2

of these monomers results in a decline of this inhibition and a subsequent modulation of actin dynamics (Lavoie et al., 1993b; Benndorf et al., 1994; Guay et al., 1997; Wieske et al., 2001). The larger oligomeric forms of Hsp27 are necessary for the chaperoning functions of Hsp27 (Lavoie et al., 1995). *In vitro*, Ehrnsperger et al. (1997) have shown that Hsp27 oligomers bind unfolded proteins and hold them in a folding-competent state. Using a nonphosphorylatable form of Hsp27 (serines replaced with alanine), Prévaille et al. (1998) have shown that the large oligomers are also important for protection from TNF $\alpha$ -induced necrotic cell death. The hyperaggregated forms of Hsp27 maintain the ability to reduce intracellular reactive oxygen species and increase glutathione levels to combat the oxidative stress associated with TNF $\alpha$  treatment in culture (Prévaille et al., 1998).

In addition, Hsp27 interferes with apoptotic cell death pathways induced by Fas/APO-1 receptor activation (Mehlen et al., 1996b; Charette et al., 2000). The binding of FAS/APO-1 with its ligand, Fas-L, results in the activation of two death pathways, one fast and one slow (Charette et al., 2000). The fast apoptotic pathway involves the recruitment of Fas-associated death domain (FADD) and the activation of procaspase-8 and procaspase-3 leading to cell death (Chinnaiyan et al., 1995; Muzio et al., 1996; reviewed in Cohen, 1997). The slow apoptotic pathway involves the Fas-binding protein, death associated protein (Daxx) and the activation of apoptosis signal regulated kinase 1 (Ask1) and subsequent stress activated protein kinase/Jun N-terminal kinase (SAPK/JNK) activation (Chang et al., 1998). The inhibition of the fast apoptotic pathway by Hsp27 is not currently correlated with any change in Hsp27 oligomerization or phosphorylation state (reviewed in Arrigo et al., 2002). However, the phosphorylated

dimeric form of Hsp27 binds to Daxx and disrupts the Daxx-dependent apoptotic pathway (Charette et al., 2000).

Mitochondrial-mediated apoptosis involves the release of cytochrome c from the mitochondria, apoptosome formation and the activation of procaspase-9 and procaspase-3 (reviewed in Beere, 2001). Hsp27 inhibits mitochondrial-mediated apoptosis by interacting with and sequestering cytochrome c once released from the mitochondria (Bruey et al., 2000; Concannon et al., 2001). Hsp27 also sequesters procaspase-3, inhibiting its activation (Concannon et al., 2001). The form of Hsp27 that interacts with both cytochrome c and procaspase-3 is undetermined.

Given the induction and expression of Hsp27 in various mammalian cell types including neurons *in vitro*, along with its specific protective functions, there is a potential protective role for Hsp27, *in vivo*, following central nervous system (CNS) injury.

### **Hsp27 in the Mammalian Central Nervous System**

The expression and distribution of Hsp27 in the CNS is particularly striking during development and in the adult under normal conditions and after acute pathophysiological challenge. In the CNS, Hsp27 is expressed constitutively in well-defined subsets of neurons but only occasionally in neuroglia. In contrast, expression of Hsp27 in neurons and neuroglia is markedly increased in response to physiological challenges and in various models of nervous system injury, suggesting specific cell-type, stress-dependent expression of Hsp27.

### ***Developmental and Constitutive Expression of Hsp27***

During development, Hsp27 is detected at the seventeenth week of gestation in neurons and non-neuronal cells in human brains (Aquino et al., 1996). In the mouse, Hsp27 is detected in spinal cord neurons and cerebellar Purkinje cells of mouse at embryonic day 16 (Gernold et al., 1993) although Armstrong et al. (2001a,b) were unable to confirm this finding. During postnatal development, Hsp27 has a distinct pattern of expression in cerebellar Purkinje cells (Armstrong et al., 2001a). A phase of limited expression early in postnatal development is followed by a widespread expression of Hsp27 in most Purkinje cells at postnatal day 6. Global expression is gradually reduced leaving the restricted parasagittal banding of Hsp27 in a select population of Purkinje neurons in the adult (Armstrong et al., 2000). In addition, Hsp27 is transiently expressed in neurons of the inferior colliculus and thalamus during postnatal development (Armstrong et al., 2001b). Sustained expression of Hsp27 into adulthood is detected in spinal cord motor neurons and neurons of many cranial nerve nuclei including the motor, sensory and mesencephalic nuclei of the trigeminal nerve, facial nucleus, nucleus ambiguus, dorsal motor nucleus of the vagus and hypoglossal nucleus (Armstrong et al., 2001b).

In the adult rat CNS, the distribution of Hsp27 has been described in detail (Plumier et al., 1997c). Hsp27 is not normally expressed in neurons of the forebrain; however, a small number of arcuate neurons are Hsp27-positive. In contrast to the adult mouse, no Hsp27 is detected in rat cerebellar Purkinje neurons. Hsp27 is detected normally in many motor neurons of the oculomotor nuclei (oculomotor, trochlear, and abducens) of the brain stem, although this expression is minimal to non-existent in the mouse (Armstrong et al., 2001b). Hsp27 is observed in the motor neurons of the trigeminal, facial, vagal,

and hypoglossal cranial nerve nuclei and is normally expressed at high levels in anterior horn motor neurons in the spinal cord.

***Inducible Expression of Hsp27 in the Central Nervous System in Various Models of Acute Stress***

***Heat shock***

In the brain, Blake et al. (1990a) were the first to detect the heat-induced expression of Hsp27 mRNA by Northern analysis. Western analysis indicated increased levels of Hsp27 protein in the cerebral cortex, hippocampus and cerebellum of rats from 8 to 16 hours following hyperthermic treatment (Inaguma et al., 1995). In the cerebellum, the hyperthermic-induced expression of Hsp27 is localized to Bergmann glia using immunohistochemistry (Bechtold and Brown, 2000). Following hyperthermic treatment, Hsp27 is reported to be transported along the radial fibers of the Bergmann glia and is detected in perisynaptic glial processes in the molecular layer of the cerebellum. Bechtold and Brown (2000) propose that this expression of Hsp27 contributes to protective mechanisms at the level of the synapse.

***Kainic acid-induced status epilepticus***

Kainic acid is a powerful excitatory neurotoxic analogue of glutamate (Olney et al., 1974) that causes cell death in various regions of the rat CNS including the hippocampus, amygdala, entorhinal and piriform cortex. The damage is similar to that seen with temporal lobe epilepsy (Ben-Ari, 1985). Following kainic acid-induced status epilepticus Hsp27 is detected primarily in astrocytes in the affected areas including the



hippocampus, entorhinal and piriform cortex indicating a role for these cells in protection or recovery of adjacent neurons following induced status epilepticus (Plumier et al., 1996). Kato et al. (1999b) detected Hsp27 in astrocytes and in neurons of the hippocampus, piriform cortex and entorhinal cortex indicating that some neurons in the affected regions express Hsp27.

### *Cortical spreading depression*

Cortical spreading depression (CSD) is characterized by a transient disruption of neural activity in the brain that spreads from a central location (Leao, 1944). The wave of neural activity spreads to adjacent neurons causing sodium, calcium, and chloride ions to enter the neurons, resulting in a burst of action potentials followed by electrical silence. Experimentally, spreading depression occurs either as a consequence of ischemic injury in the brain or is triggered by direct application of potassium chloride (KCl) to the cortex (Matsushima et al., 1996). In this experimental paradigm, brains are resistant to subsequent ischemic injury as indicated by reduced infarct size (Matsushima et al., 1998). It is interesting to note that following KCl-induced CSD, Hsp27 expression in astrocytes throughout most of the ipsilateral cortex (Plumier et al., 1997b) correlates with the neuroprotective effect.

Blocking of CSD with MK-801, an N-methyl-D-aspartate receptor antagonist, suppresses the expression of Hsp27 throughout the cortex, suggesting that glutamate release plays a role in the expression of Hsp27 in astrocytes (Plumier et al., 1997b). Thus, KCl-induced CSD up-regulates Hsp27 in astrocytes, raising the possibility that increased Hsp27 reduces stroke-like injury.

### *Ischemia and preconditioning*

In the brain, ischemia is the result of insufficient cerebral blood flow causing decreases in cerebral oxygen content. At the cellular level, oxidative stress caused by ischemia results in the death of neurons in the ischemic core surrounded by a penumbra of injured neurons and glial cells that may further degenerate or survive during reperfusion (Dirnagl et al., 1999; Sharp et al., 2000). Ischemic injury is also associated with the subsequent development of CSD (Matsushima et al., 1996). Various animal models of ischemia are used to study the cellular and molecular responses to stroke-like injury in the brain. The study of Hsp27 expression in the brain following ischemic injury has led to the intriguing hypothesis that Hsp27 expression in astrocytes and the protection of astrocyte function may be important in neuron survival. In the rat brain, following focal cerebral ischemia caused by middle cerebral artery occlusion, Hsp27 mRNA and protein are elevated (Higashi et al., 1994; Wagstaff et al., 1996) and Hsp27 mRNA is detected over a broad area of the ipsilateral cortex but not in the ischemic core (Higashi et al., 1994; Imura et al., 1999). Immunohistochemical studies have localized the expression of Hsp27 to astrocytes throughout the ipsilateral cortex and part of the contralateral cortex (Kato et al., 1995; Currie et al., 2000). Following cortical photothrombotic injury, Hsp27 is expressed in astrocytes in the penumbra and throughout the ipsilateral cortex (Plumier et al., 1997a). The widespread astrocytic expression of Hsp27 following ischemic injury correlates with ischemia-induced CSD and indicates that Hsp27 may play a role in the astroglial response to ischemic stress (Imura et al., 1999).

Brief ischemia preconditions the brain to be more resistant to subsequent episodes of severe ischemic injury (Kato et al. 1994a; Kitagawa et al., 1997; Puisieux et al., 2000).

For example, in a model of ischemic preconditioning, a 10 min occlusion of the middle cerebral artery produces significant tolerance to subsequent permanent middle cerebral artery occlusion (Barone et al., 1998). From 1 to 7 days after the preconditioning stimulus there is a significant reduction in infarct size and neurological deficits following permanent middle cerebral artery occlusion. In this model, Hsp27 is expressed throughout the ipsilateral cortex, mainly in astrocytes, for up to 4 weeks after preconditioning (Currie et al., 2000). Interestingly, preconditioning with KCl-induced CSD also results in a significant resistance to subsequent ischemic injury (Kobayashi et al., 1995; Matsushima et al., 1996). Ischemic preconditioning and KCl-induced CSD both induce a prolonged expression of Hsp27 primarily in astrocytes suggesting the intriguing possibility that Hsp27 protects astrocyte function during and after ischemia and improves neuronal survival through astrocyte-neuron interactions. In support of this idea, evidence suggests that astrocytes enhance the survival of neurons after oxidative injury *in vitro* in mixed astrocyte-neuron cultures (Desagher et al., 1996; Xu et al., 1999). Thus, Hsp27 may confer tolerance to astrocytes against ischemic injury by preserving functions such as antioxidant activity or glutamate uptake that may, in turn, protect neurons in the cortex from more severe injury.

### *Axotomy*

In general, axonal disruption in the CNS results in the apoptotic cell death of the injured neurons. Following peripheral nerve injury, however, most neurons continue to survive and begin a process of regeneration (reviewed in Sofroniew, 1999). In order to find appropriate methods to counteract neuron cell death following CNS injury, it has

become increasingly important to understand the molecular mechanisms underlying neuronal cell death and survival in response to axonal injury.

Following various peripheral nerve injuries, Hsp27 is strongly induced and expressed. After cervical resection of the vagus nerve, Hsp27 is up-regulated in both sensory neurons of the nodose ganglion and motor neurons of the dorsal motor nucleus of the vagus and nucleus ambiguus (Hopkins et al., 1998). Hsp27 is detected at high levels in most injured neuronal cell bodies from 2 to 90 days following vagotomy. After transection of the sciatic nerve, Hsp27 expression is up-regulated and persists in dorsal root ganglion (DRG) neurons and in ventral horn motor neurons for months (Costigan et al., 1998). Following sciatic and vagus nerve transection, most of the associated axotomized neurons survive for months after the injury and the survival of injured neurons correlates with up-regulated Hsp27 expression (Hopkins et al., 1998; Costigan et al., 1998).

In the CNS, lesioning of axons in the fimbria fornix up-regulates the expression of Hsp27 primarily in astrocytes of the hippocampus and medial septum, with some neurons of the medial septum expressing Hsp27 by 10 days post lesion (Anguelova and Smirnova, 2000). It has been suggested that increased expression of Hsp27 in neurons after peripheral nerve axotomy or central fimbria fornix lesion may contribute to the prevention of neuronal cell death (Costigan et al., 1998; Anguelova and Smirnova, 2000). In support of this, experiments involving the peripheral nervous system (PNS) have shown that Hsp27 expression is correlated with the survival of DRG neurons lesioned at post-natal day 0 (p0). Following lesioning at p0, 75% of DRG neurons die by apoptosis by 7 days. However, all surviving DRG neurons are Hsp27-positive (Lewis et al., 1999).

It is an intriguing possibility to consider that Hsp27-positive neurons represent a specific population of cells that are preferentially spared following both peripheral and central nerve injury.

### ***Inducible expression of Hsp27 in neurodegenerative disease***

Hsp27 is found at increased levels mostly in reactive astrocytes or neuroglia in neurodegenerative diseases of the CNS, suggesting that the expression of this stress-induced protein is disease-related (Brzyska et al., 1998). While little is known about the role of Hsp27 in neurodegenerative diseases, their detection leads one to consider whether such expression is beneficial and slows the degenerative process, or detrimental and indicative of the severity of the disease process.

Alzheimer's disease is a progressive neurodegenerative disorder that is characterized neuroanatomically by amyloid beta peptides that assemble into hyperphosphorylated tau neurofibrillary tangles that are deposited as plaques during neuritic degeneration, with subsequent neuron loss in the affected cortex (Bornemann and Straufenbiel, 2000). The expression of Hsp27 is detected in large numbers of reactive and degenerative glial cells in areas rich in plaques (Renkawek et al., 1994). In addition, Hsp27 is found in plaques (Shinohara et al., 1993; Stege et al., 1999). Hsp27-positive degenerating neurons are also more frequent in Alzheimer's disease brains (Shinohara et al., 1993). Hsp27 expression appears to increase with the severity of the Alzheimer's disease-related morphological changes and with the duration of the associated dementia (Renkawek et al., 1994). The increased accumulations of Hsp27 appear to be part of reactive processes of glial cells and neurons under pathologic conditions (Shinohara et

al., 1993). Thus, Renkawek et al. (1994) propose that the induction and increased expression of Hsp27 in astrocytes is a response to the cellular stress associated with sustained neurodegenerative processes. It has also been proposed that reactive astrocytes may play a role in the pathogenesis of Alzheimer's disease (Frederickson, 1992).

There are suggestions that chronic oxidative stress may also be important in the development of Alzheimer's disease (Pappolla et al., 1996). Oxidative stress clearly increases the expression of antioxidant enzymes and Hsps (Omar and Pappolla, 1993) suggesting that the expression of Hsp27 is a marker of oxidative stress in Alzheimer's disease.

Given that Hsp27 acts as a molecular chaperone, preventing aggregation of unfolded peptides, increased levels of Hsp27 may reflect a response to prevent amyloid fibril formation and toxicity. However, association of another sHsp,  $\alpha$ B-crystallin, with amyloid fibrils actually increases the toxicity of these fibrils (Steger et al., 1999).

Hsp27 is expressed at increased levels in the brains of Parkinson's disease patients with dementia (Renkawek et al., 1999). Parkinson's disease involves the selective degeneration of dopamine producing neurons in the substantia nigra resulting in a progressive loss in the ability to initiate or sustain coordinated movement. Symptoms include tremor, muscle rigidity and bradykinesia (slowing of movement and a loss of spontaneous movement) (reviewed in Sian et al., 1999). In the later stages of Parkinson's disease, Alzheimer's disease-like dementia becomes a prominent feature. In the brains of these patients, there is reactive gliosis and increased expression of Hsp27 in the cortex similar to that observed in Alzheimer's disease. However, there is little or no detectable expression (above that of controls) of Hsp27 in substantia nigra neurons of Parkinson's

disease patients without dementia (Renkawek et al., 1994; Renkawek et al., 1999). It is apparent that Hsp27 is not induced in the early stages of Parkinson's disease, even though degeneration of the dopaminergic substantia nigra neurons may be due to increased free radical formation in these cells (Han et al., 1999; Zhang et al., 1999). Renkawek et al. (1999) have proposed that the pathology of dementia in Parkinson's disease and Alzheimer's disease are related although the function of Hsp27 in reactive astrocytes in dementia is not yet clear.

In multiple sclerosis, increased expression of Hsp27 is found in and on the margins of sclerotic lesions (Aquino et al., 1997). Multiple sclerosis is a chronic, often progressive, inflammatory demyelinating disease of the CNS (Birnbaum, 1995; van Noort, 1996). Hsp27 is found in fibrous astrocytes in the region of the sclerotic lesion and in oligodendrocytes on the margin of the lesion. Hsp27 has been detected in multiple sclerosis myelin but not in control myelin (Aquino et al., 1997). This lead to the suggestion that the increased myelin-associated expression of Hsp27 (and other Hsps) may initially prevent destruction of the myelin sheath, but the prolonged expression of Hsps may exacerbate the disease. Other studies have implicated Hsps in the pathology of multiple sclerosis (van Noort et al., 1995; 1998). One suggestion has been that an immune response to the Hsps of an infectious agent results in a cross-reactive immune response to CNS myelin associated Hsps (reviewed in Birnbaum, 1995) and the prolonged up-regulated expression of Hsps provides additional immune responsive targets that contribute to the progression of multiple sclerosis (Aquino et al., 1997). It is interesting to note that expression of  $\alpha$ B-crystallin, another member of the sHsp family, is elevated in oligodendrocytes and astrocytes in the region of multiple sclerosis lesions

(van Noort et al., 1995). This protein elicits an immune response by T cells isolated from multiple sclerosis and control patients, lending further support to the hypothesis that expression of Hsps is involved in the progression of this inflammatory disease.

Alexander's disease is characterized by progressive psychomotor retardation and is frequently accompanied by seizures and a variable degree of megalencephaly (Borrett and Becker, 1985). Histologically, there is a considerable degree of gliosis and lack of myelination, but the most significant diagnostic feature is the presence of Rosenthal fibers found associated with skeins of intermediate filaments within reactive and neoplastic astrocytes (Iwaki et al., 1993; Head et al., 1993). The presence of Rosenthal fibers in astroglia and the occurrence of these fibers in astrocytomas and glial scars lead to the hypothesis that Alexander's disease represents a dysfunction of astrocytes (Borrett et al., 1985; Head et al., 1993). CNS tissue from patients who suffered from Alexander's disease contained elevated levels of Hsp27 and Hsp27 was detected in Rosenthal fibers in astrocytes (Head et al., 1993; Iwaki et al., 1993). Head et al. (1993) proposed that the conditions resulting in Rosenthal fiber formation are chronic and appear to be irreversible as opposed to more transitory stress such as heat shock. A possible explanation for Rosenthal fiber formation is the chronic over expression of Hsp27 (Head et al., 1993; Iwaki et al., 1993). Rosenthal fibers are present in large numbers in Alexander's disease, but it is unclear if these inclusions are beneficial to the astrocytes or whether Rosenthal fibers promote the development of Alexander's disease. Further study is needed to determine the function of astrocytic Hsp27 in this fatal neurological disorder.

The possibility that the expression of Hsp27 is altered in Creutzfeldt-Jakob disease has not been addressed directly. Creutzfeldt-Jakob disease is a lethal prion



disease in humans and is a member of the family of transmissible spongiform encephalopathies like scrapies in sheep. Neuropathological features include late onset dementia, in some cases ataxia, and spongiform degeneration of brain tissue and gliosis (reviewed in Prusiner, 1994). Interestingly, Hsp27 and Hsp70 (both highly inducible under stress conditions) are not induced by heat shock in scrapie infected mouse neuroblastoma cells. In these cells, constitutively expressed Hsc70 does not translocate to the nucleus with heat shock and remains localized to various intensely labeled regions of the cytoplasm (Tatzelt et al., 1995; 1998). The role that this apparent unresponsiveness after heat shock in scrapie infected cells plays in prion disease pathology remains to be determined. Although no study has looked specifically at the expression and induction of Hsp27 in prion diseased brains, Hsps with chaperone function could play a role in this lethal degenerative disease.

### **Focus of the Thesis Work**

The review of the current literature relating to the induction and expression of Hsp27 in the mammalian CNS identifies several important areas for investigation. The work in this thesis further explores the cell type-specific and stress-dependent induction of Hsp27 in the mammalian CNS *in vivo*, and investigates the relationship between Hsp27 expression and neuron survival using two specific models of cellular stress: the classic heat shock induction of Hsp27 and the induction of Hsp27 in the rat visual system following axotomy of the optic nerve. In addition, to determine the influence of a known neuroprotective molecule on the induction and expression of Hsp27 following neuronal

injury, an investigation of the effects of an exogenously applied neurotrophin, brain derived neurotrophic factor (BDNF), on the expression of endogenous Hsp27 in axotomized retinal ganglion cells (RGCs) was undertaken.

### ***Rationale for the models of stress***

#### ***Heat shock***

Experimental heat shock or hyperthermic treatment in adult rats typically consists of an increase in core body temperature to 42°C for 15 minutes (Currie and White, 1981). Whether the temperature increase is brought about extrinsically by heating an anesthetized animal (Krueger et al., 1999) or a non-anesthetized animal (Khan and Brown, 2002), or intrinsically by administration of drugs such as methamphetamine (Goto et al., 1993), morphological evidence of cell loss, tissue damage, or apoptotic cell death in the adult rat CNS has not been reported. There is considerable evidence suggesting that prior hyperthermic treatment may protect cells from subsequent more stressful insults (Barbe et al., 1988; Chopp et al., 1989; Kitagawa et al., 1991a,b; Samali and Cotter, 1996; Karunanithi et al., 1999). An understanding of the induction and cell type-specific expression of Hsp27 following hyperthermic treatment will enhance our understanding of the protective effects of heat shock at the cellular level. The work in this thesis (Chapter 2) represents the first report of the immunohistochemical localization of Hsp27 in the rat CNS following heat shock treatment.

### *Optic nerve transection*

Due to the limited access to fiber tracts within the CNS there are few models of axotomy that specifically injure an isolated population of neurons. However, the optic nerve is easily accessible and transection of the optic nerve causes specific injury to retinal ganglion cells (RGCs), the sensory output neurons of the retina (Villegas-Pérez et al., 1993). By 14 days following transection of the optic nerve, most RGCs have died by apoptosis (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994). Axotomy of the optic nerve also affects the optic nerve and tract causing Wallerian degeneration of axons distal to the cut site and degeneration of RGC axon nerve terminals in the retinorecipient layers of the superior colliculus (Thanos and Thiel, 1991; Meller et al., 1993).

Transection of the optic nerve is a convenient model of axotomy used to study the molecular mechanisms involved in neuronal apoptosis, survival and axonal degeneration in the injured adult CNS. Using Western analysis and immunohistochemical techniques, the induction and cell type specific expression of Hsp27 in the retina and superior colliculus have been determined to further define the molecular mechanisms involved in the CNS response to axonal injury.

### *Brain derived neurotrophic factor treatment of injured retinal ganglion cells*

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family of trophic molecules that are important for differentiation, survival and maintenance of neurons during development of the CNS and in the adult (Mansour-Robaey et al., 1994; Conover and Yancopoulos, 1997). BDNF interacts with the cell surface transmembrane receptor TrkB (tyrosine receptor kinase B) to elicit intracellular signaling leading to the

transcription of genes associated with neuron maintenance and survival (reviewed in Heumann, 1994; Segal and Greenberg, 1996; Klöcker et al., 2000).

BDNF is an important trophic molecule for RGCs. Both BDNF and the TrkB receptor are present in the ganglion cell layer on RGCs (Jelsma et al., 1993; Vecino et al., 2002) and in the retinorecipient layers of the superior colliculus (Allendoerfer et al., 1994; reviewed in von Bartheld, 1998; Frost et al., 2001). BDNF is important for the establishment and survival of a stable population of RGCs during retino-collicular innervation during development (Ma et al., 1998; Spalding et al., 1998) and for the prolonged survival of RGCs following superior colliculus ablation in the neonatal rat (Cui and Harvey et al., 1995). In the adult rat, intraocular administration of exogenous BDNF at the time of optic nerve transection prolongs RGCs survival (May and Thanos, 1993; Mansour-Robaey et al., 1994; Peinado-Ramón et al., 1996; Koeberle and Ball, 2002).

Hsp27 is shown to be protective in neurons following trophic withdrawal in culture; however, little is known about the effects of trophic factor application on the injury-induced expression of Hsp27. Therefore, the expression of Hsp27 in injured RGC following intraocular administration of BDNF was examined. The results of this work will help to further define the interactions of various important neuron survival pathways that are activated in sensory neurons in response to injury and during neurotrophic treatment.

**Specific Objectives of the Thesis**

1. To determine the cell type-specific expression of Hsp27 in the adult rat brain, brain stem and spinal cord following hyperthermic treatment (Chapter 2).
2. To determine the cell type-specific expression of Hsp27 in the rat visual system following optic nerve transection (Chapter 3).
3. To determine the effects of BDNF administration on the expression of Hsp27 in injured rat retinal ganglion cells (Chapter 4).

## **The Visual System of the Rodent**

*To provide a context for the results of experiments involving the expression of Hsp27 in the visual system following optic nerve transection (Chapter 3) and BDNF administration (Chapter 4), a general overview of the organization of the retina and the rat visual system are presented.*

### ***The retina***

The mammalian eye develops embryonically as an outpouching of the diencephalon (Moore, 1977). The neural component of the eye, the retina, is considered to be an isolated part of the CNS and is responsible for the conversion of light energy into nerve impulses. The nerve impulses are transmitted to the brain via retinal ganglion cell (RGC) axons that travel in the optic nerve.

The retina is a multilayered structure and the general histological organization of the retinal layers in cross section (Fig 1.3), from external (scleral) to internal (vitreal), is as follows: 1) the retinal pigment epithelium (RPE; limited in albino rats); 2) the receptor segment layer (RSL), containing the receptor portion of the photoreceptor cells; 3) the outer (external) limiting membrane, (OLM, not indicated) containing Müller cell (retinal glial cell) processes; 4) the outer nuclear layer (ONL) containing the cell bodies of photoreceptor cells; 5) the outer plexiform layer (OPL) containing the bases of the photoreceptor cells, the outer processes of bipolar cells and the processes of horizontal cells; 6) inner nuclear layer (INL) containing the cell bodies of bipolar cells, horizontal

**Figure 1.3: Histological organization of the rat retina. A, hematoxylin and eosin stained radial section of the adult rat retina showing the organization of the retinal layers. RPE, retinal pigment epithelium; RSL, receptor segment layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. B, primary visual pathway in the retina. First order neurons are the photoreceptor cells in the ONL. Second order neurons are the bipolar cells in the INL and the output neuron of the retina (third order neurons) are the retinal ganglion cells (RGCs) in the GCL. fl, fibre layer.**

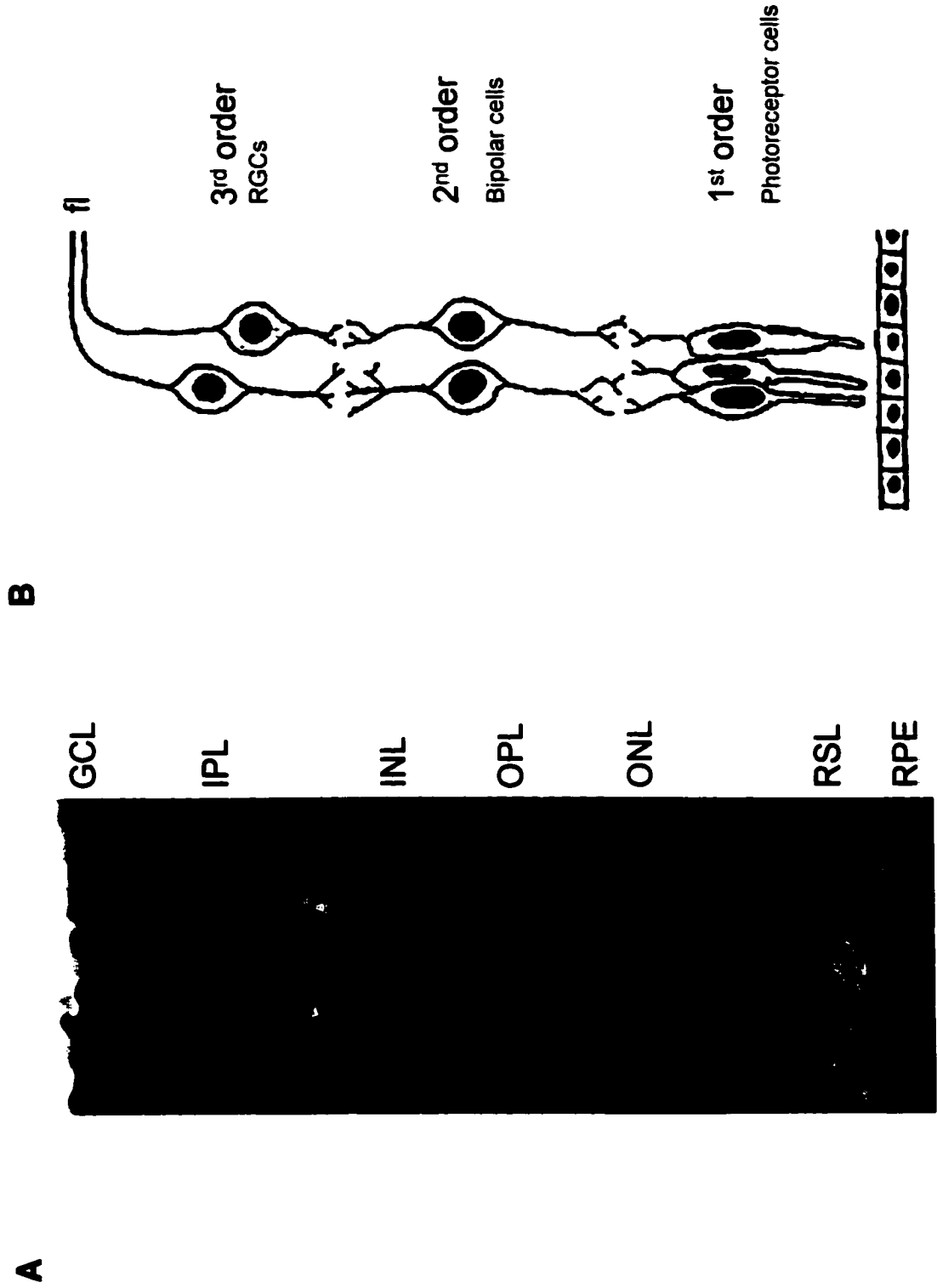


Figure 1.3



cells and amacrine cells. Müller cell bodies are also located here; 7) the inner plexiform layer (IPL) containing the processes of amacrine cells, the axons of bipolar cells and the dendrites of RGCs; 8) the ganglion cell layer (GCL) containing the cell bodies of RGCs and displaced amacrine cells; 9) the fiber layer (fl) containing the unmyelinated axons of RGCs that converge at the optic disc to form the ON; 10) the inner limiting membrane (ILM, not indicated) contains Müller cell process end feet and separates the retina from the vitreous body.

### ***The primary visual pathway***

The primary visual pathway in the rat is illustrated in Figure 1.3B and 1.4. The first order neurons of the visual system are the photoreceptor cells that reside in the ONL. The second order neurons in this pathway are the bipolar cells that reside in the INL and synapse with the dendrites of the RGCs in the IPL. The unmyelinated axons of RGCs travel in the fiber layer, converge on the optic disc and enter the head of the optic nerve where all axons become myelinated by oligodendrocytes (Sefton and Lam, 1984; Sefton et al., 1985). The optic nerve extends to the optic chiasm where in the albino rat, approximately 98.5% of RGC axons cross to the contralateral optic tract (reviewed in Sefton and Dreher, 1995).

Virtually all RGC axons project to the superior colliculus (SC) (reviewed in Sefton and Dreher, 1995). The SC is a horizontally laminated structure and its layers from the surface are: zonal (Z); superficial gray (SuG); optic (Op); intermediate gray (InG); intermediate white (InWh); deep gray (DpG) and deep white (DpWh) (Paxinos

**Figure 1.4:** Diagrammatic representation of the primary visual pathway originating from the retinal ganglion cells (RGCs) of the right eye, viewed as if facing the front of the rat. The axons exit the eye and form the optic nerve. The majority of RGC axons cross at the optic chiasm and form the optic tract projecting to the superficial gray (SuG), optic (Op) and intermediate gray (InG) layers the superior colliculus (SC). Adapted from Sefton and Dreher (1995) and Paxinos and Watson (Figure 44, 1998).

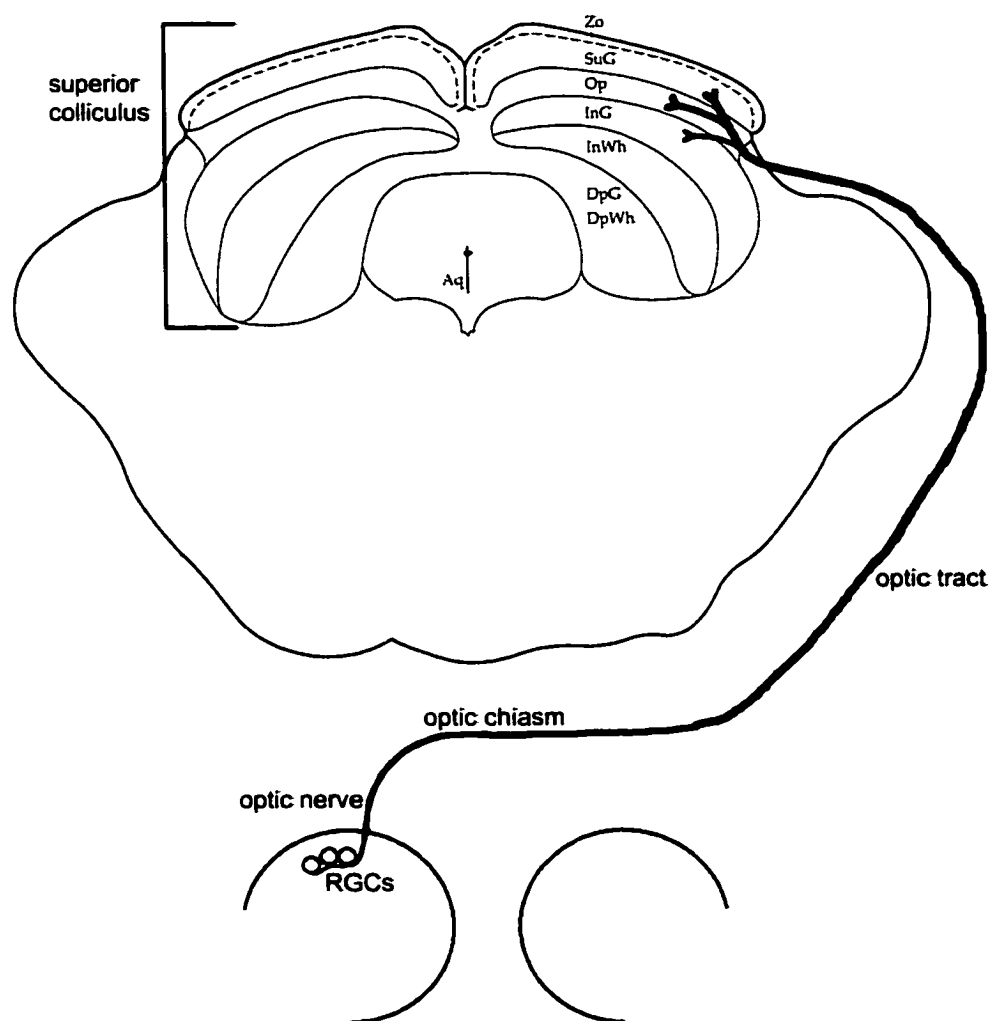


Figure 1.4

and Watson, 1998 (Figures 42-52)). RGC axons enter through the Op and form connections with neurons in the visual layers of the SC including, the SuG, Op and InG (Linden and Perry, 1983; reviewed in Sefton and Dreher, 1995). Sensory information is relayed from the visual layers of the SC to the dorsal lateral geniculate and ventrolateral geniculate nuclei of the thalamus and then to the visual cortex (reviewed in Sefton and Dreher, 1995). Although the SC is the major target of RGCs, retinofugal axons also terminate in the dorsolateral geniculate nucleus, ventrolateral geniculate nucleus, the intergeniculate leaflet and several pretectal nuclei including, the olivary pretectum, the posterior pretectum and the nucleus of the optic tract (reviewed in Sefton and Dreher, 1995).

**CHAPTER 2:**

**HYPERTHERMIC INDUCTION OF THE 27-KDA HEAT SHOCK PROTEIN  
(HSP27) IN NEUROGLIA AND NEURONS OF THE RAT CENTRAL NERVOUS  
SYSTEM**

**This chapter is published in the Journal of Comparative Neurology, 2000, 428:495-510.**

## **Introduction**

Hsps are an evolutionarily conserved group of proteins that are highly inducible by a wide variety of stressors. Some of the Hsps are constitutively expressed and others are highly inducible by metabolic stressors. Of great interest are observations demonstrating that once a heat shock response has been induced, the cells or organs can show remarkable resistance to subsequent metabolic stress. For example, the highly inducible member of the 70 kDa family of Hsps, Hsp70, has been shown to be protective following ischemic injury (Marber et al., 1995; Plumier et al., 1995). Similarly, a member of the small heat shock family, Hsp27 (Hsp25 in mouse) has been shown to play a role in cellular repair and mechanisms of protection against cell stress. As well, overexpression of Hsp27 in NIH/3T3 cells confers thermoresistance (Landry et al., 1989; Lavoie et al., 1993a) and increases resistance to oxidative stress and cytotoxicity induced by TNF $\alpha$  treatment (Mehlen et al., 1995a,b). With respect to cellular function, during stress Hsp27 is considered to be a molecular chaperone that participates in protein folding in an ATP-independent manner and is thought to bind to denatured proteins (Jakob et al., 1993; Jakob and Buchner, 1994).

In the CNS, Hsp27 is not normally detected in the cerebral cortex, but is constitutively expressed in many sensory and motor neurons of brain stem cranial nerve nuclei and spinal cord of the adult rat (Plumier et al., 1997c). Hsp27 is highly induced in forebrain glial cells following ischemic injury (Kato et al., 1994a; Plumier et al., 1997a), kainic acid-induced status epilepticus (Plumier et al., 1996) or potassium chloride-induced cortical spreading depression (Plumier et al., 1997b). From these studies, it was suggested with neuronal injury, glutamate release and excitotoxicity were preferentially

inducing Hsp27 in activated or reactive glia. Hsp27 is also selectively and strongly up-regulated in sensory and motor neurons after cervical vagotomy (Hopkins et al., 1998) or after lesions of the sciatic nerve (Costigan et al., 1998, Lewis et al., 1999). Taken together, these studies show that Hsp27 is highly inducible in a variety of different types of cells and that the responses of a given cell type, especially in the nervous system, vary depending upon the specific nature of the stress.

Hsp27 can be up-regulated in a cell-type and a stress specific manner in the rat nervous system following various forms of injury but, the effects of whole body hyperthermia are unknown. Therefore, we investigated the expression of Hsp27 in specific cell types in the brain following a brief, generalized hyperthermic treatment.

## **Materials and Methods**

### ***Animals***

Male Sprague-Dawley rats (275-340g; Charles River, Québec, Canada) were cared for in accordance with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed on a temperature-controlled heating pad (50°C) until body temperature, monitored with a rectal thermometer, reached 42°C. Core body temperature was maintained between 42 and 42.5°C for 15 minutes. Animals were then allowed to recover for 1.5 hours to 6 days. Control animals were anesthetized with sodium pentobarbital but not heated. Naïve control animals were not anesthetized or heated.

### ***Perfusion and tissue fixation***

At the selected times following heat shock, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially through the ascending aorta with 100mM phosphate-buffered saline (PBS) at room temperature for 5 minutes followed by cold (4°C) 100 mM phosphate buffer containing 4% paraformaldehyde for 15 minutes. Brains were removed from the skull and postfixed for approximately 24 hours at 4°C in phosphate buffer containing 4% paraformaldehyde followed by immersion in 30% sucrose made up in 100 mM phosphate buffer. Serial coronal 40 µm sections were cut on a freezing microtome then stored in Millonig's buffer (pH 7.4) until processed for immunohistochemistry.

### ***Western blot analysis***

Tissue was homogenized in 0.32 M sucrose and protein concentration was determined according to the method of Lowry et al. (1951). Samples containing 40 µg of protein were solubilized in sodium dodecyl sulfate sample buffer [10% glycerol, 5% β-mercaptoethanol, 3% SDS in upper Tris buffer (0.125 M Tris-HCl pH = 8.8/0.1% SDS)], boiled for 10 minutes and then loaded onto a polyacrylamide gel (2.5% upper gel, 12% running gel) according to the method of Laemmli (1970). Mid-range protein molecular weight markers (Promega Inc. Madison WI: 6 µl per lane) were treated in the same way and loaded in parallel. Samples were electrophoresed at 75V through the upper stacking gel and then at 175V for an additional 6 hours. After electrophoresis, proteins were electroblotted onto an Immobilon-P membrane (Millipore, Mississauga, ON) at 100mA for 18 hr. Membranes were air dried. For immunostaining, membranes were first wet



with methanol, rinsed with phosphate buffered saline (PBS; pH 7.4) and then washed for 10 minutes in PBS/0.2% Tween 80 at 37°C. Membranes were blocked for one hour at 37°C in 5% skim milk/1% bovine serum albumin in PBS. Primary rabbit polyclonal antibody specific for the murine 25 kDa Hsp, Hsp25, and the rat 27 kDa Hsp, Hsp27, (Plumier et al., 1996; SPA-801, StressGen Biotechnologies, Victoria, BC, Canada) was diluted 1:1000 in 5% skim milk/1% bovine serum albumin 1X PBS. Membranes were incubated with primary antibody at 4°C for 18 hours with gentle agitation. Membranes were washed in PBS/0.2% Tween 80 at 37°C and then incubated for 1 hour at 37°C with peroxidase conjugated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) diluted 1:500 in PBS/0.2% Tween 80. Blots were washed with PBS/0.2% Tween 80 at 37°C. The second day, peroxidase conjugated antibody was detected using the electrogenerated chemiluminescence Western Blot Detection Kit and Hyperfilm ECL. (Amersham, Arlington Height, IL). Equal loading of the lanes was confirmed using Amido Black to counterstain for proteins.

### ***Hsp27 immunohistochemistry***

Immunohistochemical detection of Hsp27 in rat brain was performed as previously described (Plumier et al., 1996, 1997c). Briefly, free-floating 40 µm-thick coronal sections (1:6 series throughout the brain) from hyperthermic treated and control rat brains were washed in PBS (pH 7.4) containing 0.1% Triton X-100 (PBS-Triton X). Following a 30-minute incubation in 1% hydrogen peroxide in PBS-Triton X, sections were washed in PBS-Triton X and incubated overnight at 4°C with the Hsp25 rabbit polyclonal antibody (1:20000; StressGen Biotechnologies Corp., Victoria, BC) made up

in PBS-Triton X containing 2% goat serum. The next day, sections were washed in PBS-Triton X and incubated for one hour in PBS-Triton X containing biotinylated secondary goat antibody raised against rabbit IgG (1:400; Vector Laboratories Inc. Burlingame, CA). Sections were washed with PBS-Triton X then incubated for one hour in avidin-biotin-horseradish peroxidase complex (1:1000; Vector Laboratories Inc. Burlingame, CA). Following three PBS-Triton X washes, sections were incubated in PBS-Triton X containing 0.3 mg/100 ml glucose oxidase, 40 mg/ml ammonium chloride and 200 mg/ml  $\beta$ -D(+)-glucose (Sigma Chemical Co., St. Louis, MO) and 0.05% diaminobenzidinetetrahydrochloride (Sigma Chemical Co., St. Louis, MO) for 15 minutes. After washing, sections were floated onto gelatinized slides and air dried overnight. Sections were then processed through a graded series of alcohols, defatted with xylene and coverslipped using Entallen (E. Merck, Darmstadt, Germany).

### ***Hsp27 and double-labeling immunohistochemistry***

To further characterize the nature of the Hsp27 immunoreactivity (IR) cells, double-labeling immunohistochemistry was carried out using several different neuronal and glia markers. For calbindin labeling of cerebellar Purkinje neurons and Hsp27 labeling of Bergmann glia, selected cerebellar sections were washed in PBS-Triton X then incubated 1 hour in PBS-Triton X containing 10% donkey serum (Pel-Freez). Sections were incubated overnight at 4°C in PBS-Triton X with the Hsp27 rabbit polyclonal antibody (1:5000; StressGen Biotechnologies Corp., Victoria, BC) and calbindin-D mouse monoclonal antibody (1:5000; Sigma Immuno Chemicals, St. Louis, MO). After washing with PBS-Triton X, sections were incubated overnight at 4°C in

PBS (no Triton-X) containing Cy-2 conjugated donkey anti-mouse IgG (1:400; Jackson Laboratories) and Cy-3 conjugated donkey anti-rabbit IgG (1:400; Jackson Laboratories, Bar Harbor, ME). The sections were rinsed several times in PBS then floated onto gelatinized slides, air dried overnight and coverslipped with Citifluor (Glycerol/PBS solution; Marivac, Halifax, NS).

The protein gene product, PGP 9.5 is a neuron and neuroendocrine specific marker that is expressed throughout the central and peripheral nervous system (Trowern et al., 1996). For PGP 9.5 labeling of subfornical organ and the area postrema neurons, selected sections were treated as above with PBS-Triton X, 10 % goat serum, and incubated overnight at 4°C in PBS-Triton X with 2% goat serum and the mouse monoclonal PGP 9.5 (1:1000; UltraClone Limited, Isle of Wight, England) and the Hsp27 rabbit polyclonal antibody (1:2000; StressGen Biotechnologies Corp. Victoria, BC). After washing with PBS-Triton X, sections were incubated for 3 hours in PBS (no Triton-X) containing Alexa goat anti-mouse 488 (1:400) and Alexa goat anti-rabbit 546 (1:1000; Molecular Probes, Eugene, OR). The sections were rinsed several times in PBS (no Triton-X) then floated onto gelatinized slides, air dried overnight and coverslipped with gelatin.

The following neuroglial markers were used: a mouse monoclonal antibody specific for vimentin (1:2000; Sigma Immuno Chemicals, St. Louis, MO), a mouse monoclonal microglial specific marker, anti-rat CD11b/c (OX-42, 1:5000; CedarLane, Hornby, ON), and a mouse monoclonal antibody specific for glial fibrillary acidic protein (GFAP, 1:1000; Dimension Laboratories, Mississauga, ON). Briefly, sections were washed in PBS-Triton X then incubated 1 hour in PBS-Triton X containing either 10%

donkey serum (for donkey secondary) or 10% goat serum (for goat secondary). Sections were incubated overnight at 4°C in PBS-Triton X containing the Hsp27 rabbit polyclonal antibody (1:2000; StressGen Biotechnologies Corp. Victoria, BC) and the appropriate glial marker (see above) and either 2% goat serum or 2% donkey serum. After washing in PBS-Triton X, sections were incubated 2 nights at 4°C in PBS (no Triton-X) containing either Cy-2 conjugated donkey anti-mouse IgG (1:800) and Cy-3 conjugated donkey anti-rabbit IgG (1:400; Jackson Laboratories, Bar Harbor, ME) or 3 hours in PBS (no Triton-X) containing Alexa goat anti-mouse 488 (1:400) and Alexa goat anti-rabbit 546 (1:1000; Molecular Probes, Eugene, OR). The sections were rinsed several times in PBS (no Triton-X) then floated onto gelatinized slides, air dried overnight and coverslipped with Citifluor (Glycerol/PBS solution; Marivac, Halifax, NS).

### ***Immunohistochemical controls***

To control for non-specific immunostaining, sections were incubated with the omission of each primary antibody. In addition, brain tissue sections from non-hyperthermic treated animals were included for all immunohistochemical reactions.

### ***Microscopy and image processing***

Sections were examined using bright-field and fluorescence microscopy on a Zeiss Axioplan microscope. Selected stained sections were scanned using a Diagnostic Instruments Inc. Spot II digital camera mounted to a Zeiss Axioplan 2 microscope and images captured digitally using Adobe Photoshop 5.0 software. Selected sections were scanned using a Zeiss LSM510 laser scanning microscope. Prints and photographic

plates were made using a Kodak 8650 printer with continuous tone dye sublimation. Other than brightness and contrast, captured images were not adjusted.

## **Results**

Whole body hyperthermia caused up-regulation of Hsp27 in several types of neuroglia throughout the gray and white matter of the CNS, in the ependyma and choroid plexus as well as in neurons of the hypothalamus, circumventricular organs and the dorsal vagal complex. The increases in levels of Hsp27 were time dependent, reaching peaks within 24 hours on the basis of Western blot analysis and immunohistochemistry before gradually declining. In anesthetized control animals, no changes in Hsp27 levels were observed.

### ***Western blot analysis***

Western blot analysis confirmed that there were high constitutive levels of Hsp27 in the brainstem while there was little or no constitutive expression of Hsp27 in the cerebellum, hippocampal formation, or cerebral cortex of control animals (Figure 2.1). The expression of Hsp27 in each of these areas was increased 24 hours after hyperthermic treatment (Figure 2.1). No immuno-reaction was detected when the Hsp27 primary antibody was omitted. Amido Black counterstaining of proteins confirmed relative equal loading of the lanes (not shown).

### ***Neuroglia***

***Astrocytes:*** Hyperthermia induced an increase in Hsp27 IR in glia throughout the

CNS. Although widespread, the increase was not uniform, being relatively stronger in the hippocampal formation (Figure 2.2) and the lateral septal area (Figure 2.3B). Hsp27 IR was first detected in glia 1.5 to 3 hours after hyperthermic treatment, was robust by 6 hours and appeared to reach a peak by 24 hours after hyperthermic treatment (Figures 2.2, 2.3B, 2.4B). Figure 2.2A shows Hsp27 IR in the cerebral cortex and hippocampal formation of a control animal. In controls, Hsp27 IR was found in occasional astrocytes in the gray matter and, more frequently, in glia in the corpus callosum (Figure 2.2A; Plumier et al., 1997c). Figure 2.2B shows the striking increase in Hsp27 IR in the cerebral cortex, hippocampal formation, and corpus callosum 24 hours after hyperthermia. Figure 2.2C and D illustrate the time-dependent induction of Hsp27 in the hippocampal formation after heat shock. By 3 hours, Hsp27 IR was present in many glia. After 24 hours, many more glia were Hsp27-positive and their processes were more extensively stained (Figure 2.2D). After 6 days, fewer glia were stained in the hippocampal formation but those that were had strongly stained processes (not shown). The morphology of Hsp27-positive glia indicated that most were astrocytes. This was confirmed by double immunostaining with GFAP (Figure 2.6 A-C). Some Hsp27-positive glia were also positive for vimentin (not shown). In the hippocampus, no Hsp27 positive cells were positive for OX-42.

*Bergmann glia:* Heat shock resulted in a strong induction of Hsp27 in Bergmann glia, reaching a maximum after 24 hours (Figures 2.4B, 2.5). The long apical processes of Bergmann glia showed a time-dependent proximal to distal increase in Hsp27 IR (Figure 2.5). Within 3 hours after heat shock, Hsp27 IR was present in cell somata at the interface of the Purkinje cell and granule cell layers of the cerebellar cortex (Figure

2.5A). As shown in figure 2.5, increases in Hsp27 IR in the apical processes increased from proximal to distal reaching the pial surface by 24 hours after hyperthermia. By 6 days, Hsp27 IR was substantially less but some strongly stained cells and their processes remained (Figure 2.5D). In the cerebellum, double-labeling for GFAP (Figure 2.6G-I) or Hsp27 and Calbindin (Figure 2.6J-L) confirmed that Bergmann glia were Hsp27-positive. There were, however, other Hsp27 IR cells in the cerebellum that resembled glia but which were not GFAP immunoreactive (Figure 2.6I).

### ***Ependyma and choroid plexus***

***Ependyma:*** In control animals, Hsp27 IR was patchy and diffuse (Figures 2.3A, 2.4A). Heat shock resulted in strong Hsp27 IR in the ependyma (Figures 2.3B-C, 2.4B). In the dorsal part of the ependymal lining of the IIIrd ventricle (Figure 2.3C, D), the stained cells resembled those in the lateral and IVth ventricles while in the ventral region, Hsp27 IR was present in tanycytes, the specialized ependymal cells found lining the lower part of the ventricle, and their processes that extend into the parenchyma of the hypothalamus (Figure 2.3C, inset).

In addition, there was strong Hsp27 IR in cells that were closely associated with the ependyma and the adjacent hypothalamus (Figure 2.3D). These cells were small, and round with few processes and were present by 24 hours following hyperthermia. Interestingly, all of these cells stained very strongly for vimentin (Figure 2.6D-F) but were negative for GFAP, and OX-42 (not shown). Similar cells were also present in the rostral diencephalon adjacent to the IIIrd ventricle.

In control animals, the ependyma of the IVth ventricle showed occasional Hsp27-

positive cell bodies. Within 1.5 hours Hsp27 IR was present in many ependymal cell bodies with a progressive increase in the number of Hsp27 IR ependymal cells observed with time after hyperthermic treatment. A maximum appeared to be reached by 24 hours (Figures 2.3C, 2.4B) and Hsp27 IR in cells of the ependyma remained high by 6 days (Figure 2.3D).

*Choroid plexus:* In control animals, the choroid plexus of the lateral and IVth ventricles contained few Hsp27 IR cells (Figure 2.4C). After heat shock, Hsp27 IR increased over time reaching a maximum by 24 hours (Figure 2.4D). Hsp27 IR in the choroid plexus decreased over time but was still relatively strong 6 days after heat shock.

### *Neurons*

*Hippocampal formation:* Hsp27 IR was detected in some dentate granule cells of the dentate gyrus from 3 hours to 24 hours following hyperthermic treatment (Figure 2.2C,D, arrows respectively). Hsp27-positive neurons were not observed in the dentate gyrus at 6 days after heat shock. No Hsp27 IR was detected in pyramidal cells of the hippocampus at any time following heat shock.

*Hypothalamus:* Hsp27 IR was induced in the hypothalamus after heat shock. Hsp27 IR was observed in neurons of the paraventricular (Figure 2.7A) nucleus and, in many neurons of the dorsomedial nucleus, pars compacta of the hypothalamus. (Figure 2.7B).

*Circumventricular organs:* The subfornical organ and area postrema both showed time-dependent increases in staining of neurons (Figure 2.8). In control animals, there was no Hsp27 IR in neurons in either of these structures (Figure 2.8A, C). In contrast, in



both structures Hsp27 IR in neurons was first detected at 1.5 hours and was strong 3 hours after heat shock, reaching a maximum by 24 hours (Figure 2.8B, D). A decrease was evident by 4 days when only a few Hsp27 IR cell bodies were present in the subfornical organ and area postrema. By 6 days, Hsp27 IR had returned to control levels with few or no stained neurons. Double-labeling with the neuronal marker PGP 9.5 verified that the Hsp27 IR cells in the subfornical organ (Figure 2.9 A-C) and the area postrema (Figure 2.9D-F) were neurons.

*Dorsal motor nucleus of the vagus nerve:* In control animals, staining of the DMV was rather sporadic and variable. In some rats, the DMV contained few or no Hsp27-positive motor neurons while in others, there were a few strongly staining neurons and numerous lightly stained Hsp27 IR neurons. Nonetheless, overall, there was a tendency for an increase in motor neuron staining in the DMV after hyperthermia (Figure 2.10). There was no clear relationship between time after heat shock and degree of DMV staining although there were indications that Hsp27 IR was increased as early as 1.5 hours after heat shock.

*Nucleus of the tractus solitarius:* In control animals, there was Hsp27 staining of axons but no staining of neuron cell bodies in the NTS, similar to that reported by Plumier et al. (1997c). At 24 hours after heat shock, a small number of Hsp27 IR neurons were present in the caudal NTS, especially the medial subnucleus (Figure 2.10).

**Figure 2.1:** Western blot analysis of proteins from selected regions of control and 24 hours post-heat shock rat brain. Proteins from the brainstem, cerebellum, hippocampal formation, and cerebral cortex were separated by one-dimensional gel electrophoresis and transferred to immobilon membranes. The membranes were reacted with a polyclonal rabbit antibody raised against the murine 25-kDa Hsp, which recognizes the rat homolog protein Hsp27. Molecular weight markers (kDa) are indicated on the right.

Abbreviations: C, control; HS, 24 hours after heat shock.

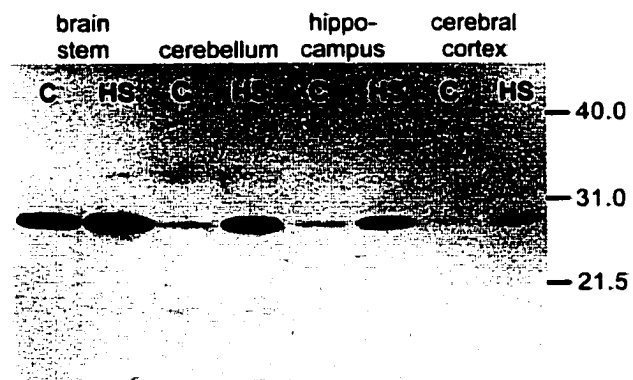


Figure 2.1

**Figure 2.2:** Hsp27 immunoreactivity (Hsp27 IR) in the cerebral cortex and hippocampal formation. **A:** Coronal section from control showing Hsp27 IR in only a few glia cells (arrows). **B:** Section from rat brain 24 hours after heat shock treatment showing widespread Hsp27 IR in many glia and a few neurons. Box indicates region shown in panel D. **C:** Hilus, 3 hours after heat shock showing Hsp27 IR in glia and in occasional granule cells of the dentate gyrus (arrows). **D:** Hilus, 24 hours after heat shock showing Hsp27 IR in glial cells bodies and fine processes as well as some neurons of the dentate gyrus (arrows). Abbreviations: CA3c, cornu ammonis region 3c; CC, corpus callosum; Cor, cerebral cortex; GC, granule cells of the dentate gyrus; Hil, hilus of the dentate gyrus; Lmol, lacunosum moleculare; Or, stratum oriens; Py, pyramidal cell layer; Rad, stratum radiatum. Scale bars A and B, 400  $\mu\text{m}$ ; C and D, 100  $\mu\text{m}$ .

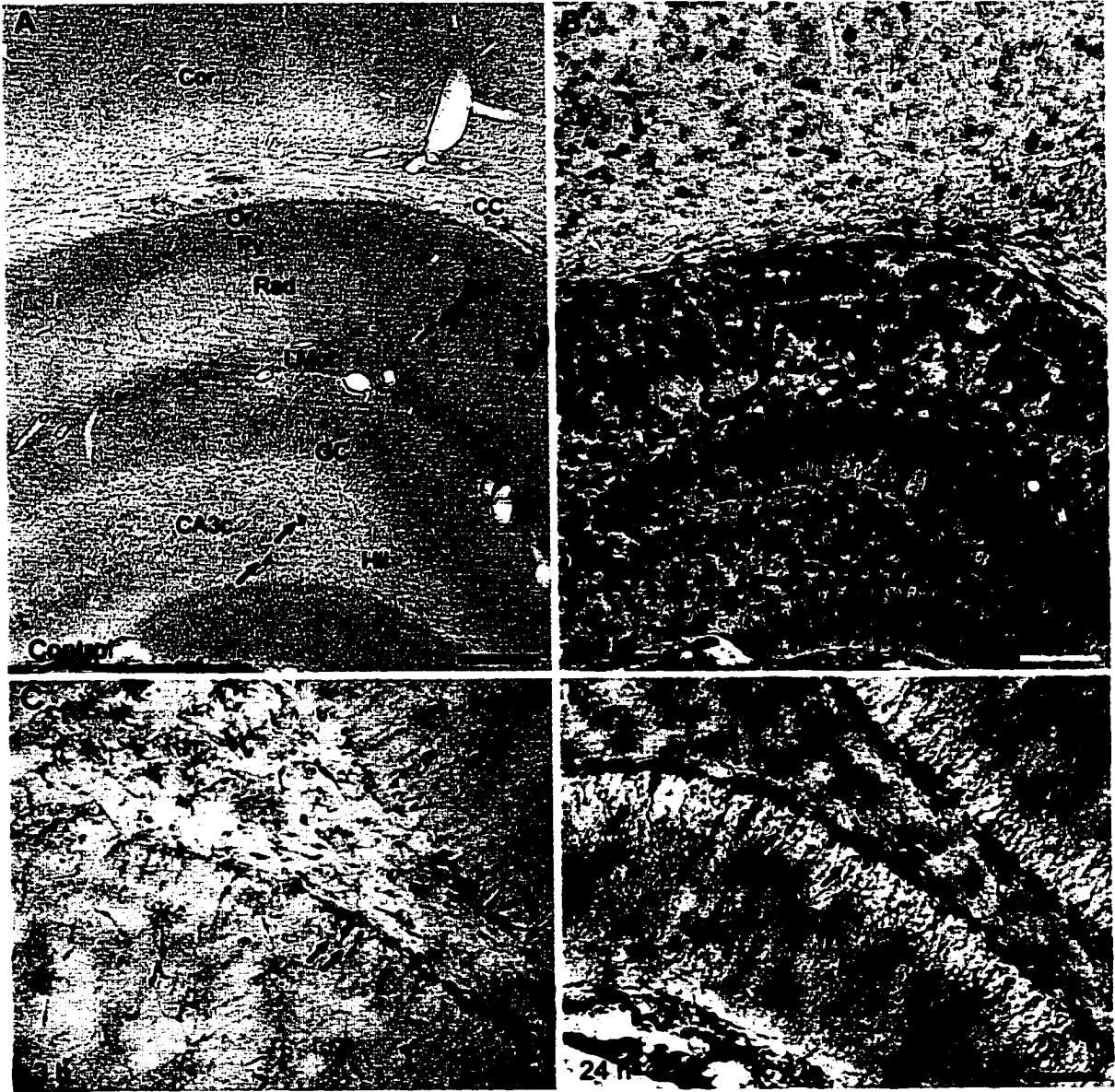


Figure 2.2

**Figure 2.3:** Hsp27 immunoreactivity (Hsp27 IR) in the ependyma of the forebrain and diencephalon. **A:** Control forebrain and lateral ventricle. **B:** Forebrain and lateral ventricle 24 hours after heat shock. **C:** Diencephalon and third ventricle 24 hours after heat shock. Rectangle shows area enlarged in inset. Note the transition from ependyma to tanycytes lining the third ventricle. **D:** Third ventricle 6 days after heat shock. Note that at 6 days after heat shock, Hsp27 IR was still very strong in ependymal cells and in small round cells adjacent to the lining of the third ventricle. Abbreviations: III, third ventricle; CC, corpus callosum; CPu, caudate putamen; LS, lateral septal area; LV, lateral ventricle. Scale bars in A, B, and C, 400  $\mu\text{m}$ ; inset in C, 50  $\mu\text{m}$ ; and in D, 100 $\mu\text{m}$ .

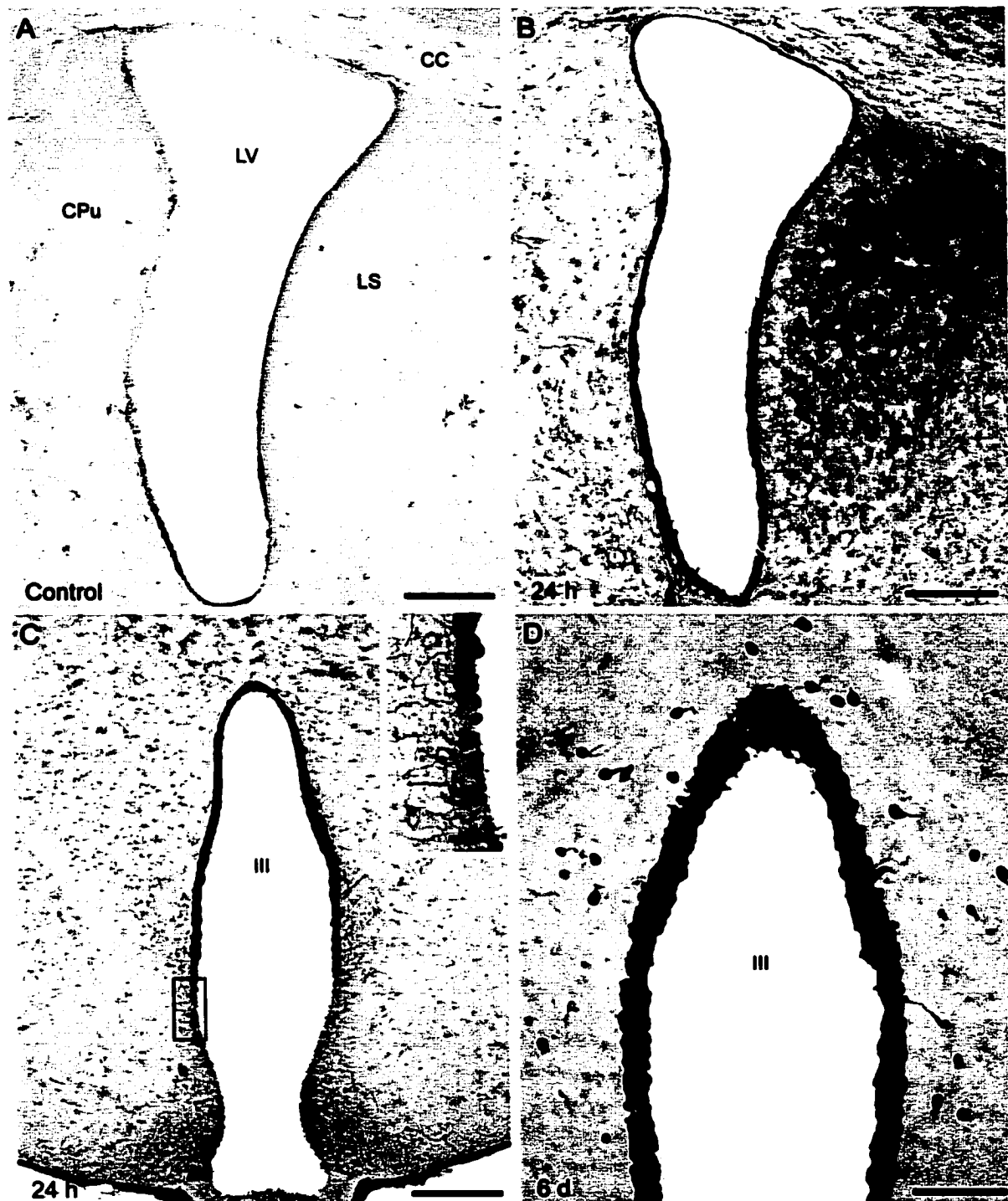


Figure 2.3

**Figure 2.4:** Hsp27 immunoreactivity (Hsp27 IR) at the level of the pons (A and B) and medulla oblongata (C and D). **A:** Control. **B:** 24 hours after heat shock. Note the strong increase in Hsp27 IR in glia, especially in Bergmann glia and in ependymal cells of the fourth ventricle. **C:** Choroid plexus in the IVth ventricle of a control animal. **D:** 24 hours after heat shock. Note the strong Hsp27-immunoreactive cells in the choroid plexus. Abbreviations: IV, fourth ventricle; G, granule cell layer; M, molecular layer; MesV, mesencephalic nucleus of the trigeminal nerve; PC, Purkinje cell layer; Vm, trigeminal motor nucleus; wm, white matter. Scale bars A and B, 400  $\mu\text{m}$ ; C and D 200  $\mu\text{m}$ .



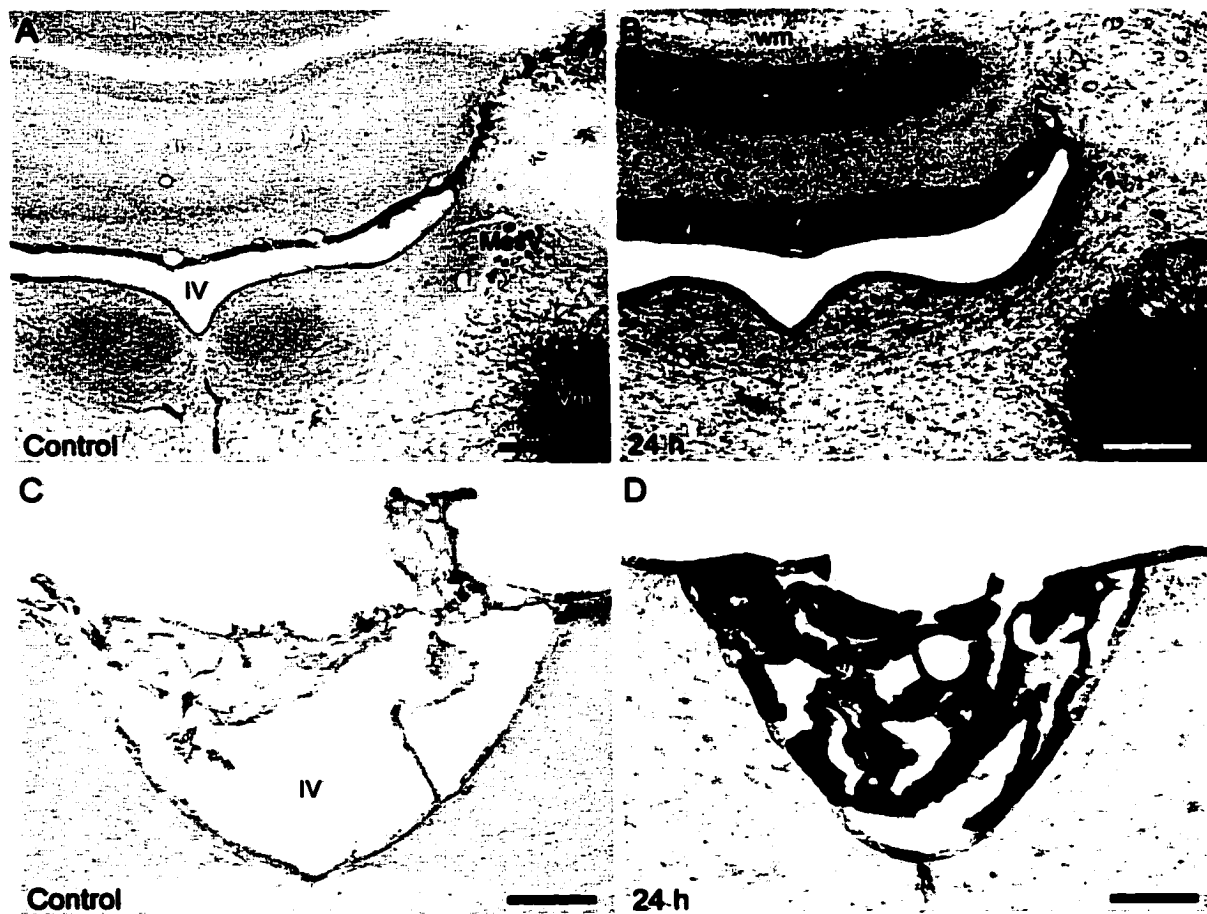


Figure 2.4

**Figure 2.5:** Hsp27 immunoreactivity (Hsp27 IR) in the cerebellum at different intervals after heat shock. **A:** 3 hours **B:** 6 hours. **C:** 24 hours. **D:** 6 days. Note that in Bergmann glia, Hsp27 IR was first present in somata (A) and that the Hsp27 IR increased from proximal to distal over time (B and C). Note also the banding pattern in C and D. Abbreviations: G, granule cell layer; M, molecular layer; PC, Purkinje cell layer. Scale bars, 100  $\mu$ m.

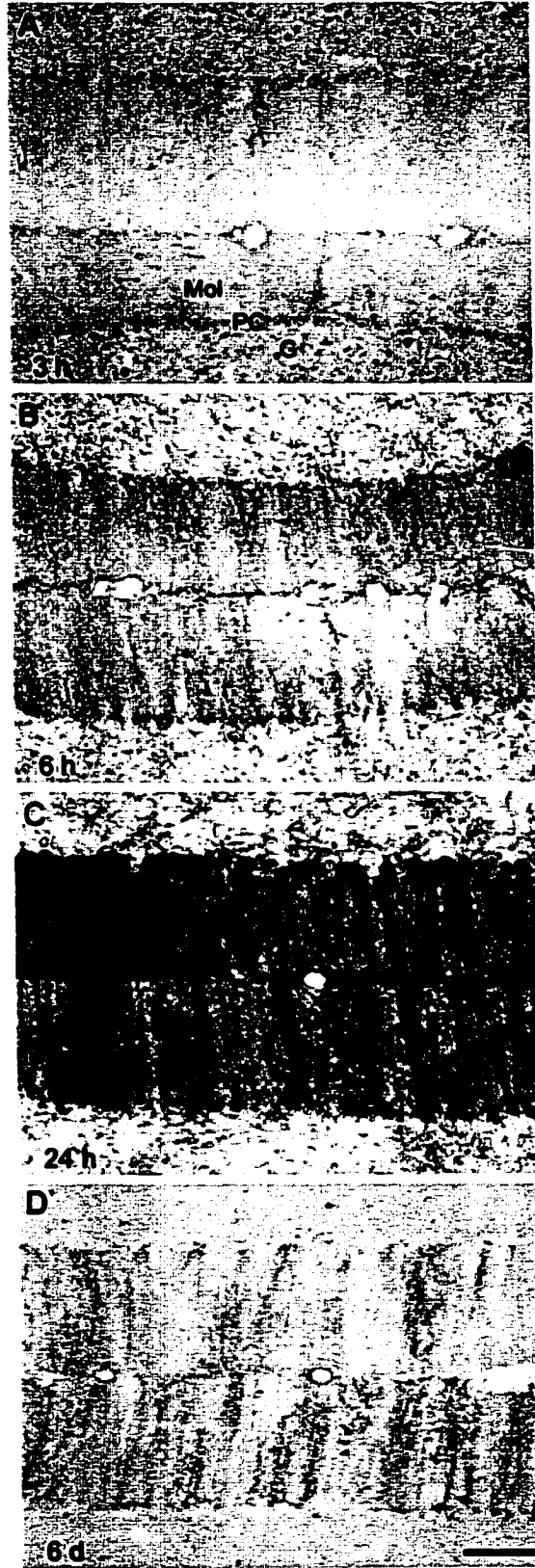


Figure 2.5

**Figure 2.6:** Immunofluorescent micrographs of double-labeling immunohistochemistry for Hsp27 and GFAP (A-C and G-I), vimentin (D-F) or Calbindin (J-L) in various regions of the rat brain following heat shock treatment. The combined double-labeling images in C, F, I and L show Hsp27 immunoreactivity (Hsp27 IR) red (Cy-3; Alexa 543 in F) and GFAP (Cy-2), vimentin (Alexa 488) or calbindin (Cy-2) in green. Double-labeled cells appear yellow. **A-C:** Confocal micrographs of hilus of the dentate gyrus 24 hours following heat shock treatment. Arrows indicate some of many double-labeled glial cells. Note that some Hsp27 positive cells do not stain for GFAP. **D-F:** Confocal micrographs of vimentin (D) and Hsp27 (E) in the hypothalamus adjacent to the IIIrd ventricle 6 days following heat shock. Note double-labeling of the ependymal lining and unidentified cells off the IIIrd ventricle (arrows). Not all vimentin positive cells adjacent to the IIIrd ventricle were Hsp27 positive. **G-I:** Micrographs of GFAP (G) and Hsp27 (H) in the cerebellar cortex 24 h following hyperthermic treatment. The combined image in I shows that Bergmann cell processes (green in G) are also Hsp27 IR (red in H). Arrows indicate some of the many double-labeled processes. Note, the Hsp27-positive cell in the molecular layer that does not stain for GFAP. **J-L:** Micrographs of calbindin (J) and Hsp27 (K) in the cerebellar cortex 24 hours following hyperthermic treatment. The combined image in L shows that the Hsp27-positive cells (red in K) are Bergmann glia in close proximity to the somata of calbindin-positive Purkinje neurons (green in J). Abbreviation: III, third ventricle. Scale 50µm.

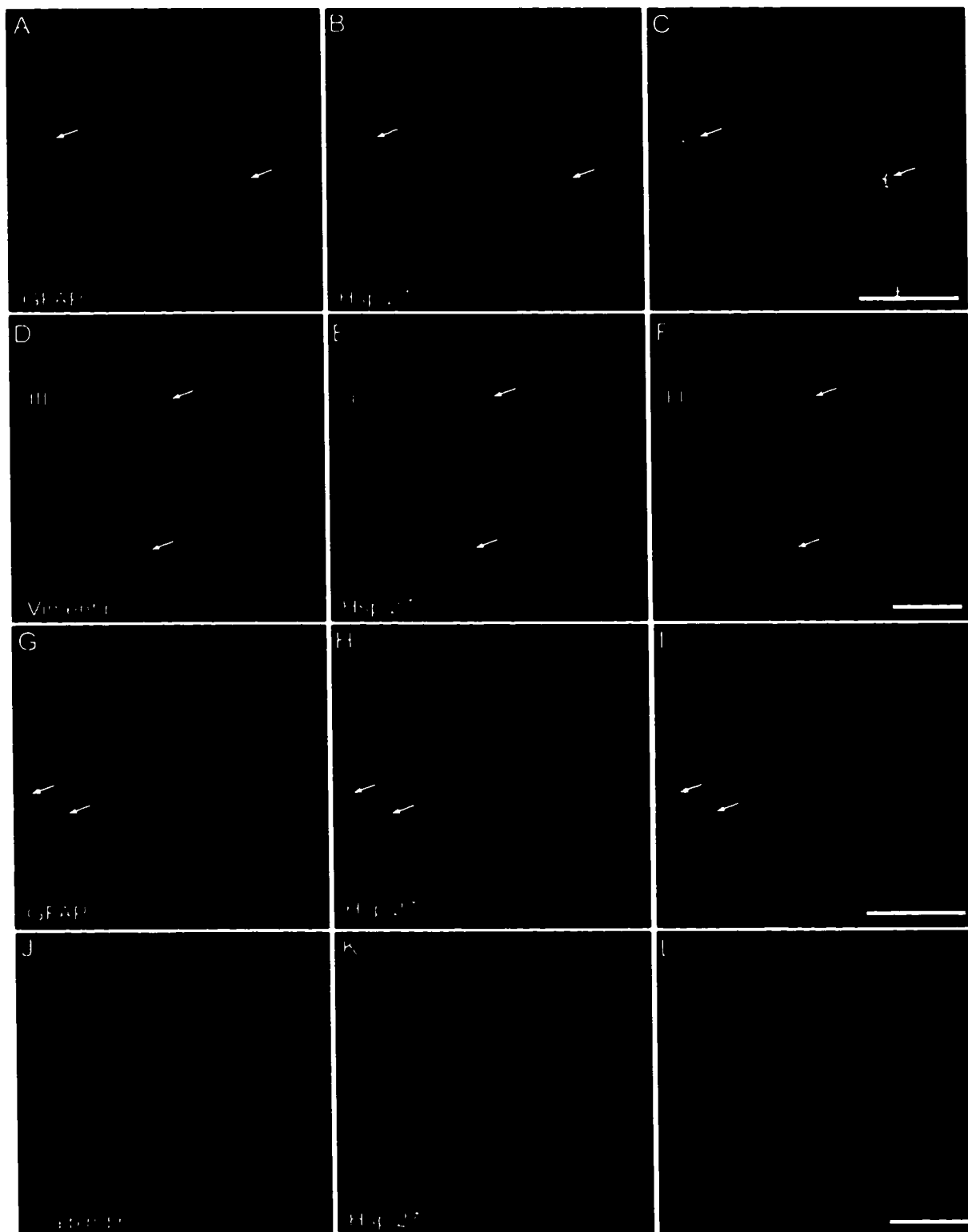


Figure 2.6

**Figure 2.7:** Hsp27 immunoreactivity (Hsp27 IR) in the paraventricular nucleus of the hypothalamus (A) and the dorsomedial hypothalamic nucleus (B) 24 hours following hyperthermic treatment. Abbreviation: III, third ventricle. Scale bar in A, 100  $\mu\text{m}$ ; and in B, 50 $\mu\text{m}$ .

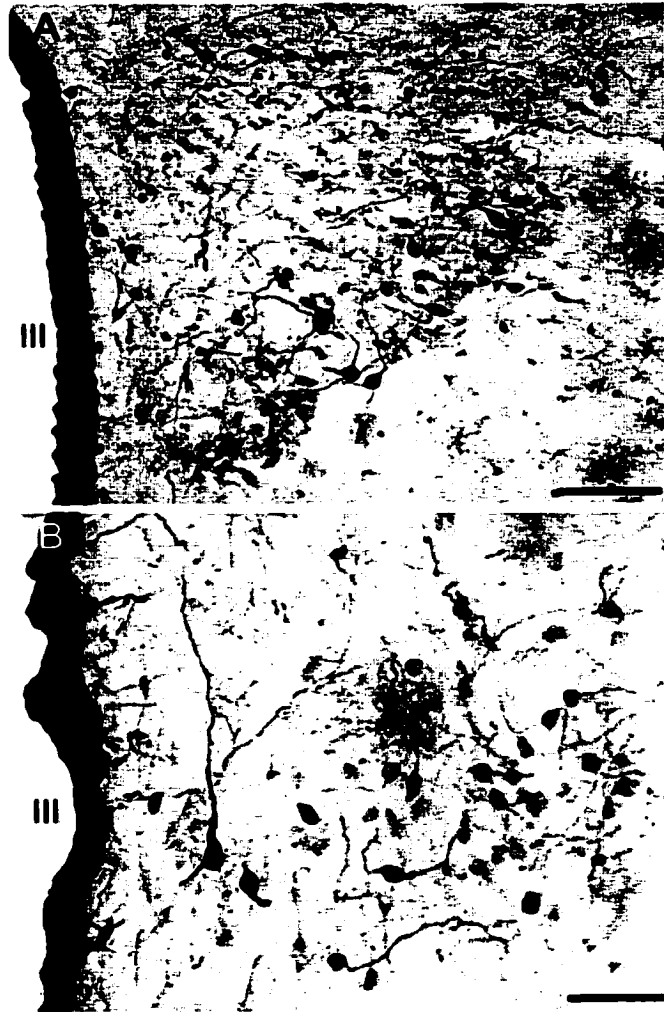


Figure 2.7

**Figure 2.8:** Hsp27 immunoreactivity (Hsp27 IR) in the subfornical organ (A and B) and the area postrema (C and D). **A:** Control subfornical organ. **B:** Hsp27 IR in the subfornical organ 24 h after hyperthermic treatment. **C:** Control area postrema. **D:** Hsp27 IR in the area postrema 24 h after heat shock treatment. Scale bars, 100  $\mu$ m.



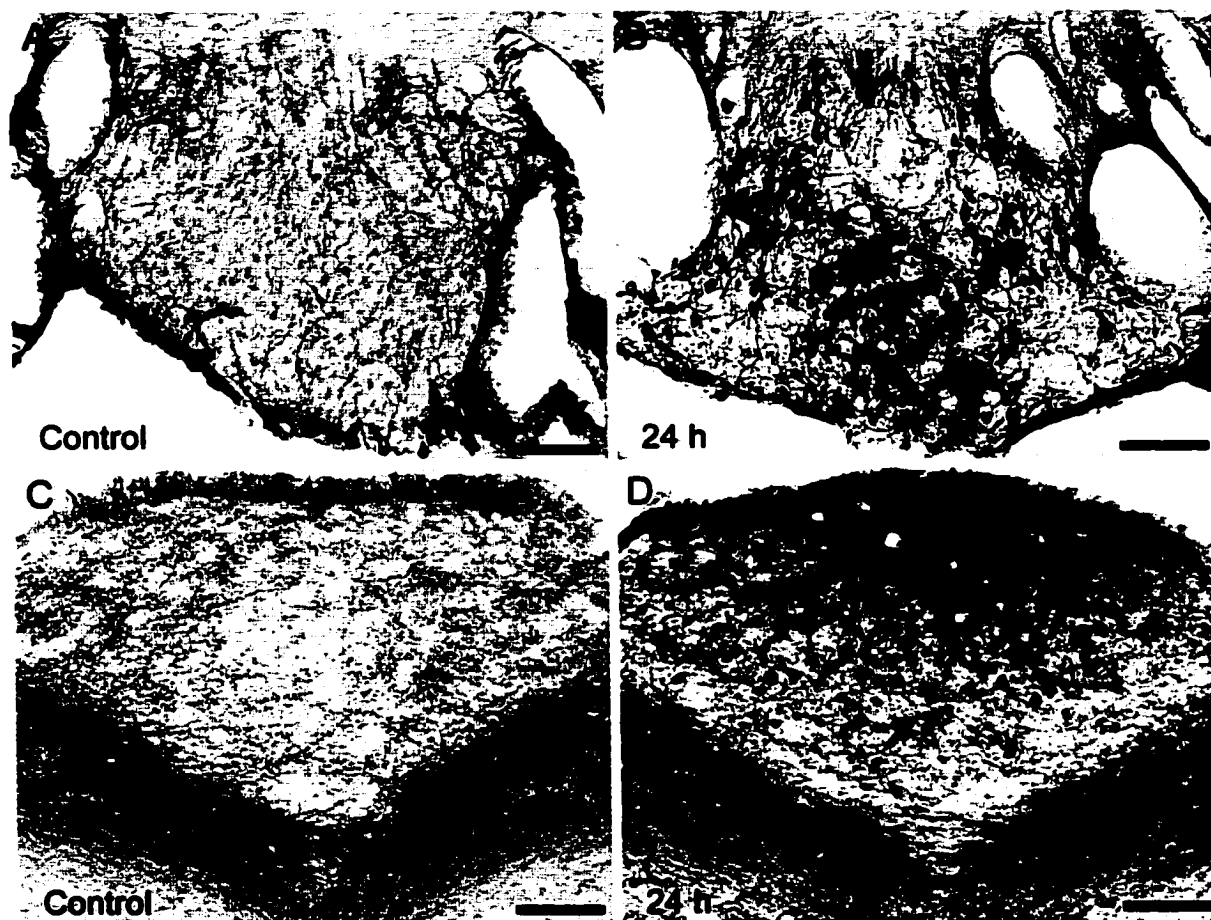


Figure 2.8

**Figure 2.9:** Confocal laser scanning microscope images of double-labeling immunohistochemistry for Hsp27 and PGP 9.5 in the subfornical organ (A-C) and the area postrema (D-F) 24 h following hyperthermic treatment. The combined double-labeling images in C and F shows PGP 9.5 in red (Alexa 543) and Hsp27IR in green (Alexa 488) with some double-labeled neurons in yellow (indicated by arrows). Asterisk indicates PGP 9.5 labeled neurons that are not double-labeled for Hsp27. Scale bars, 20 $\mu$ m.

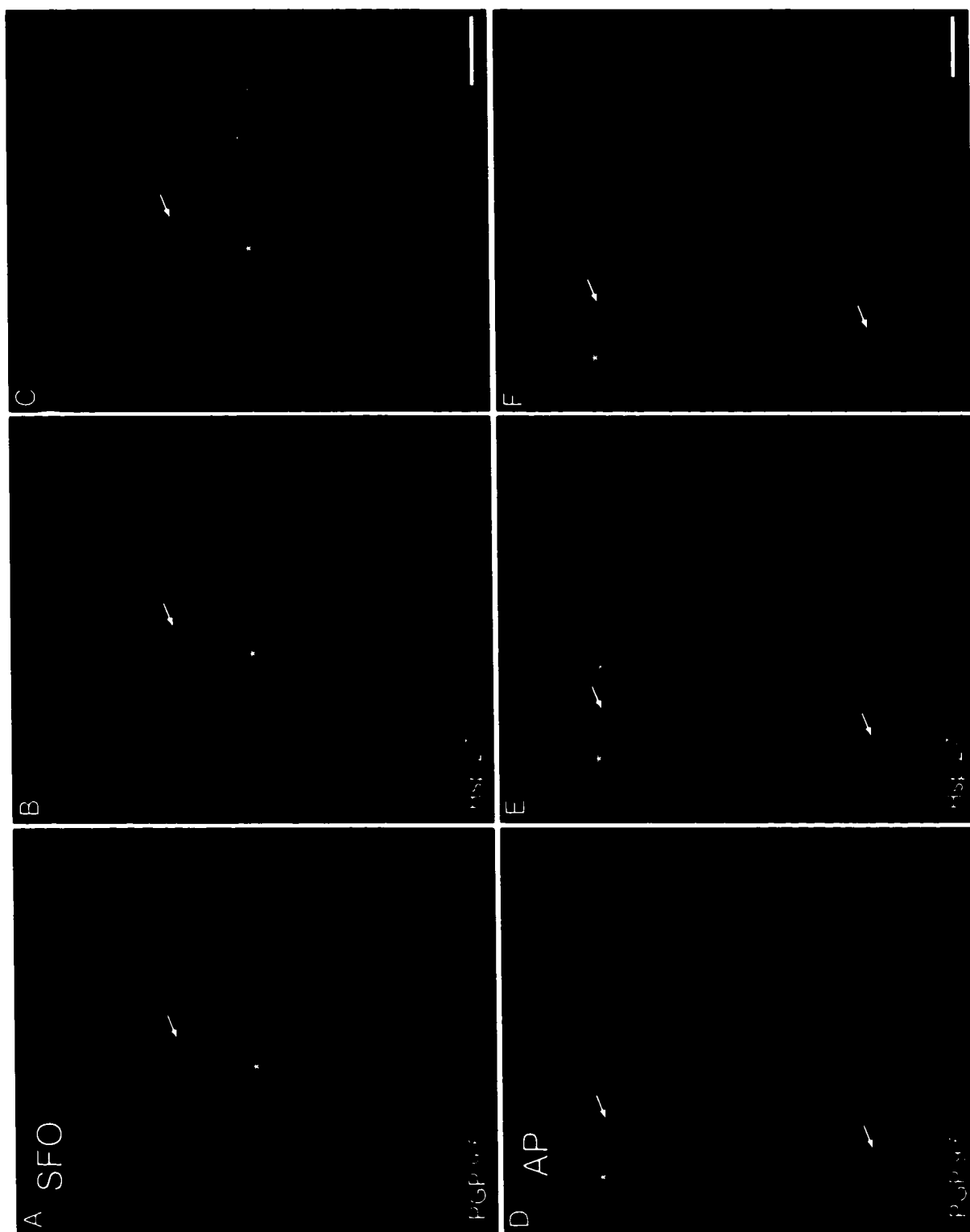


Figure 2.9

**Figure 2.10:** Hsp27 immunoreactivity (Hsp27 IR) in the nucleus of the tractus solitarius and the dorsal motor nucleus of the vagus nerve. **A:** Control. Note the constitutive expression of Hsp27 in the hypoglossal nucleus as previously reported by Plumier et al. (1996). **B:** 24 hours after heat shock. Note the Hsp27-positive neurons in the area postrema, dorsal motor nucleus of the vagus nerve and nucleus of the tractus solitarius (indicated by arrows). Abbreviations: XII, hypoglossal nucleus; AP, area postrema; cc, central canal; DMV, dorsal motor nucleus of the vagus nerve; Gr, gracile nucleus and tract; NTS, nucleus of the tractus solitarius. Scale bar, 200  $\mu\text{m}$ .

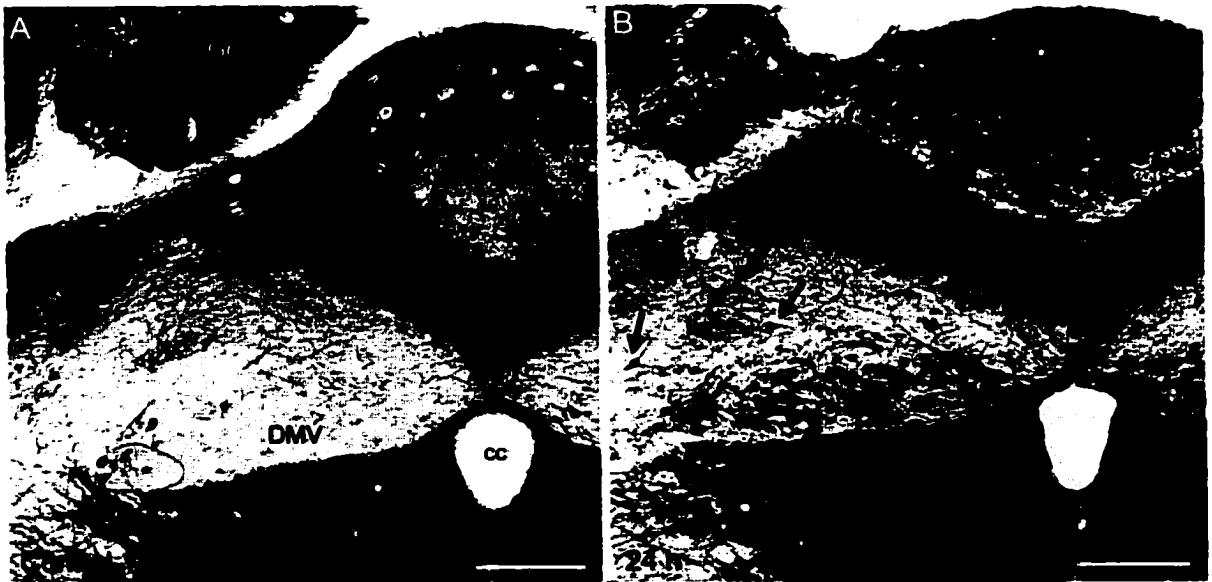


Figure 2.10

## **Discussion**

Heat shock or hyperthermic treatment has been used to induce the expression of Hsps in a wide variety of species, including rat and mouse. Whether the temperature increase is brought about extrinsically by heating an anesthetized animal (Currie and White, 1981; Krueger et al., 1999) or intrinsically by administration of compounds such as methamphetamine (Goto et al., 1993), no morphological evidence of cell loss or tissue degeneration in the CNS has been reported. In fact, there is considerable evidence suggesting that prior hyperthermic treatment may protect cells from subsequent more stressful insults (Barbe et al., 1988; Chopp et al., 1989; Kitagawa et al., 1991a,b; Samali and Cotter, 1996).

In the present study, Western analysis revealed that Hsp27 was induced above control levels in the cerebral cortex, hippocampal formation, cerebellum and brainstem after hyperthermia. Immunohistochemistry revealed two distinct patterns of Hsp27-IR. Firstly, whole body hyperthermia caused a generalized, time-dependent, non-neuronal increase of Hsp27 in astrocytes throughout the CNS, as well as in Bergmann glia, the ependyma and the choroid plexus. Secondly, hyperthermic treatment induced Hsp27 specifically in neurons of the hippocampal formation, circumventricular organs, the hypothalamus and the dorsal vagal complex. The neuronal response was more restricted, being prominent in neurons and neural systems that are involved in regulation of physiological homeostasis, including body fluid regulation and neuroendocrine responses to stress.

Hsp27 is expressed in a variety of circumstances. In the rat, Hsp27 is constitutively expressed in many motor and sensory neurons of the brainstem and spinal

cord, but is absent in the cerebral cortex (Plumier et al., 1997c). During development, Hsp27 is transiently expressed in Purkinje cells in the embryonic rat cerebellum (Gernold et al., 1993) while in the adult mouse cerebellum this protein is detected in a subset of Purkinje cells (Armstrong et al., 2000). Hsp27 is highly inducible in astrocytes after seizure activity (Plumier et al., 1996), ischemic injury (Plumier et al., 1997a; Currie et al., 2000) and cortical spreading depression (Plumier et al., 1997b). In addition, Hsp27 is inducible specifically in neurons after peripheral injury to the vagus or sciatic nerves (Hopkins et al., 1998, Lewis et al., 1999). Thus, the constitutive and developmental expression of Hsp27 is specific, being localized to well-defined subsets of neurons and glia. Injury or stress-induced expression of Hsp27 in the cerebral cortex appears as a generalized response in astrocytes while after direct injury of peripheral axons, it is restricted to neurons.

### ***Cellular functions of Hsp27***

Evidence suggests that Hsp27 acts as a molecular chaperone that binds unfolded proteins during heat shock, preventing non-specific aggregation of proteins (Jakob et al., 1993; Merck et al., 1993). Hsp27 also regulates actin filament organization and stabilizes actin filaments during oxidative stress (Lavoie et al., 1993a, b, 1995; Huot et al., 1996, 1997). The protective effect of Hsp27 may be related to an increase in intracellular glutathione levels which suggests that sHsps regulate the level of antioxidative enzymes. Similarly, overexpression of human Hsp27 reduces the intracellular generation of reactive oxygen species induced by treatment with TNF $\alpha$  (Mehlen et al., 1995a,b; Mehlen et al., 1996a). Moreover, Hsp27 overexpression inhibits apoptosis induced by

Fas/APO-1 receptor activation or treatment with staurosporine, a protein kinase C inhibitor (Mehlen et al., 1996b; Samali and Cotter, 1996). Recently, the overexpression of Hsp27 has been shown to inhibit etoposide-induced apoptosis by the inhibition of caspase activation (Garrido et al., 1999).

The functions of Hsp27 may be regulated by its phosphorylation state and whether it is in a monomer or multimer form (Lavoie et al., 1995). Under stressful conditions, such as stimulation with hydrogen peroxide or cytokines, activation of the p38 mitogen-activated protein (MAP) kinase causes increased phosphorylation of Hsp27 (Huot et al., 1997; Guay et al., 1997). Hsp27 phosphorylation favors the shift of multimer Hsp27 to monomer Hsp27 (Lavoie et al., 1995) and increases cell survival against lethal heat shock or cell death induced by hydrogen peroxide (Lavoie et al., 1995; Huot et al., 1996). Recently, overexpression of Hsp27 in cultured dorsal root ganglion neurons has been shown to protect neurons from apoptosis after withdrawal of nerve growth factor. In addition, an established neuronal cell line overexpressing Hsp27 was protected from retinoic acid-induced apoptosis (Wagstaff et al., 1999). Whether constitutive Hsp27 in neurons or induced Hsp27 in either neurons or astrocytes regulates apoptosis *in vivo* has not yet been established. However, Lewis et al. (1999) have demonstrated that, following transection of the sciatic nerve in postnatal day 0 rats, all surviving axotomized neurons were Hsp27-positive.

#### ***Hyperthermic induction of Hsp27 in neuroglia and non-neuronal cells***

***Macroglia.*** In normal adult rats, Hsp27 is constitutively expressed in a very limited population of glia in the CNS, primarily in the corpus callosum near the midline



and in the subjacent decussation of the fornix (Plumier et al., 1997c). Occasionally, single astrocytes in the brain parenchyma are also Hsp27 IR. In contrast to the virtual absence of Hsp27 in neuroglia in most of the brain, hyperthermia rapidly induced the expression of Hsp27 in a variety of types of neuroglia, including astrocytes and Bergmann glia, as well as in ependymal cells and cells of the choroid plexus. The expression of Hsp27 in glia may be indicative of their activation. In fact, Hsp27 appears to be a sensitive marker for the study of reactive gliosis (Plumier et al., 1997b). In Bergmann glia, the progression over time of Hsp27 IR along the apical processes of these cells suggests that Hsp27 protein was being transported from the cell body along radial fibers distally. Similar heat-induced expression and transport of Hsp27 distally in Bergmann glia was recently reported by Bechtold and Brown (2000).

*Ependyma and choroid plexus.* The generalized and global expression of Hsp27 in astrocytes, Bergmann glia, and several specialized non-neuronal cells may be related to these cells sharing a common developmental origin. In fact, each of these specialized cell types (astrocytes, Bergmann glia, as well as ependymal cells and cells of the choroid plexus) express the rat protein target for the antiproliferative antibody (TAPA, also known as CD81), reflecting the role of TAPA in establishing and maintaining cellular contacts of different types of macroglia (Sullivan and Geisert, 1998). Similarly, rat type 1 interleukin-1 receptor (IL-1R1), a target for systemic interleukin, is associated with barrier-related cells including the leptomeninges, ependyma, especially non-tanycytic portions of the third ventricle, and choroid plexus (Ericsson et al., 1995). In keeping with this, Hsp27 was less strongly induced in non-tanycytic portions of the third ventricle. Interestingly, intracerebroventricular administration of corticotropin-releasing factor

results in an increase in *c-fos* mRNA in ependymal cells (Imaki et al., 1993), cells that express the cytokine receptor IL-1R1 (Ericsson et al., 1995). Thus, widespread induction of Hsp27 in each of these specialized cell types is consistent with the presence of similar receptors and proteins indicating a similar developmental origin (Ericsson et al., 1995; Sullivan and Geisert, 1998).

*Subependymal cells.* A number of small Hsp27-positive cells with few or no processes were associated with the brain parenchyma subjacent to the ependyma of the third ventricle. In order to characterize these cells, double labeling studies were carried out. The cells also stained positively for vimentin but not for GFAP, nestin or OX-42. Their location, morphology and immunohistochemical profile raise the possibility that they are stem cells derived from the lining of the third ventricle. It is also noteworthy that they are found subjacent to the non-tanycytic portion of the third ventricle ependyma. These subependymal Hsp27-positive cells have similarities to ependymal and subependymal cells identified as multi-potent neural stem cells with a broad capacity for differentiation into either neuroglia or neurons (Johansson et al., 1999; Momma et al., 2000).

#### ***Hyperthermic induction of Hsp27 in neurons***

The physiological challenge presented by an increase in core body temperature to 42°C is associated with tachycardia, hypertension followed by hypotension (Kregel et al., 1988; Currie et al., 1990; Tsay et al., 1999), and profuse salivation, a component of behavioral thermoregulation in the rat (Stricker and Hainsworth, 1970). After hyperthermic treatment, select neurons of the hippocampal formation, hypothalamus,

brainstem and circumventricular organs expressed high levels of Hsp27. The central autonomic structures containing neurons that were Hsp27-positive is strikingly similar to those that showed increased Fos expression in response to inflammatory stimuli, e.g., fever (Ericsson et al., 1994; Sagar et al., 1995; Elmquist and Saper, 1996; Elmquist et al., 1996; Takahashi et al., 1997; Tkacs et al., 1997), and dehydration (Morien et al., 1999), as well as neuroendocrine stress that results in activation of the hypothalamic-pituitary-adrenal axis (Sawchenko et al., 2000). Exposure to an ambient temperature of 42°C induces the expression of the activity dependent transcription factor c-fos in the paraventricular nucleus of the hypothalamus (Tsay et al., 1999). Because the Hsp27-positive neurons represent a subset of those that are typically active in the neuroendocrine stress response, they may be more specific for the physiological challenges presented by hyperthermia or osmotic shock as opposed to those of generalized neuroendocrine stress responses.

After hyperthermia, Hsp27 was expressed in neurons in the paraventricular nucleus of the hypothalamus, the dorsomedial hypothalamic nucleus and the subfornical organ. The expression of Hsp27 in the dorsomedial nucleus of the hypothalamus may be related to the involvement of this area in thermoregulation (Kobayashi et al., 1999). Interestingly, hyperthermia also induces the expression of Hsp70 mRNA in the compact subnucleus of the dorsomedial hypothalamus (Blake et al., 1990b). As well, the dorsomedial and paraventricular nuclei of the hypothalamus respond to inflammatory stimuli, stress and administration of corticotropin-releasing hormone with increased Fos (Imaki et al., 1993; Elmquist et al., 1996). It is noteworthy that the dorsal medial hypothalamus and the subfornical organ both project heavily to the paraventricular

nucleus (Miselis, 1981; Thompson et al., 1996). On the basis of these connections, Thompson et al. (1996) have argued that the dorsomedial nucleus is capable of influencing subfornical organ inputs to the paraventricular nucleus, thereby playing a role, albeit complex, in body fluid homeostasis. However, because these hypothalamic and subfornical organ neurons also participate in baroreflex and cardiovascular control (Potts et al., 1999) and are activated by inflammatory stimuli (Ericsson et al., 1994; Sagar et al., 1995; Elmquist and Saper, 1996; Elmquist et al., 1996; Takahashi et al., 1997; Tkacs et al., 1997), it remains to be determined which components of the physiological responses to hyperthermia (cardiovascular regulation, dehydration or fever) are related to the up-regulation of Hsp27 in neurons. Thus, the expression of Hsp27 in regions of the adult rat CNS involved in the monitoring and maintenance of body fluid homeostasis may be part of that regulatory mechanism, perhaps with an interaction between temperature regulation and thirst.

The expression of Hsp27 in neurons in central autonomic structures of the medulla oblongata such as the dorsal vagal complex may be related to the roles of the nucleus of the tractus solitarius in processing afferent information from the upper alimentary tract and the dorsal motor nucleus of the vagus in the efferent control of gastric motility and secretion. Neurons in these nuclei may also be affected by the increased salivation and swallowing accompanying hyperthermia (Stricker and Hainsworth, 1970).

Induction of Hsp27 in the neurons of the dentate granule cells of the hippocampal formation and in the hypothalamus is consistent with changes in their activity following physiological challenges. It has previously been shown that these and other areas are also

affected by hyperthermia (Morimoto and Murakami, 1985; Blake et al., 1990). For example, following peripheral (whole body) thermal stimulation, Morimoto and Murakami (1985) identified increased metabolic activity as shown by increased levels of [<sup>14</sup>C] deoxyglucose in the hippocampus and the anterior hypothalamic region. Subsequently, Blake et al. (1990b) showed that *in vivo* hyperthermia induced the expression of Hsp70 mRNA in the dentate gyrus, and the dorsomedial and paraventricular nuclei of the hypothalamus.

### ***Regulation of Hsp27 expression***

At present, it is known that Hsp27 is expressed developmentally, constitutively, and pathophysiologically. In the brain, most injuries induce Hsp27 in astrocytes, while direct neuronal injury induces Hsp27 specifically in neurons. As discussed above, hyperthermia induced Hsp27 in glia and non-neuronal cells as well as in specific neurons involved in central autonomic function. In the present study, hyperthermia and its sequelae may induce Hsp27 in non-neuronal cells and in neurons through different signaling pathways that lead to activation of the heat shock transcription factor. Possibilities include thermal, osmotic and autonomic regulatory activation. In cells, increased temperature leads to changes in protein stability and increased levels of denatured or coagulated protein causing activation of the heat shock transcription factor, HSF1 (Morimoto, 1993; Cotto and Morimoto, 1999) and, consequently, a generalized induction of Hsp27.

Conceivably, the stress of dehydration during hyperthermia could induce Hsp27 in response to osmotic regulation as in the case of Hsp70 in hepatocytes (Schliess et al.,

1999). In fact, hyper-osmotic or hypo-osmotic stress of mammalian cells in culture can activate HSF1 (Caruccio et al., 1997). Bergmann glia (Golgi epithelial cells) are exquisitely sensitive to changes in plasma osmolality, increasing their content of taurine and other amino acids, in response to hypo-osmotic stress (Nagelhus et al., 1993). Whether the induction of Hsp27 is due to the heat *per se* or osmotic stress, the end result is metabolic instability leading to protein denaturation and activation of the HSF1. Alternatively, the selective induction of Hsp27 in neurons of the central autonomic areas controlling body temperature and fluid balance, suggests an activity-dependent neuronal activation of Hsp27 expression.

After hyperthermia, the neuronal expression of Hsp27 appears to peak by 24 hours and by six days is declining and approaching background levels. In contrast, after direct injury to the sciatic or vagus nerves (Costigan et al., 1998; Hopkins et al., 1998), Hsp27 up-regulation occurs within 24-48 hours and the increased levels of Hsp27 persist in many neurons for up to 90 days (Hopkins et al., 1998). Such results suggest that the duration of expression and the rate of degradation are variable and may be dependent on the nature of the injury or stress.

## **Conclusions**

The present results provide further evidence of the cell type and stress-specific nature of Hsp27 induction in the adult rat CNS. Not only does hyperthermic treatment induce Hsp27 expression in several types of glia, but also induces it in specific neuronal populations important for body fluid homeostasis and thermoregulation. These results suggest regulatory roles for Hsp27 in select populations of neurons and in glial function

**under the stressful conditions of hyperthermia. Thus, in the mammalian brain the expression of Hsp27 is regulated developmentally, constitutively, pathophysiologically, and possibly by activation of specific neural circuits.**

**CHAPTER 3:**

**INJURY TO RETINAL GANGLION CELLS INDUCES EXPRESSION OF THE  
SMALL HEAT SHOCK PROTEIN HSP27 IN THE RAT VISUAL SYSTEM**

**This chapter has been published in Neuroscience, 2002, 110:653-665.**



## **Introduction**

Neuronal injury by axotomy of the optic nerve causes specific damage to the retinal ganglion cells (RGCs) that results in the apoptotic death of approximately 90% of these neurons by 14 days after the injury (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994). There is a continuing search for factors that enhance or prolong the survival of these cells so that novel treatments can be developed for diseases resulting from CNS neuronal death. Hsp27 may be one of these factors. The expression of Hsp27 in the PNS in surviving neurons following axotomy (Hopkins et al., 1998; Costigan et al., 1998; Lewis et al., 1999) and its anti-apoptotic effects when over-expressed in cell culture (Wagstaff et al., 1999) support the hypothesis that Hsp27 plays an important role in cell survival following axotomy or trophic factor withdrawal.

Hsp studies in the retina, *in vivo*, have focused largely on the inducible expression of Hsp70. Hsp70 has been detected in photoreceptor cells following hyperthermic treatment (Barbe et al., 1988) or bright-light exposure (Liu et al, 1998; Tytell et al., 1994). *In vitro*, Hsp70 has been detected in RGCs following hyperthermic treatment (Caprioli et al., 1996). These RGCs showed increased tolerance to hypoxic and excitotoxic injury. Further, increased levels of antibodies to Hsp27 have been identified in the serum of patients with glaucoma (Tezel et al., 1998; Wax et al., 2001). When applied to human retinal tissue, Hsp27 antibodies enter RGCs by an endocytic mechanism and trigger apoptosis, indicating that Hsp27 protein itself may inhibit apoptosis when not blocked by its antibody (Tezel and Wax, 2000). In addition, immunohistochemical studies have detected the increased expression of Hsp27 in RGCs

and their associated nerve fiber layer, and the optic nerve head of glaucomous human eyes post mortem (Tezel et al., 2000).

To investigate the relationship between Hsp27 expression and RGC injury *in vivo*, we have used Western blotting and immunohistochemical techniques to study the induction and cell-specific expression of Hsp27 in various regions of the rat visual system from 1 to 28 days after optic nerve transection.

## **Materials and Methods**

### ***Animals***

Female Sprague-Dawley rats (175-250 g; Charles River, St. Constant, QC) were cared for in accordance with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. For all surgical procedures, rats were anaesthetized with a mixture of ketamine (62.5 mg/kg), xylazine (3.25 mg/kg) and acepromazine (0.62 mg/kg) in 0.9% saline, administered by an intramuscular injection.

### ***Optic nerve transection***

Following the methods of Mansour-Robaey et al. (1994) and Clarke et al. (1998), the left optic nerve was transected with microscissors at approximately 0.5 mm from the eye. Preservation of the blood supply was confirmed by microscopic examination of the retina through the dilated pupil. Animals recovered to 1, 4, 7, 10, 14 and 28 days following transection (n = 4 at each time). Sham control animals were exposed to the same surgical procedures but the optic nerve was not cut (n = 4). Sham control retinas were examined at 4 days following the sham operation. The right eye of each animal was used as an internal control.

### ***Retinal ganglion cell labeling***

One week prior to optic nerve transection, Fluorogold (2% FG in 0.9% saline; Fluorochrome, Fluorochrome Inc., Denver, CO) soaked Gelfoam (UpJohn, Don Mills, ON) was applied to the surface of both superior colliculi to label the cell bodies of RGCs by retrograde transport (Vidal-Sanz et al., 1988; Villegas-Pérez et al., 1993).

### ***Retinal tissue processing***

At the end of the experimental period, rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially with 0.9% saline in 0.1 M PB followed by 4% paraformaldehyde in 0.1 M PB. The left (experimental) and right (internal control) eyes were removed, post-fixed in 4% paraformaldehyde overnight, gelatin embedded and sectioned through the optic disc on a freezing microtome at 30  $\mu$ m.

### ***Superior colliculus tissue processing***

RGCs were not labeled with FG in a separate group of animals for study of the superior colliculus (SC) following optic nerve transection. Animals were perfused as above at 1, 4, 7, 10, 14 and 28 days following optic nerve transection (n = 2 in each group). Sham operated controls were perfused at 4 days following sham operation (n = 2). Brains were removed and post-fixed in 4% paraformaldehyde overnight. Coronal sections were cut at 30  $\mu$ m on a freezing microtome.

### ***Western analysis***

Western analysis was performed according to methods previously described (Chapter 2). Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and transcardially perfused with 0.9% saline in 100 mM phosphate buffer (PB). The right (control) and left retina and the right and left superior colliculi were isolated from rats 10 days (n = 2) following optic nerve transection. Tissue was homogenized in 100

mM PB containing Complete™ mini protease inhibitor cocktail (Boehringer Mannheim, Germany). Protein concentration was determined according to the method of Lowry et al. (1951). Samples containing 10 µg of protein were solubilized in sodium dodecyl sulfate sample buffer, boiled for 10 minutes and then loaded onto a polyacrylamide gel (2.5% upper gel, 12% running gel) according to the method of Laemmli (1970). Samples were electrophoresed at 75 V then proteins were electroblotted onto an Immobilon-P membrane (Millipore, Mississauga, ON) at 30 V for 18 hr. Membranes were air-dried. For immunostaining, membranes were wet with methanol, rinsed with phosphate buffered saline (PBS; pH = 7.4) and blocked for one hour at 37°C in 5% skim milk/1% bovine serum albumin in PBS. Primary rabbit polyclonal antibody specific for the murine Hsp25 and rat Hsp27, (Plumier et al., 1996; Chapter 2; SPA-801, StressGen Biotechnologies, Victoria, BC) was diluted 1:1000 in 5% skim milk/1% bovine serum albumin 1X PBS. Membranes were incubated with primary antibody at 4°C for 18 hours with gentle agitation. Membranes were washed in PBS/0.2% Tween 80 at 37°C and then incubated for 1 hour at 37°C with peroxidase conjugated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) diluted 1:2000 in 5% skim milk/1% bovine serum albumin 1X PBS. Blots were then washed with PBS/0.2% Tween 80 at 37°C. The peroxidase-conjugated antibody was detected using the electrogenerated chemiluminescence Western Blot Detection Kit and Hyperfilm ECL (Amersham, Arlington Height, IL). Equal loading of the lanes was confirmed using Amido Black counterstain for proteins.

### ***Immunohistochemistry***

Radial sections of retina through the optic disk (5 sections per animal) and serial coronal sections through the entire SC were used for Hsp27 immunohistochemical analysis following the methods stated in Chapter 2. Primary rabbit polyclonal antibody specific for the murine Hsp25 and the rat Hsp27 (SPA-801, StressGen Biotechnologies, Victoria, BC) was diluted 1:10 000 in PBS 0.1% Triton-X (PBS-X) containing 2% goat serum. The biotinylated goat secondary antibody raised against rabbit IgG was diluted 1:400 (Vector Laboratories Inc. Burlingame, CA) in PBS-X. Sections were washed with PBS-X then incubated for one hour in avidin-biotin-horseradish peroxidase complex (1:1000; Vector Laboratories Inc.). Following PBS-X washes, sections were incubated in PBS-X containing 0.3 mg/100 ml glucose oxidase, 40 mg/ml ammonium chloride and 200 mg/ml B-D(+) glucose (Sigma Chemical Co., St. Louis, MO) and 0.05% diaminobenzidine-tetrahydrochloride (Sigma Chemical Co.) for 20 minutes. After washing, sections were floated on to gelatinized slides, air dried overnight and coverslipped using Entallen (E.Merck, Darmstadt, Germany).

### ***Immunofluorescence***

Sections were selected as above and treated with PBS-X, 10 % goat serum, and incubated overnight at 4°C in PBS-X with 2% goat serum and Hsp25/27 rabbit polyclonal antibody (1:1000; StressGen Biotechnologies Corp., Victoria, BC). After three 10 minute rinses, sections were incubated for 3 hours in PBS (no Triton-X) containing Alexa goat anti-rabbit 546 or 488 (1:500; Molecular Probes, Eugene, OR). The sections were rinsed several times in PBS then floated onto gelatinized slides, air

dried overnight and coverslipped with Fluoromount (BDH Chemicals, Poole, England). Hsp27-immunopositive cell profiles were counted in all 5 sections/animal in all four animals per group. Hsp27-positive cells were confirmed as RGCs by changing to the UV filter to detect Fluorogold labeling.

*Glial fibrillary associated protein:* Radial sections from the retina and coronal sections from the SC were treated as above, being washed in PBS-X, then blocked for 1 hour with 10% horse serum in PBS-X. Sections were incubated overnight at 4°C in PBS-X with 2% horse serum and the glial fibrillary associated protein (GFAP) monoclonal antibody (1:200; Nova Castra, Halifax, NS). After three 10 minute PBS-X washes, sections were incubated for 3 hours in PBS (no Triton-X) containing Alexa goat anti-mouse 546 or 448 (1:400; Molecular Probes, Eugene, OR). The sections were rinsed several times in PBS then floated onto gelatinized slides, air dried overnight and coverslipped with Fluoromount.

#### ***Microscopy and image processing***

Sections were examined using bright-field and fluorescence microscopy on a Zeiss Axioplan microscope. Selected immunofluorescence and immunohistochemically stained sections were scanned using a Diagnostic Instruments Inc. Spot II digital camera mounted to a Zeiss Axioplan 2 microscope and images captured digitally using Adobe Photoshop 5.0 software. Prints and photographic plates were made using a Kodak 8650 printer with continuous tone dye sublimation. Other than brightness and contrast, images were not adjusted.

***Statistical analysis***

Fluorogold and Hsp27 positive RGCs were counted for each animal (total of 5 sections) and the data are presented as means  $\pm$  standard error of the mean for each group (n = 4 animals per group). Mann-Whitney U test comparing means at the various times was performed with significance set at  $p < 0.05$ .



## **Results**

### ***Fluorogold labeling***

Fluorogold-positive retinal ganglion cell profiles were examined (Figure 3.1) to confirm the extent of cell loss following optic nerve transection. FG-positive RGCs were counted in 5 non-adjacent radial sections through the optic disk (Figure 3.2). FG-positive RGCs were distinguished from FG-positive microglia in the injured retina by cell morphology, diffuse punctate FG-staining and location in the retina. There was no significant difference between the number of FG-labeled RGCs in right eye controls ( $4430 \pm 144$ ) and the left eye at 1 day ( $3624 \pm 289$ ) or the left eye at 4 days ( $3973 \pm 228$ ) following optic nerve transection or in the sham operated controls ( $4151 \pm 265$ ). The number of FG-positive RGCs declined sharply to  $2117 \pm 158$  and  $853 \pm 127$  at 7 and 10 days, respectively. By 14 days following optic nerve transection, the number of FG labeled RGCs was reduced by approximately 90% ( $489 \pm 83$ ) compared to sham operated controls and by 28 days the number of RGCs had decreased by 95% ( $281 \pm 42$ ). The rate of cell loss was similar to that reported by Berkelaar et al. (1994), Mansour-Robaey et al. (1994) and Koeberle and Ball (1998).

### ***Western analysis***

*Retina:* Western analysis confirmed increased levels of Hsp27 protein in injured left retinas at 10 days following optic nerve transection (Figure 3.3). Hsp27 protein was detected in right eye control retinal tissue.

*Superior colliculus:* Denervation of the right SC by left ON transection resulted in an increase in the level of Hsp27 expression in the right SC at 10 days following optic nerve transection (Figure 3.3). Low levels of Hsp27 expression were detected in the left SC 10 days following optic nerve transection. In sham-operated animals, no difference in the low levels of Hsp27 was detected between left and right SC (data not shown).

### ***Immunohistochemical analysis***

*Hsp27 in the retina:* Hsp27 immunoreactivity was not detected in RGCs of control (right, unoperated) retinas or in the RGCs of sham operated (left) retinas. Similarly, there was no Hsp27 immunoreactivity detected at one day following optic nerve transection (left retina) using either DAB immunohistochemistry or immunofluorescence. However, by 4 days following optic nerve transection, Hsp27 was detected in the cytoplasm of the cell bodies, dendrites and axons in a subset of RGCs (Figure 3.4). Omission of the primary antibody in the immunohistochemical reaction resulted in no immunoreactivity in RGCs of injured retina. Diffuse fluorescence was detected in the inner and outer plexiform layers and the photoreceptor layer in both right (control) and left (injured retina; see Figure 3.4A) with the omission of the primary antibody indicating the non-specific nature of the fluorescence in these regions.

Hsp27 was detected in an average of  $123 \pm 8$  RGCs (per 5 retinal sections) as confirmed by FG double-labeling (Figure 3.4). Fewer than 50 Hsp27-positive cells (per 5 retinal sections) were detectable at 7 days ( $40 \pm 6$ ). This number was further reduced to  $22 \pm 3$  by 28 days following injury (Figure 3.5). The mean number of Hsp27-positive RGCs was determined as a percentage of surviving FG-positive RGCs (Figure 3.6).

Interestingly, by 28 days following optic nerve transection, the percentage of FG-positive RGCs expressing Hsp27 increased significantly to 8% ( $p < 0.05$ ) (Figure 3.6). Hsp27 expression was also increased in the nerve fiber layer of the retina (asterisks, Figure 3.4) and optic nerve head (not shown) following optic nerve transection. No Hsp27-positive Müller cells were observed at any time examined.

*Optic tract:* Hsp27 immunoreactivity was detected in the optic tract of control and sham operated animals in cells with astroglial morphology (Figure 3.7A). Hsp27 immunoreactivity was increased in glial cells in the optic tract as early as 4 days (Figure 3.7B) and remained high to 90 days ( $n = 2$ ) following optic nerve transection. Hsp27 appears to be localized in the cytoplasm and fine processes of these astroglia with the nucleus being Hsp27-negative (Figure 3.7B inset).

*Superior Colliculus:* Expression of Hsp27 was detected in RGC target tissue, specifically the optic layer of the right SC, from 4 (Figures 3.8, 3.9B, 3.10A) to 28 days following optic nerve transection. Double-labeling with GFAP confirmed that Hsp27-positive cells in the SC were astrocytes (Figure 3.9). The number of Hsp27-positive astrocytes declined by 28 days following optic nerve transection (Figure 3.10C); however, Hsp27 expression was still detected in a few astrocytes in the optic layer of the SC at 30 and 90 days following optic nerve transection ( $n = 2$  for each group).

*GFAP in the retina:* Little or no GFAP immunoreactivity was detected in the retina of control right eyes or in the sham operated controls. At 14 and 28 days following

optic nerve transection, GFAP was detected in Müller cell processes as previously reported by Leon et al. (2000). Despite the indication of glial activation by the induced expression of GFAP in Müller cells following axotomy, none of these activated glia expressed Hsp27 at any time examined following optic nerve transection. The lack of detection of Hsp27 immunoreactivity in Müller cells appears to be in direct contrast to the strong expression of Hsp27 in astrocytes of the SC detected as early as 4 days following axotomy (see below).

*GFAP in the Superior Colliculus:* GFAP was detected at increased levels in the superficial gray, optic and intermediate gray layers of the right SC, from 4 to 28 days (Figure 3.10B, D). In contrast to Hsp27, GFAP immunoreactivity remained high to 28 days following optic nerve transection (Figure 3.10D).

**Figure 3.1:** Radial section of rat retina with retinal ganglion cells (RGCs) labeled from the superior colliculus (SC) with Fluorogold (FG). **A:** Left retina at 4 days following optic nerve transection. **B:** Left retina 14 days following optic nerve transection shows the decrease in the number of FG-positive RGCs. Arrows indicate some of the FG-positive RGCs in the ganglion cell layer (GCL). Asterisks indicate numerous microglia that have become FG-positive through phagocytosis of dying RGCs. Scale bar, 50  $\mu$ m. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RSL, receptor segment layer.

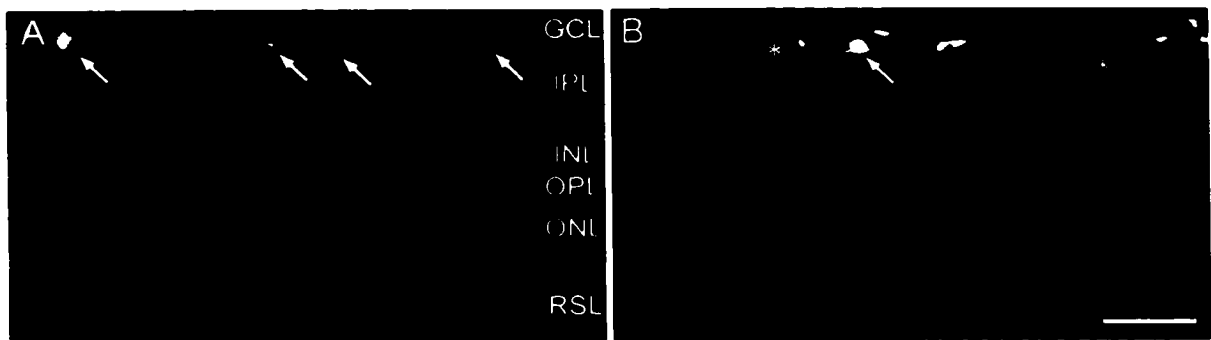


Figure 3.1

**Figure 3.2: Number of Fluorogold (FG)-positive retinal ganglion cells (RGCs) following optic nerve transection (total counts from 5 non-adjacent radial sections through the optic nerve per rat retina reported as a mean of  $n = 4$  animals for each group). There is no significant difference in the number of FG-positive RGCs surviving in retinas of sham operated or at 0 (right eye controls) 1, or 4 days following optic nerve transection. By 7 and 10 days, the number of FG-positive RGCs has decreased to 50% and 25%, respectively; by 28 days, as few as 5% of FG-positive RGCs remain.**

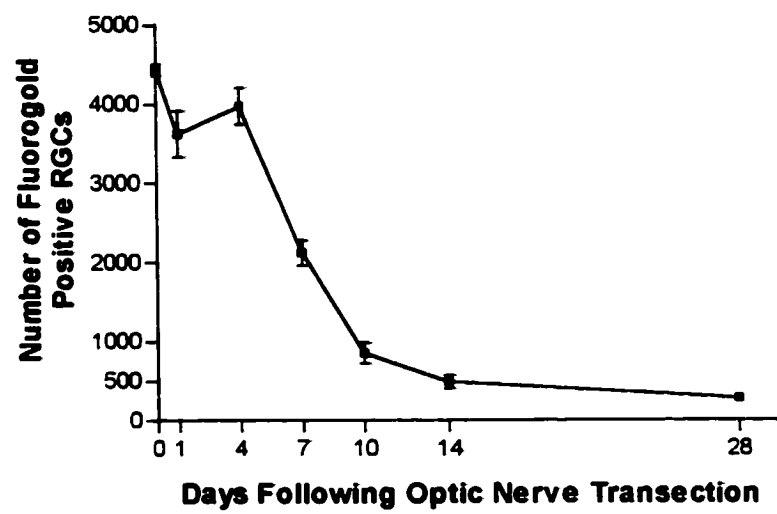


Figure 3.2



**Figure 3.3:** Western blot analysis of proteins isolated from retina and superior colliculus (SC) tissue homogenates at 10 days following optic nerve transection. Increased levels of Hsp27 are detected in the left (L) retina and right (R) SC following optic nerve transection. Note the detection of Hsp27 protein in uninjured right retina and left SC.

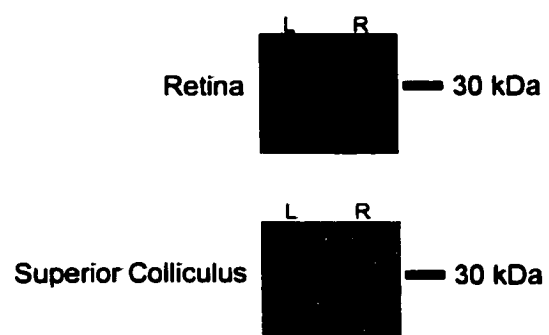


Figure 3.3

**Figure 3.4:** Radial retinal section showing immunohistochemical localization of Hsp27 in rat retinal ganglion cells (RGCs) 4 days following optic nerve transection. **A:** Hsp27 immunofluorescence in cells in the ganglion cell layer. Note that in the central arrowed RGC (in the plane of focus) Hsp27 immunofluorescence is cytoplasmic and not nuclear. **B:** Retrograde labeling with Fluorogold (FG) from the SC confirms that Hsp27-positive cells in the ganglion cell layer are RGCs. Arrows indicate FG-labeled RGCs that are Hsp27-positive. Asterisks indicate Hsp27 immunoreactivity in RGC axonal layer. Scale bar, 20  $\mu\text{m}$ .

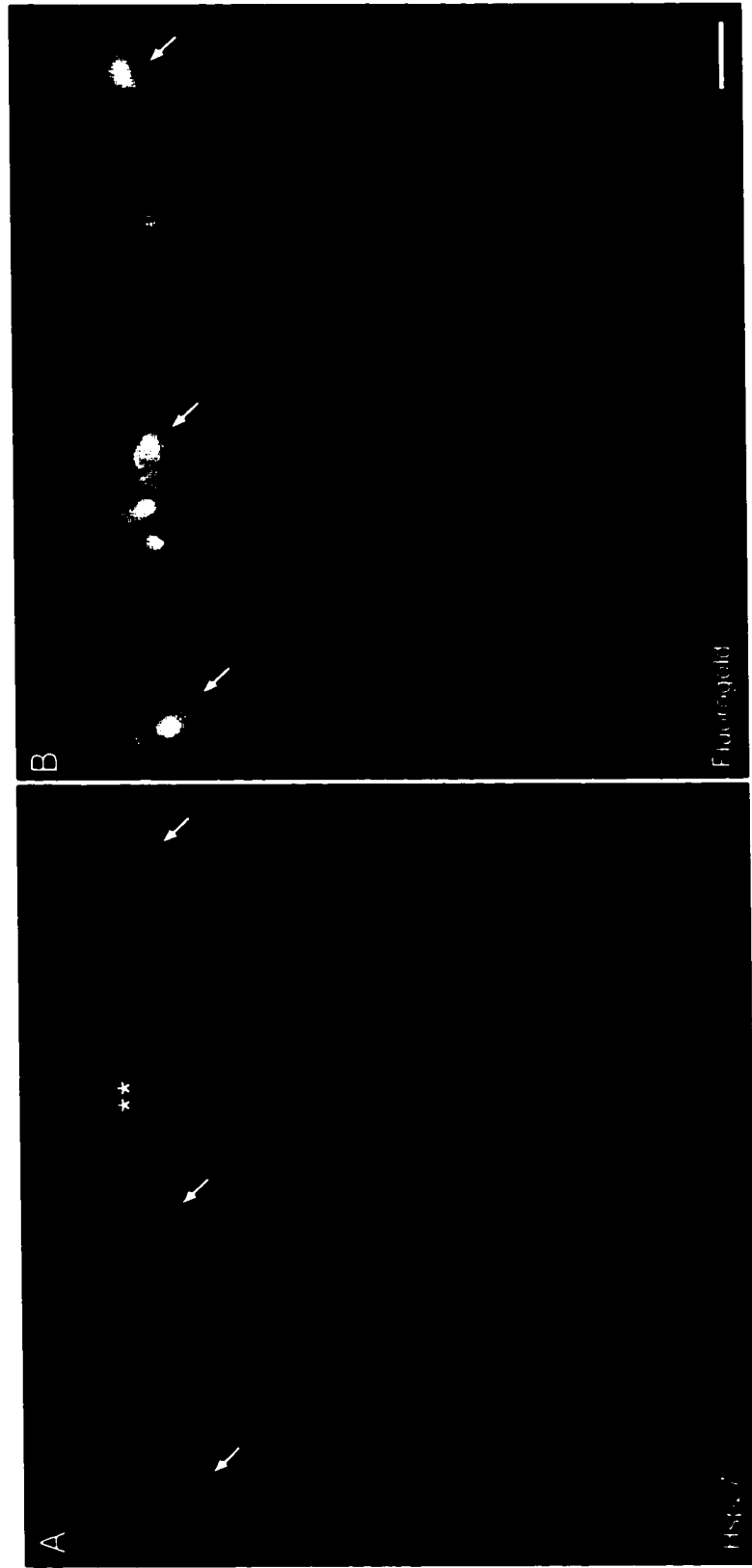


Figure 3.4

**Figure 3.5:** Number of Hsp27-positive retinal ganglion cells (RGCs) at various days following optic nerve transection (5 non-adjacent radial sections through the optic disk per rat retina, n = 4 animals for each group). No Hsp27-positive RGCs are detected in right eye control retinas (0 days). In sham operated animals, an average of 1 Hsp27 positive RGC was detected in the group. From 4 to 28 days following optic nerve transection, the data indicate a significant increase in the number of Hsp27-positive RGC cell profiles ( $p < 0.05$ ) when compared to sham operated controls. There is no significant change in the number of Hsp27-positive RGC cell profiles from 10-28 days following optic nerve transection. Asterisk  $p < 0.05$ .

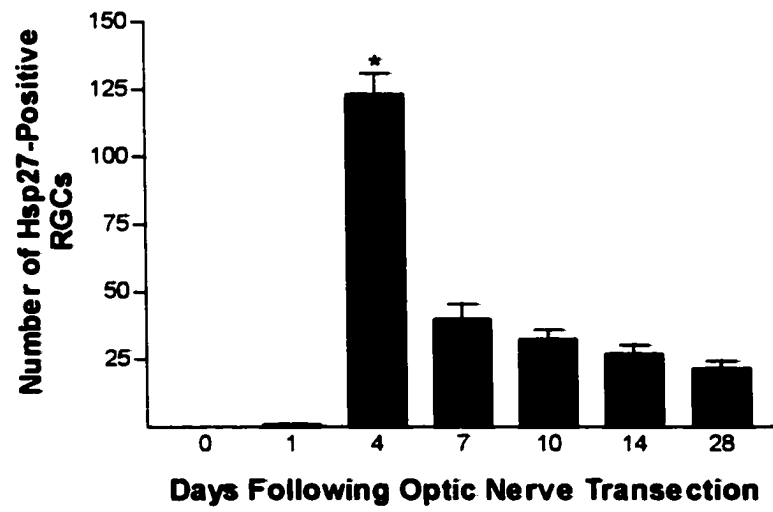


Figure 3.5

**Figure 3.6: Percentage of Fluorogold (FG)-labeled retinal ganglion cells (RGCs) expressing Hsp27 from 4 – 28 days following optic nerve transection. The percentage of RGCs that express Hsp27 significantly increases ( $p < 0.05$ ) from 10 to 28 days compared with 7 days. These data indicate that the rate of decline of Hsp27 RGCs is slower than the rate of decline of the general FG-labeled RGC population. Asterisk,  $p < 0.05$ .**

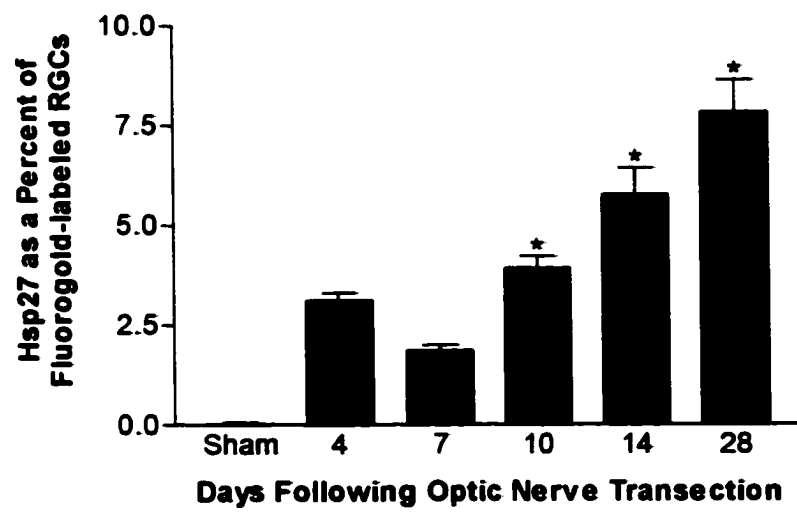


Figure 3.6



**Figure 3.7:** Immunohistochemical localization of Hsp27 in the optic tract following optic nerve transection (coronal section at the level of the dorsomedial hypothalamic nucleus at Bregma - 3.14 mm, Figure 32, Paxinos and Watson, 1998). **A:** Sham operated contralateral optic tract. Hsp27 is detected in some cells with astroglial morphology within the tract. **B:** 4 days following optic nerve transection Hsp27 expression is increased in the contralateral optic tract. **B inset:** High magnification photomicrograph showing localization of Hsp27 immunoreactivity to the cell bodies and processes of astroglia in the optic tract at 4 days following optic nerve transection. Arrows indicate two Hsp27-positive astroglia with Hsp27-negative nuclei. Scale bar in A, 200  $\mu\text{m}$  (for A and B); scale bar in inset, 25  $\mu\text{m}$ .

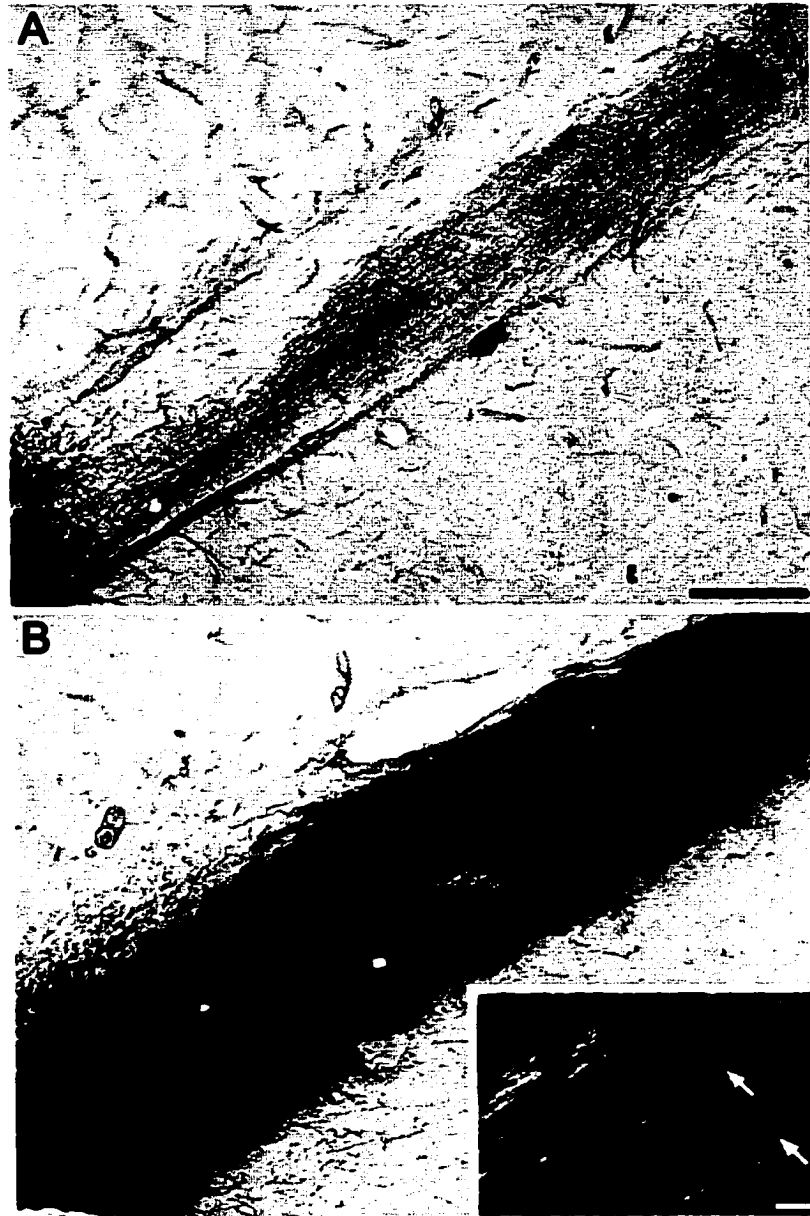


Figure 3.7

**Figure 3.8:** Immunohistochemical localization of Hsp27 and GFAP in astrocytes of the superior colliculus (SC) at 4 days following left optic nerve transection (coronal sections at Bregma - 6.30 mm, Figure 45, Paxinos and Watson, 1998). **A:** Left SC shows little or no expression of Hsp27. Hsp27-positive cells are detected in the optic layer (OP) of the right SC. **B:** Left SC shows low constitutive levels GFAP. Right SC shows increased number of GFAP-positive astrocytes throughout all layers by 4 days following transection of the left optic nerve. Zo, zonal layer; SuG, superior gray layer; OP, optic layer; InG, intermediate gray layer. Scale bar, 200  $\mu$ m.

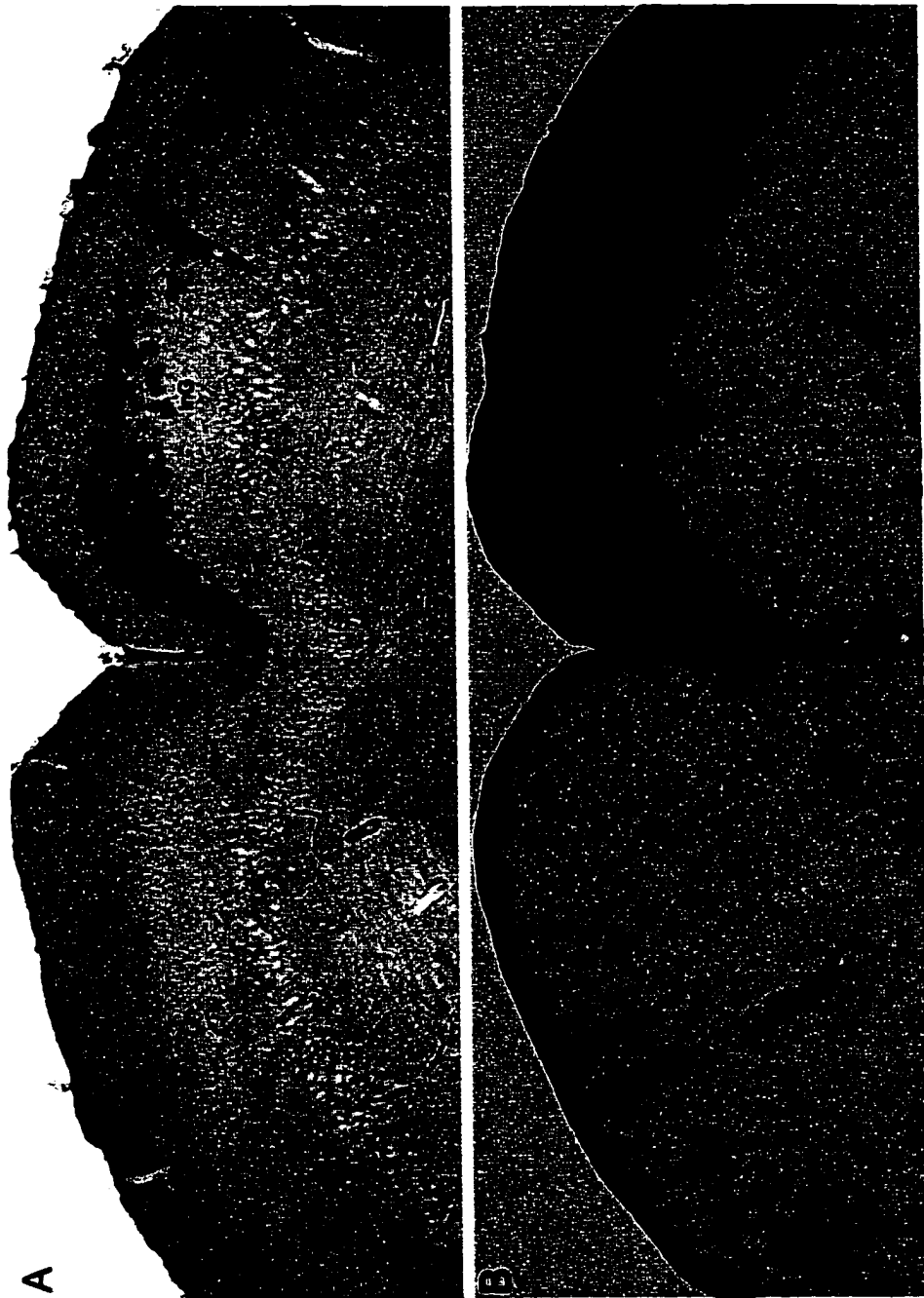


Figure 3.8

**Figure 3.9:** Immunohistochemical co-localization of Hsp27 and GFAP immunofluorescence in astrocytes of the superior colliculus (SC). **A:** GFAP-positive astrocytes (Alexa 488, green) in the SC 4 days following optic nerve transection. **B:** Hsp27-positive cells (Alexa 546, red) in the deafferented SC. **C:** Merged composite photomicrograph showing double label confirming that Hsp27-positive cells are GFAP-positive astrocytes. Scale bar, 50  $\mu\text{m}$ .

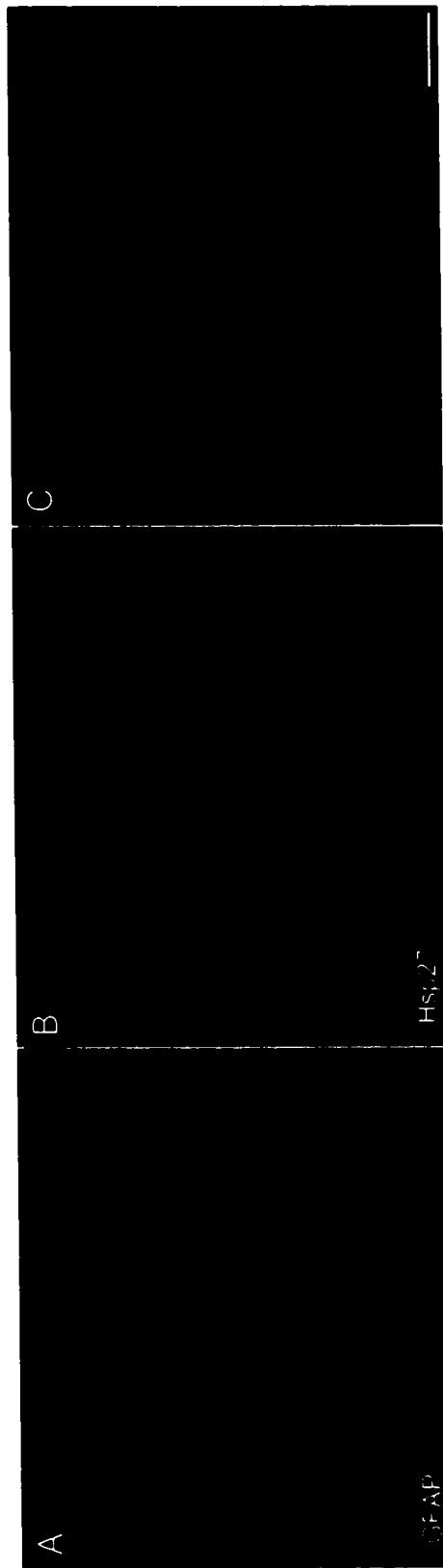


Figure 3.9

**Figure 3.10:** Coronal section midway through the rostral/caudal extent of the superior colliculus (SC) showing immunofluorescent labeling of astrocytes in the deafferented SC at 4 days and 28 days following optic nerve transection. **A, C:** Hsp27 at 4 and 28 days, respectively. **B, D:** GFAP at 4 and 28 days, respectively. Note that GFAP expression remains high to 28 days throughout the visual layers of the SC whereas Hsp27 expression is limited to the optic layer of the SC and declines at 28 days. Scale bar, 100  $\mu$ m.

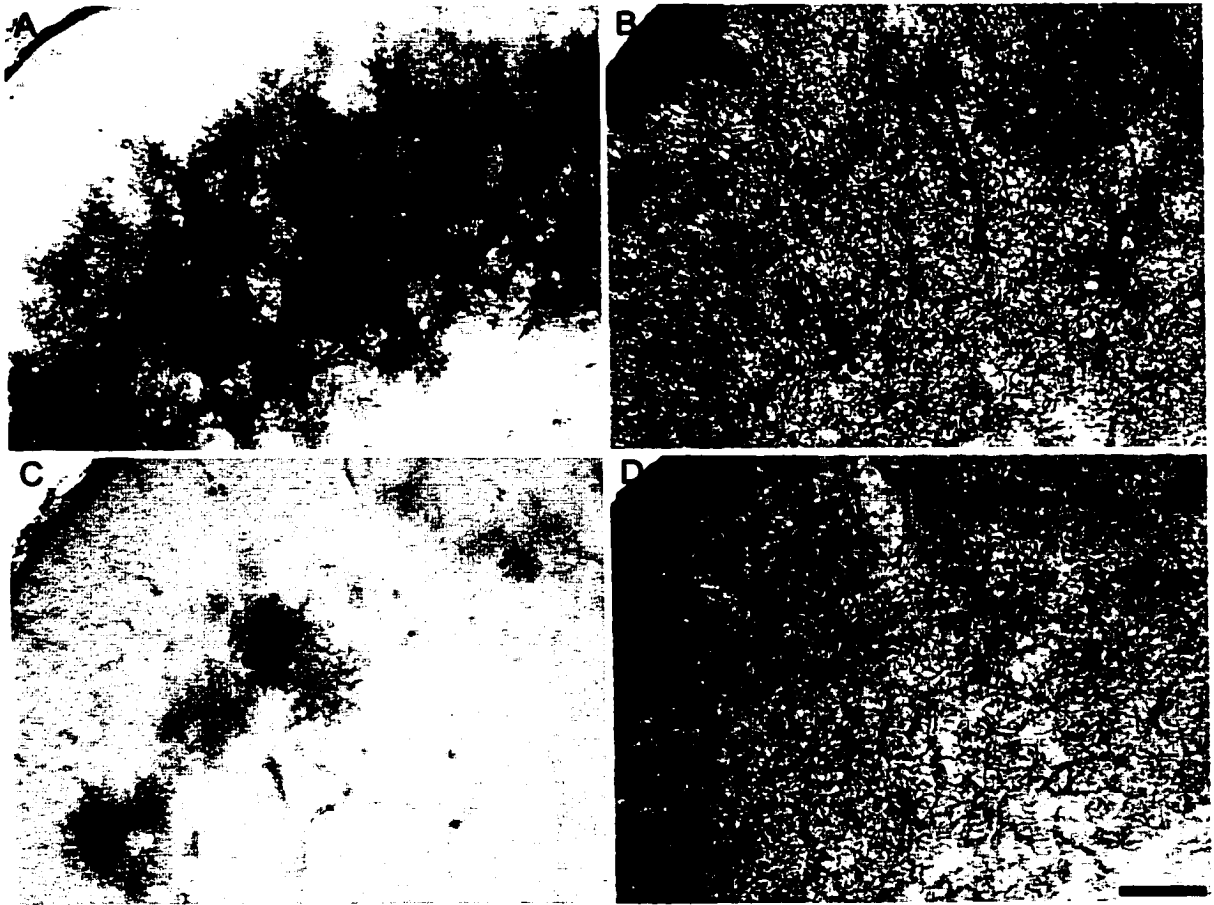


Figure 3.10



## **Discussion**

Axotomy of the optic nerve results in the apoptotic cell death of a significant number of RGCs by 14 days following transection (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994). Hsp27 is an inhibitor of apoptosis (Garrido et al., 1999; Bruey et al., 2000; Pandey et al., 2000). In the present study, Hsp27 was detected in a limited subset of injured RGCs and there was a significant increase in the percentage of surviving RGCs that were Hsp27-positive by 28 days following optic nerve transection. In addition, Hsp27 immunoreactivity was increased in the nerve fiber layer of the retina and was increased in glial cells of the optic tract. Hsp27 was also detected in GFAP-positive astrocytes in the right deafferented SC from 4 to 28 days following axotomy.

### ***Neuronal expression of Hsp27 following axotomy***

Hsp27 is normally expressed in many sensory and motor neurons of the brainstem and spinal cord of the adult rodent (Plumier et al., 1997c; Armstrong et al., 2001b). Here, using immunohistochemistry, little or no constitutive expression of Hsp27 is detected in RGC neurons of the uninjured adult rat retina. However, following transection of the optic nerve, Hsp27 immunoreactivity was detected in a subset of RGCs at 4 days (Figure 3.5) and continuing to 28 days following axotomy.

The expression of Hsp27 in a limited subset of RGCs following axotomy is in contrast to studies showing a high number of neurons expressing Hsp27 following peripheral nerve injury. While peripheral neurons seem to respond uniformly to axotomy with rapid expression of Hsp27, only a minority of the central neurons respond with the

expression of Hsp27. Axotomy of the vagus nerve (Hopkins et al., 1998) and transection of the sciatic nerve and dorsal roots (Costigan et al., 1998; Lewis et al., 1999) results in Hsp27 expression in most neuron cell bodies from 2 to 90 days following axonal transection. The majority of these neurons survive with little cell loss to 4 months following peripheral nerve injury (Coggeshall et al., 1997, Costigan et al., 1998). In the CNS, fimbria fornix lesions induce Hsp27 expression in some medial septum neurons by 10 days (Anguelova and Smirnova, 2000). It has been suggested that increased levels of Hsp27 immunoreactivity in neurons after peripheral nerve axotomy and fimbria fornix lesion contribute to the prevention of cell death in these models (Costigan et al., 1998; Anguelova and Smirnova, 2000). In other work, the expression of Hsp27 strongly correlates with the survival of peripheral neurons following axotomy (Lewis et al., 1999). At post-natal day 0, no Hsp27 is detected in sciatic motor neurons in the ventral horn, a time when most of these neurons do not survive axotomy. However, in the neurons that do survive axotomy at post-natal day 0, all expressed Hsp27. In addition, transfection of cultured neonatal neurons with an adenoviral vector expressing Hsp27 rescued these neurons from death induced by NGF withdrawal (Lewis et al., 1999).

As RGC loss continued from 5 to 28 days following axotomy, there was an increase in the percentage of Hsp27-positive RGCs. The response of individual RGCs to axotomy is variable, in that not all RGCs die at the same time (Villegas-Pérez, et al., 1993; Berkelaar et al., 1994) and that there is a small population of RGCs that survive for months following optic nerve transection (Villegas-Pérez et al., 1993). Between 4 and 28 days after axotomy, Hsp27 is found in only 3 to 8% of the surviving FG-labeled RGCs. Such low numbers of Hsp27-positive cells might lead to the conclusion that there is no

clear relationship between Hsp27 and survival of RGCs, since most surviving neurons seem to survive without Hsp27. However, the increase in Hsp27-positive RGCs as a percentage of the FG-labeled RGCs indicates a slower rate of decline of Hsp27-positive RGCs compared to the FG-labeled population (Figure 3.6). This suggests that Hsp27 becomes increasingly important for the long-term survival of these cells. The detection of Hsp27 in a subset of RGCs following optic nerve transection may be an early indicator of a small population of cells that survive following this injury. Whether prior induction of Hsp27 in a larger population of RGCs would increase the number of RGCs surviving axotomy remains to be determined.

#### ***Hsp27 in RGC axons and astroglia of the optic tract***

Increased levels of Hsp27 immunoreactivity were detected in the nerve fiber layer of the retina (asterisks, Figure 3.4), the optic nerve head (data not shown) and in RGC dendritic processes extending into the inner plexiform layer following transection of the optic nerve. Detection of Hsp27 in the RGC axons and dendrites in addition to the soma of RGCs indicates the transport of Hsp27 from the cell body into RGC processes. The detection of Hsp27 immunoreactivity in RGC axons and the optic nerve head has also been detected in post mortem human glaucomatous eyes (Tezel et al., 2000).

Distal to the site of injury, in the optic tract, increased Hsp27 immunoreactivity was not apparent in axons but was most prominent in the associated astroglia of the optic tract (Figure 3.7B). Hsp27 immunoreactivity was detected in the cell body and processes of these astrocytes while their nuclei appeared to be unstained (Figure 3.7B, inset).

While it is reported for the first time that degeneration of axons in the optic tract causes the increased expression of Hsp27 in optic tract astrocytes, it is clear that there are other changes in these astrocytes. For example, Wallerian or anterograde degeneration of axons in the optic tract causes proliferation of the associated astrocytes (Skoff, 1975; Guénard et al., 1996) and changes in cell morphology related to gliosis (Guénard et al., 1996). In addition, anterograde degeneration of axons in the optic tract causes an increase in GFAP expression in optic tract astrocytes that begins at 4 days following eye removal (McLoon, 1986).

#### ***Hsp27 in astrocytes of the superior colliculus***

Denervation of the right SC by transection of the left optic nerve caused an increase in Hsp27 in the right SC as shown by Western analysis (Figure 3.3). Immunohistochemical analysis detected Hsp27 in GFAP-positive reactive astrocytes in the visual layers (primarily the optic layer) of the SC (Figure 3.8). Hsp27 is detected in astrocytes following kainic acid-induced seizures (Plumier et al., 1996) and cortical spreading depression (Plumier et al., 1997b). Blockade of NMDA receptors with MK-801 results in significantly less Hsp27 in cortical astrocytes following cortical spreading depression, suggesting that the induction of Hsp27 in astrocytes is related to glutamate release from neurons and uptake by astrocytes (Plumier et al., 1997b). In the present study, Hsp27 may be expressed in SC reactive astrocytes in response to aberrant release of glutamate by injured RGC axons that terminate in the SC (reviewed in Kalloniatis and Tomisich, 1999; Marc et al., 1998; reviewed in Sefton and Dreher, 1995).

Hsp27 immunoreactivity was confined to GFAP-positive astrocytes in the lower portion of the superficial gray layer, the optic layer and upper portions of the intermediate gray layer of the SC (Figure 3.8), all areas receiving direct retinal projections (Carter et al., 1991). In contrast, increased GFAP immunoreactivity was observed throughout all the visual layers of the right SC (superficial gray, optic and intermediate gray layers) following deafferentation (Figure 3.10) consistent with previous work by Schmidt-Kastner et al. (1993). Hsp27 immunoreactivity declined by 28 days following optic nerve transection; however, GFAP immunoreactivity remained high in all layers to 28 days following optic nerve transection. These findings suggest that while Hsp27 is expressed in astrocytes in the region of degenerating optic nerve terminals in the optic layer of the superior colliculus, reactive gliosis (as defined by increased GFAP expression) does not necessarily result in Hsp27 expression. Hsp27 may be a more precise marker for areas of more severe injury in the brain but does not demarcate all areas of gliosis. Interestingly, Brzyska et al. (1998) have shown that GFAP and Hsp27 expression are independently regulated in primary astrocytic cultures. *In vitro* experiments suggest that increased expression of Hsp27 in reactive astrocytes during gliosis is most likely a reaction to neuronal degeneration and not a function of gliosis (Brzyska et al., 1998). Our data in the retina and in the SC support the hypothesis that Hsp27 expression is not induced by gliosis and that GFAP and Hsp27 expression are independent events.

It is interesting that in the retina, where RGCs die by apoptosis following optic nerve transection, no Hsp27 is detected in Müller cells, the principal glial cells of the retina. However, GFAP expression is increased in these cells by 14 days following axotomy and persists to 28 days (Leon et al., 2000). In contrast, in areas of degenerating

axons and terminals, as in the optic tract and optic layer of the SC, Hsp27 was detected in astrocytes as early as 4 days following axotomy. These data further suggest the independent regulation of Hsp27 and GFAP expression. It is also suggested that the elevated expression of Hsp27 in RGC neurons is regulated by direct injury to the cell caused by axotomy, and the expression of Hsp27 in astroglia of the optic tract and superior colliculus is regulated by indirect injury related to the disruption of the RGC axons and their subsequent degeneration.

***Protein induction and Hsp27 function following optic nerve transection***

Transection of the optic nerve has been shown to cause many changes in RGC protein expression that relate to delayed cell death and apoptosis, including increased expression of c-JUN (Robinson, 1994, 1995; Hüll and Bähr, 1994; Kreutz et al., 1999), the pro-apoptotic protein Bax (Isenmann et al., 1999), and increased activation of the apoptotic mediator caspase-3 (Kermer et al., 1999). The present study demonstrates the cytoplasmic expression of Hsp27 in RGCs and in astrocytes of the optic tract and superior colliculus following optic nerve transection. Hsp27 has been shown to act as a molecular chaperone, preventing non-specific protein aggregation (Jakob et al., 1993; Merck et al., 1993) and Hsp27 regulates actin filament organization and stabilization during oxidative stress (Lavoie et al., 1993a, b, 1995; Huot et al., 1996, 1997). Cytoplasmic localization of Hsp27 is consistent with both of these functions.

In the retina, Hsp27 antibodies induce apoptosis in RGCs of isolated human retina (Tezel and Wax, 2000) indicating that Hsp27 is an important anti-apoptotic factor that may influence RGC survival and Hsp27 has been shown to protect cells from Fas/APO-1

or staurosporine induced apoptosis (Mehlen et al., 1996b; Samali and Cotter, 1996). Furthermore, Hsp27 inhibits cytochrome c-dependent activation of two important pro-apoptotic proteins, procaspase-9 (Garrido et al., 1999) and procaspase-3 (Pandey et al., 2000). In the retina, caspase inhibitors suppress apoptosis and increase survival of RGCs following optic nerve transection (Chaudhary et al., 1999). Hsp27 may play a direct role in the blockade of caspase activation in a subset of RGCs following optic nerve transection.

In addition, Kikuchi et al. (2000) report that the stress-activated kinase, p38, is detected in RGCs in its activated form following optic nerve transection. p38 is an upstream regulator of Hsp27 phosphorylation (Martin et al., 1999; Hedges et al., 1999). In its phosphorylated form, Hsp27 has been reported to protect against oxidative stress and have anti-apoptotic properties (Mehlen et al., 1996 a,b; Rogalla et al., 1999). Other investigators have indicated that Hsp27 maintains its protective function when phosphorylation is blocked (Préville et al., 1998; Martin et al., 1999; Hedges et al., 1999). The role of phosphorylation on Hsp27 function in the long-term survival of the subset of Hsp27-positive RGCs remains to be determined.

## **Conclusions**

Transection of the optic nerve is an excellent CNS model for the study of the induction and expression of Hsps in neurons and astrocytes of the rat visual system. There is a significant increase in the percentage of surviving FG-labeled RGCs that express Hsp27 by 28 days following optic nerve transection, indicating that Hsp27 may promote the survival of this subset of RGCs following injury. Hsp27 expression is

induced in the SC but is limited to astrocytes layers receiving direct retinal projections. In contrast, increased GFAP immunoreactivity, associated with glial activation, is detected in astrocytes throughout all visual layers of the SC. Our data support the view that Hsp27 expression is not induced by gliosis and that GFAP and Hsp27 expression are independent events. The detection of Hsp27 in a subset of axotomized RGCs adds to our understanding of the molecular responses of CNS neurons to injury. A more complete understanding of how neurons and astrocytes respond to injury will ultimately lead to the development of novel therapeutic strategies to enhance neuronal survival and recovery from various forms of brain injury.



**CHAPTER 4:**

**ADMINISTRATION OF BDNF SUPPRESSES THE EXPRESSION OF HSP27 IN  
RAT RETINAL GANGLION CELLS FOLLOWING AXOTOMY**

**This chapter was submitted to Neuroscience and has been accepted for publication.**

## **Introduction**

Hsps are an evolutionarily conserved group of proteins that are inducible by a wide variety of physical, pathophysiological and environmental stressors (reviewed in Morimoto et al., 1992; Morimoto, 1993). Hsps are grouped by molecular weight and amino acid sequence similarity into five main families (reviewed in Welch, 1992). Hsp27, a member of the sHsp family, plays a role in cellular repair and protective mechanisms against cell stress and apoptosis (Jakob et al., 1993; Merck et al., 1993). For example, Hsp27 over-expression in NIH/3T3 cells confers thermoresistance (Landry et al., 1989) and increases resistance to oxidative stress and cytotoxicity induced by TNF $\alpha$  treatment (Mehlen et al., 1995a,b). Over-expression of Hsp27 in cultured dorsal root ganglion neurons protects neurons from apoptosis after withdrawal of nerve growth factor. In addition, an established neuronal cell line over-expressing Hsp27 was protected from retinoic acid-induced apoptosis (Wagstaff et al., 1999). *In vivo*, Lewis et al. (1999) have demonstrated that, following transection of the sciatic nerve in post-natal day 0 rats, all surviving axotomized neurons were Hsp27-positive.

Hsp27 is highly inducible in cortical astrocytes after seizure activity (Plumier et al., 1996), ischemic injury (Plumier et al., 1997a) and cortical spreading depression (Plumier et al., 1997b). Hsp27 is also normally present in many neurons of the brainstem and spinal cord of rat and mouse (Plumier et al., 1997c; Armstrong et al., 2001b). Hsp27 is expressed at very high levels for extended periods of time in vagal sensory and motor neurons after vagal transection (Hopkins et al., 1998). These results suggest that Hsp27 plays a critical role in normal neuronal metabolism and in neuronal survival.

In the retina, antibodies to this sHsp were identified in patients with normal-pressure and high pressure glaucoma (Tezel et al., 1998). When applied to retinal tissue, Hsp27 antibodies triggered apoptosis, indicating that Hsp27 may inhibit apoptosis when not blocked by antibody binding (Tezel and Wax, 2000). More recently, Yokoyama et al. (2001) reported that electroporation of exogenous Hsp27 protein into RGCs increased resistance to apoptosis following ischemia-reperfusion injury. Hsp27 is not present at detectable levels in control, non-injured rat RGCs (Chapter 3). However, following optic nerve transection, Hsp27 is detected in a subset of RGCs from 4 to 28 days (Chapter 3, Figure 3.6). It was proposed in Chapter 3, that Hsp27 expression may contribute to the long-term survival of this subset of RGCs.

Axotomy of the optic nerve causes apoptosis in RGCs that begins at 4 days and results in the loss of most RGCs by 14 days following injury (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994; Peinado-Ramón et al., 1996). The loss of trophic support is thought to be an important factor contributing to apoptotic RGC death following optic nerve transection (reviewed in Sofroniew, 1999). Neurotrophins play a significant role in the survival of injured RGCs. BDNF is a member of the neurotrophin family and interacts with the cell surface transmembrane receptor TrkB (tyrosine receptor kinase B) to activate intracellular signaling pathways leading to the transcription of regenerative associated genes and the inhibition of apoptosis (reviewed in Heumann, 1994; Segal and Greenberg, 1996; Klöcker et al., 2000). TrkB is present in the ganglion cell layer on both RGCs and amacrine cells (Jelsma et al., 1993; Vecino et al., 2002) and *in vitro* treatment with BDNF or neurotrophin 4/5 (NT-4/5) enhances RGC survival (Cohen et al., 1994) *In vivo* studies have shown that activation of the TrkB receptor by administration of a single

dose of BDNF or NT-4/5 at the time of optic nerve transection increases the number of RGCs surviving at two weeks (Mansour-Robaey et al., 1994; Peinado-Ramón et al., 1996; Sawai et al., 1996). However, prolonged administration of neurotrophin does not appear to enhance the survival of RGCs in the long-term (Clarke et al., 1998).

The purpose of this study is to determine whether the administration of BDNF, a neurotrophin known to enhance the survival of axotomized RGCs, has an effect on the RGC expression of Hsp27, an inducible protein implicated in sensory neuron survival.

## **Materials and Methods**

### ***Animals***

Young adult female Sprague-Dawley rats (175-250 g; Charles River, St. Constant, PQ) were cared for in accordance with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. All surgical procedures were performed under general anaesthetic using a mixture of ketamine (62.5 mg/kg), xylazine (3.25 mg/kg) and acepromazine (0.62 mg/kg) in 0.9% saline, administered by intramuscular injection.

### ***Retinal ganglion cell labeling***

One week prior to experimental manipulations, Fluorogold (2% Fluorogold (FG) in 0.9% saline; Fluorochrome Inc., Denver, CO) soaked Gelfoam (UpJohn, Don Mills, ON) was applied to the surface of both superior colliculi to label the cell bodies of RGCs by retrograde transport (Vidal-Sanz et al., 1988; Villegas-Pérez et al., 1993).

### ***Optic nerve transection***

Following the methods of Mansour-Robaey et al. (1994) and Clarke et al. (1998), the left optic nerve was transected with microscissors at approximately 0.5mm from the back of the eye. Preservation of the blood supply was confirmed by microscopic examination of the retina through the pupil. Animals recovered from 0 (normal right eye) 4, 7, 10, 14 and 28 days following transection and intravitreal administration of either BDNF or vehicle (see below; n = 4 for each group).

### ***Administration of BDNF***

To determine the effect of BDNF administration on the expression of Hsp27, BDNF (5  $\mu$ l; 1  $\mu$ g/ $\mu$ l in Tris-phosphate buffer; Regeneron, Terrytown, NY) or vehicle (5  $\mu$ l Tris-phosphate buffer) was injected slowly into the vitreous chamber of the left eye by the posterior approach through the sclera and the retina, at the time of optic nerve transection (Clarke et al., 1998). Animals in these two treatment groups recovered from 0 (normal right eye), 4, 7, 10, 14 and 28 days (BDNF = 4, vehicle = 4 for each group) following optic nerve transection. BDNF and vehicle-injected non-transected controls were also included in this study. These control animals were exposed to the same surgical procedures but the optic nerve was not cut. Control animals recovered for 4 days and 10 days following surgery (n = 4 for each group).

### ***Tissue processing***

At the end of the experimental period, rats were deeply anaesthetized with a mixture of ketamine (62.5 mg/kg), xylazine (3.25 mg/kg) and acepromazine (0.62 mg/kg) in 0.9% saline, administered by intramuscular injection. Animals were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. The left (experimental) and right (normal control) eyes were removed and post-fixed for 24 hours in 4% paraformaldehyde then gelatin embedded and cut on a freezing microtome at 30 $\mu$ m.

### ***Immunohistochemistry***

***Hsp27***: Five non-adjacent radial sections of retina through the optic disk were used for Hsp27 immunohistochemical analysis following the methods stated in Chapter 3. Sections were treated with phosphate buffered saline, 0.1% Triton-X (PBS-X), 10% goat serum, and incubated overnight at 4°C in PBS-X with 2% goat serum and Hsp25 rabbit polyclonal antibody (1:1000; StressGen Biotechnologies Corp., Victoria, BC). After three-ten minute rinses, sections were incubated for three hours in PBS (no Triton-X) containing Alexa goat anti-rabbit 546 (1:500; Molecular Probes, Eugene, OR). The sections were rinsed with PBS then floated onto gelatinized slides, air-dried overnight and coverslipped with Fluoromount (BDH Chemicals, Poole, England). FG and Hsp27-immunopositive cell profiles were counted in each experimental case. Retinal ganglion cell type was confirmed by punctate FG-labeling, morphology and location within the ganglion cell layer.

***Glial fibrillary acidic protein (GFAP)***: To confirm Müller cell type several non-adjacent radial sections through the optic nerve were used from the various treatment groups and prepared as above for immunohistochemistry. Sections were incubated overnight at 4°C with 2% goat serum and the monoclonal GFAP antibody (1:200; Nova Castra, Nova Scotia, Canada) in PBS-X. After three ten minute rinses, sections were incubated for three hours in PBS containing Alexa goat anti-mouse 488 (1:500; Molecular Probes) and processed as above.

***Microscopy and image processing***

Sections were examined using fluorescence and UV microscopy on a Zeiss Axioplan microscope. Immunofluorescence stained sections were scanned using a Diagnostic Instruments Inc. Spot II digital camera mounted on a Zeiss Axioplan 2 microscope, and images were captured digitally using Adobe Photoshop 5.0 software. Prints and photographic plates were made using a Kodak 8650 printer with continuous tone dye sublimation. Other than brightness and contrast, images were not adjusted.

***Statistical analysis***

Total FG and Hsp27-positive RGCs were counted for each animal from a total of 5 sections through the optic disk per animal and the data are represented as means  $\pm$  standard error of the mean for each group (n = 4 animals per group). The non-parametric Mann Whitney U test was used to compare means between the various groups, with significance set at  $p < 0.05$ .



## **Results**

### ***Fluorogold labeling***

To compare the extent of RGC loss following optic nerve transection and concomitant administration of either BDNF or vehicle, FG-positive RGC profiles were examined in each group (Figure 4.1).

The total number of FG-positive RGCs was counted in 5 non-adjacent radial sections through the optic disk and the mean total was calculated for each group ( $n = 4$ ; Figure 4.2). At 4 days following optic nerve transection, there was no significant difference in the number of FG-positive RGCs between BDNF ( $4767 \pm 309$ ; mean  $\pm$  standard deviation) and vehicle ( $5183 \pm 289$ ) treated groups and normal right eye controls (day 0;  $4980 \pm 285$ ;  $4594 \pm 330$ ). With BDNF treatment, the number of RGCs decreased slightly, but not significantly, at 7 days ( $4186 \pm 183$ ); however, with vehicle treatment, RGC cell profiles declined significantly ( $3003 \pm 283$ ;  $p < 0.05$ ). By 10 and 14 days following optic nerve transection and BDNF treatment, RGC profile counts declined to  $3517 \pm 221$  and  $2788 \pm 417$ , respectively, compared with  $1016 \pm 69$  and  $846 \pm 69$  following vehicle treatment. By 28 days, the number of FG-positive RGCs was reduced to  $281 \pm 19$  with BDNF treatment and  $84 \pm 6$  with vehicle treatment. At 7, 10, 14 and 28 days following optic nerve transection, there was a significantly greater number ( $p < 0.05$ ) of FG-positive RGC profiles in BDNF-treated groups than vehicle-treated groups and these differences were similar to other published results (Mansour-Robaey et al., 1994). By 14 days following optic nerve transection in the vehicle-treated group, the number of surviving RGCs was reduced to 15% of right eye controls, whereas in the BDNF-treated group, approximately 50% of RGCs remained. By 28 days following

axotomy, the number of surviving RGCs was reduced significantly in both treatment groups with 5% of RGCs surviving following BDNF treatment and approximately 2% of RGCs surviving after vehicle treatment.

### ***Immunohistochemical analysis***

***Hsp27 in RGCs:*** Hsp27 immunoreactivity was not detected in RGCs of uninjured right retinas, confirming our previous report in Chapter 3. Following optic nerve transection, Hsp27 was detected in RGCs (Figure 4.3) and there was a significant increase ( $p < 0.05$ ) in the number of Hsp27-positive RGC profiles detected in the ganglion cell layer at 4, 7, 10 and 14 days in both the BDNF and vehicle-treated groups as compared to normal retina (day 0; Figure 4.4). The greatest number of Hsp27-positive RGC profiles was detected at 4 ( $51 \pm 9$ ,  $90 \pm 7$ ; BDNF and vehicle, respectively) and 7 ( $37 \pm 6$ ,  $95 \pm 14$ ; BDNF, vehicle respectively) days with numbers declining to 28 days ( $10 \pm 2$ ,  $9 \pm 2$ ; BDNF, vehicle respectively) in both the BDNF and vehicle-treated groups. The number of Hsp27-positive RGCs in the BDNF treated groups was significantly less ( $p < 0.05$ ) than in the vehicle-treated groups at 4, 7, 10 and 14 days following optic nerve transection. By 28 days following axotomy, there was no significant difference between BDNF ( $10 \pm 2$ ) and vehicle ( $9 \pm 2$ ) treated groups.

The mean number of Hsp27-positive RGCs was determined as a percentage of FG-positive RGCs (Figure 4.5). Following optic nerve transection and vehicle injection, the percentage of Hsp27-positive RGCs was significantly increased from 1.8% at 4 days to 10.5% at 28 days ( $p < 0.05$ ). With enhanced RGC survival following BDNF

treatment, the percentage of Hsp27-positive RGCs was significantly lower ( $p < 0.05$ ) than in the vehicle-treated groups, ranging from 1.1% at 4 days to 3.6% at 28 days.

Hsp27 was also detected in a small number of RGC profiles in BDNF and vehicle-injected controls at 4 ( $2 \pm 1$  and  $8 \pm 1$  respectively) and 10 days ( $3 \pm 1$  and  $7 \pm 3$ ) (Figure 4.6). The vehicle-injected control retinas had significantly greater numbers of Hsp27-positive RGC profiles ( $p < 0.05$ ) than the BDNF-injected controls at 4 days.

*Hsp27 in Müller cells:* Hsp27 immunofluorescence was detected in some GFAP-positive Müller cells in non-transected BDNF and vehicle-injected control retinas, and in transected BDNF and vehicle-treated groups (Figure 4.7). This glial expression varied in location along the radial sections but was apparent in both BDNF and vehicle-injected retinas with and without optic nerve transection (Figure 4.6). No Hsp27-positive Müller cells were detected in normal (right eye) retina.

**Figure 4.1:** Radial section of rat retina with retinal ganglion cells (RGCs) retrogradely labeled from the superior colliculus (SC) with Fluorogold (FG). **A:** FG-labeled RGCs at 10 days following optic nerve transection with concomitant vehicle injection. **B:** FG-labeled RGCs at 10 days following optic nerve transection with concomitant BDNF injection. Enhanced survival of RGCs is seen with BDNF treatment at the time of optic nerve transection. Asterisk indicates microglia in the RGC layer of injured retina. Scale bar, 50 $\mu$ m.

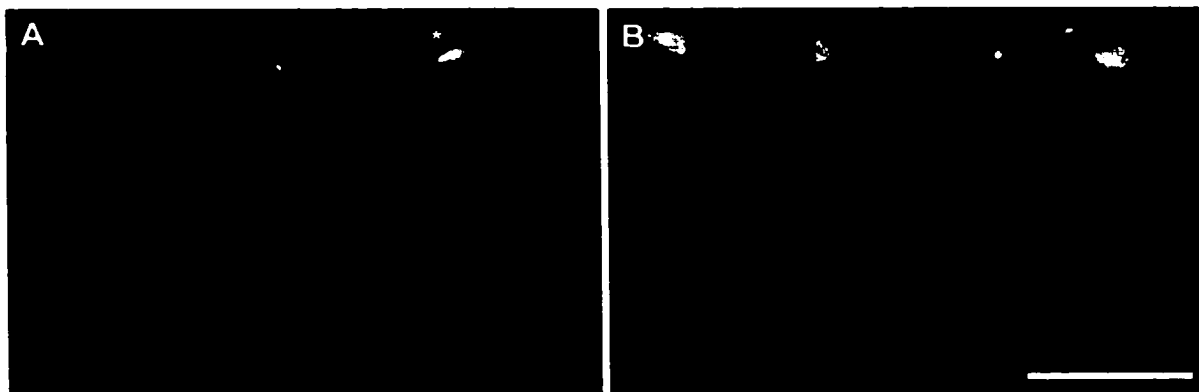


Figure 4.1

**Figure 4.2:** Number of Fluorogold (FG)-positive retinal ganglion cell (RGC) profiles following optic nerve transection and concomitant vehicle or BDNF administration (total counts from 5 non-adjacent radial sections through the optic nerve per rat retina reported as a mean of  $n = 4$  animals for each group). There is no significant difference in the number of RGC profiles counted in retinas at 0 (right eye controls) and 4 days following optic nerve transection with either vehicle or BDNF treatment. From 7 to 14 days following optic nerve transection, the number of FG-positive cell profiles is significantly decreased ( $p < 0.05$ ) in vehicle-treated retinas when compared to BDNF treated retinas.

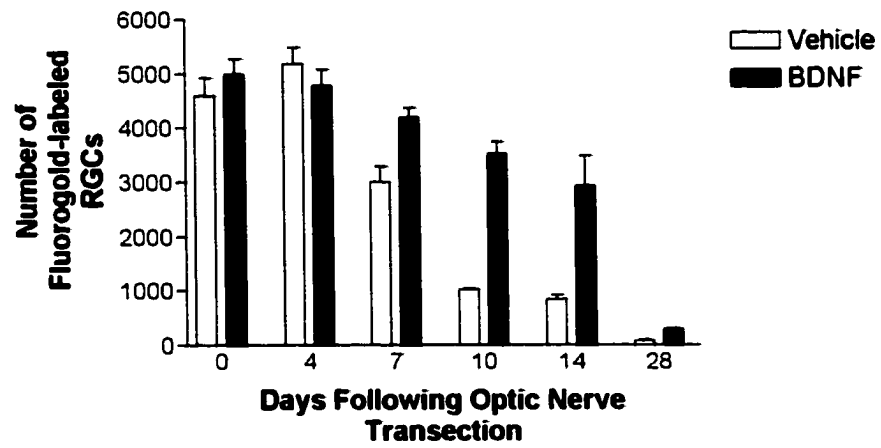


Figure 4.2

**Figure 4.3:** Radial retinal section showing immunohistochemical localization of Hsp27 in rat retinal ganglion cells (RGCs) at 7 days following optic nerve transection and concomitant vehicle treatment. **A:** Retrograde Fluorogold (FG) labeling from the superior colliculus shows RGCs in the ganglion cell layer of the retina. Arrows indicate FG-labeled RGCs that are Hsp27-positive. **B:** Hsp27 immunofluorescence in Fluorogold-positive RGCs. Scale bar, 50 $\mu$ m.



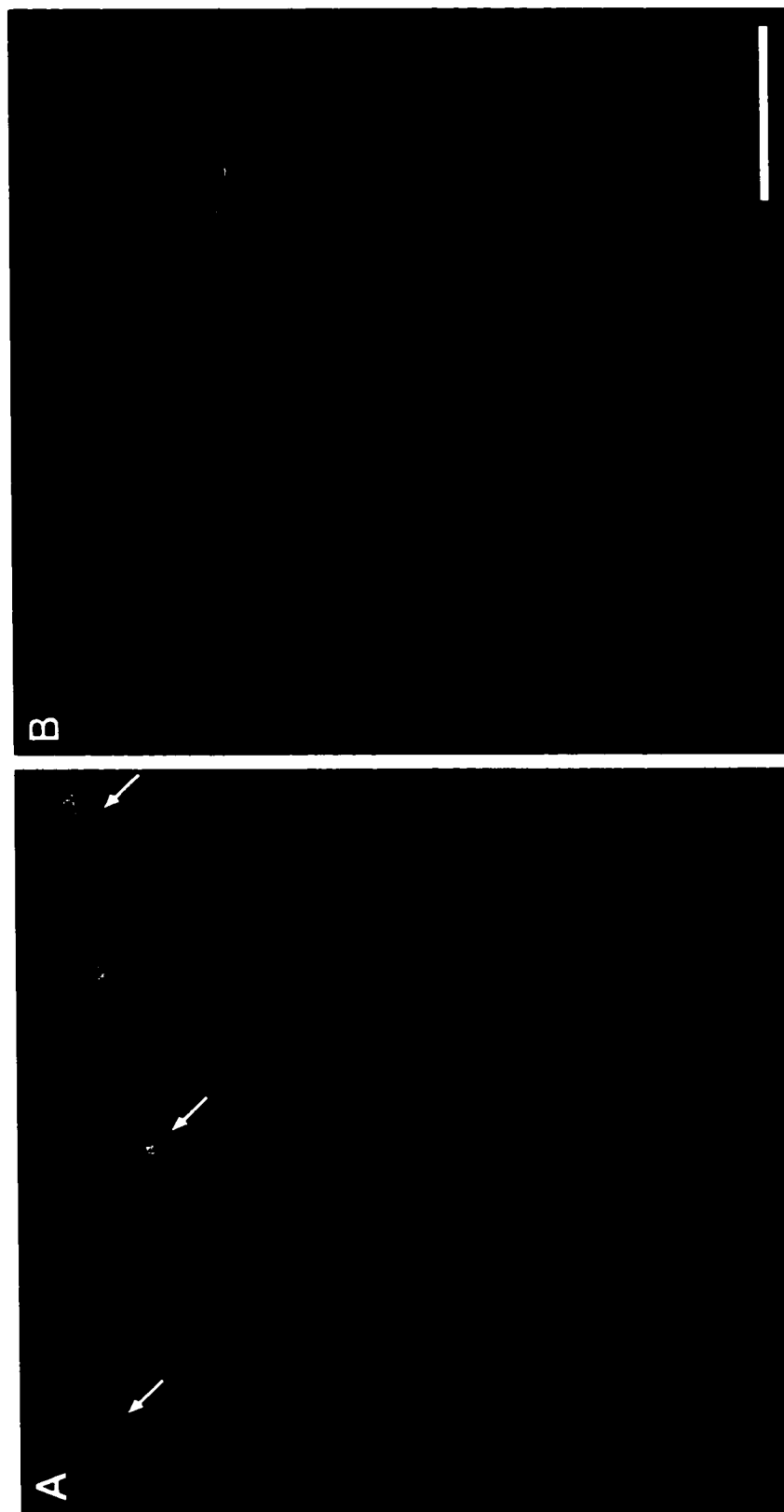


Figure 4.3

**Figure 4.4:** Number of Hsp27-positive retinal ganglion cell (RGC) profiles counted at the various days following optic nerve transection with either vehicle or BDNF treatment (5 non-adjacent radial sections through the optic disk per rat retina, n = 4 per group). No expression of Hsp27 was detected in the uninjured normal retina (day 0). The number of Hsp27-positive RGC profiles is significantly reduced ( $p < 0.05$ ) in the BDNF-treated groups at 4, 7, 10 and 14 days following optic nerve transection compared to transected vehicle-treated groups.

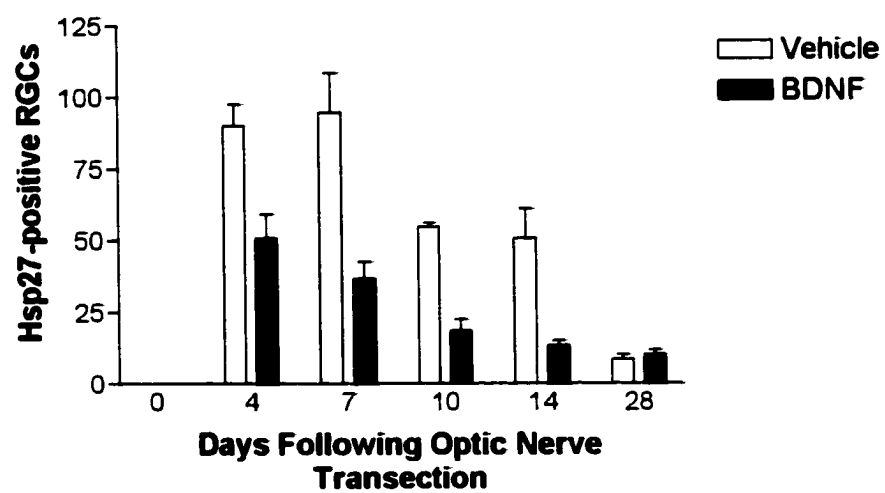


Figure 4.4

**Figure 4.5:** Percentage of Fluorogold-positive retinal ganglion cell (RGC) profiles expressing Hsp27 from 4 – 28 days following optic nerve transection and concomitant vehicle or BDNF administration. In the BDNF-treated groups, the percentage of Hsp27-positive RGCs was significantly lower ( $p < 0.05$ ) than in the vehicle-treated groups. However, the percentage of Hsp27-positive RGC profiles significantly increased by 28 days with vehicle or BDNF treatment.

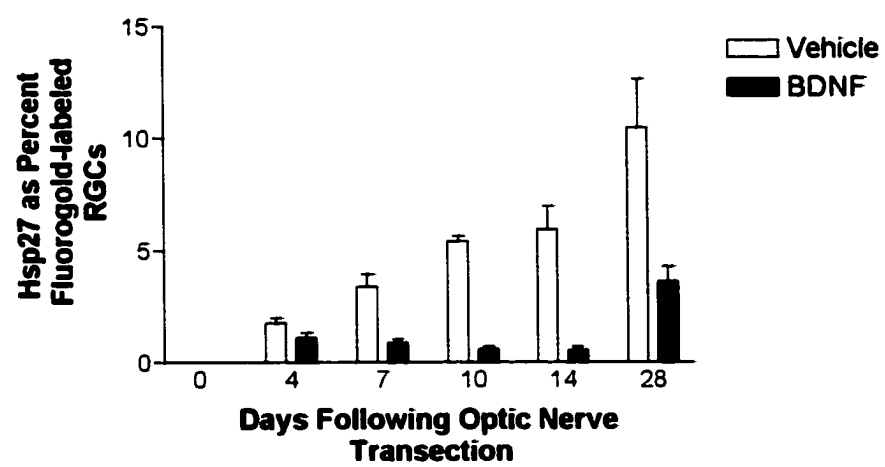


Figure 4.5

**Figure 4.6:** Number of Hsp27-positive retinal ganglion cell (RGC) profiles counted at 4 and 10 days following intraocular administration of vehicle or BDNF (no optic nerve transection). Hsp27 is detected in a small number of RGCs following intraocular injection of either vehicle or BDNF. In these retinas, where the optic nerve has not been injured, the number of Hsp27-positive RGC profiles is decreased in the BDNF-treated groups compared to vehicle-injected controls.

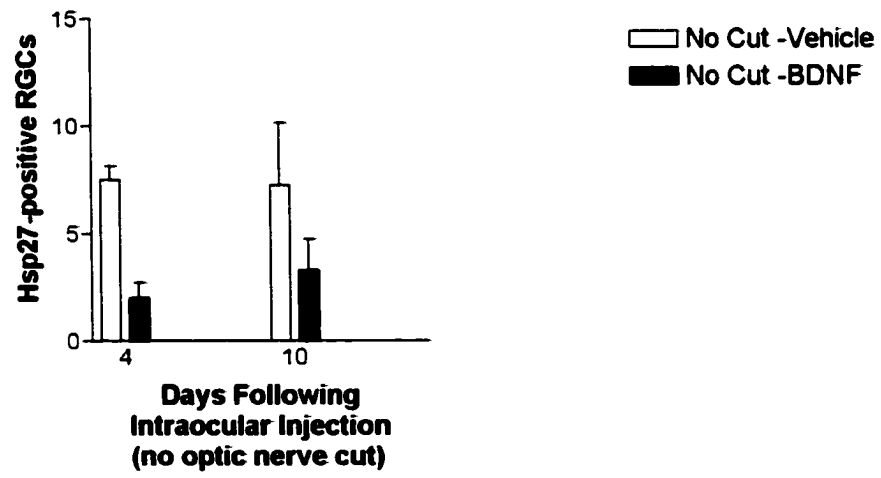


Figure 4.6

**Figure 4.7:** Immunohistochemical localization of Hsp27 in glial fibrillary acidic protein (GFAP)-positive Müller cells in a BDNF-injected retina, 4 days following optic nerve transection. **A:** Hsp27 immunofluorescent labeling of Müller cell processes (red). **B:** Fluorogold-labeled RGCs in the ganglion cell layer. **C:** GFAP-positive Müller cell processes extending through all layers of the retina. **D:** Double-labeled GFAP and Hsp27-positive Müller cells (orange) in the injured retina. The detection of Hsp27 in Müller cells correlates with the retinal response to damage induced by the intravitreal injection of either BDNF or vehicle. Arrows indicate double-labeled Müller cells. Scale bar, 50  $\mu\text{m}$ .



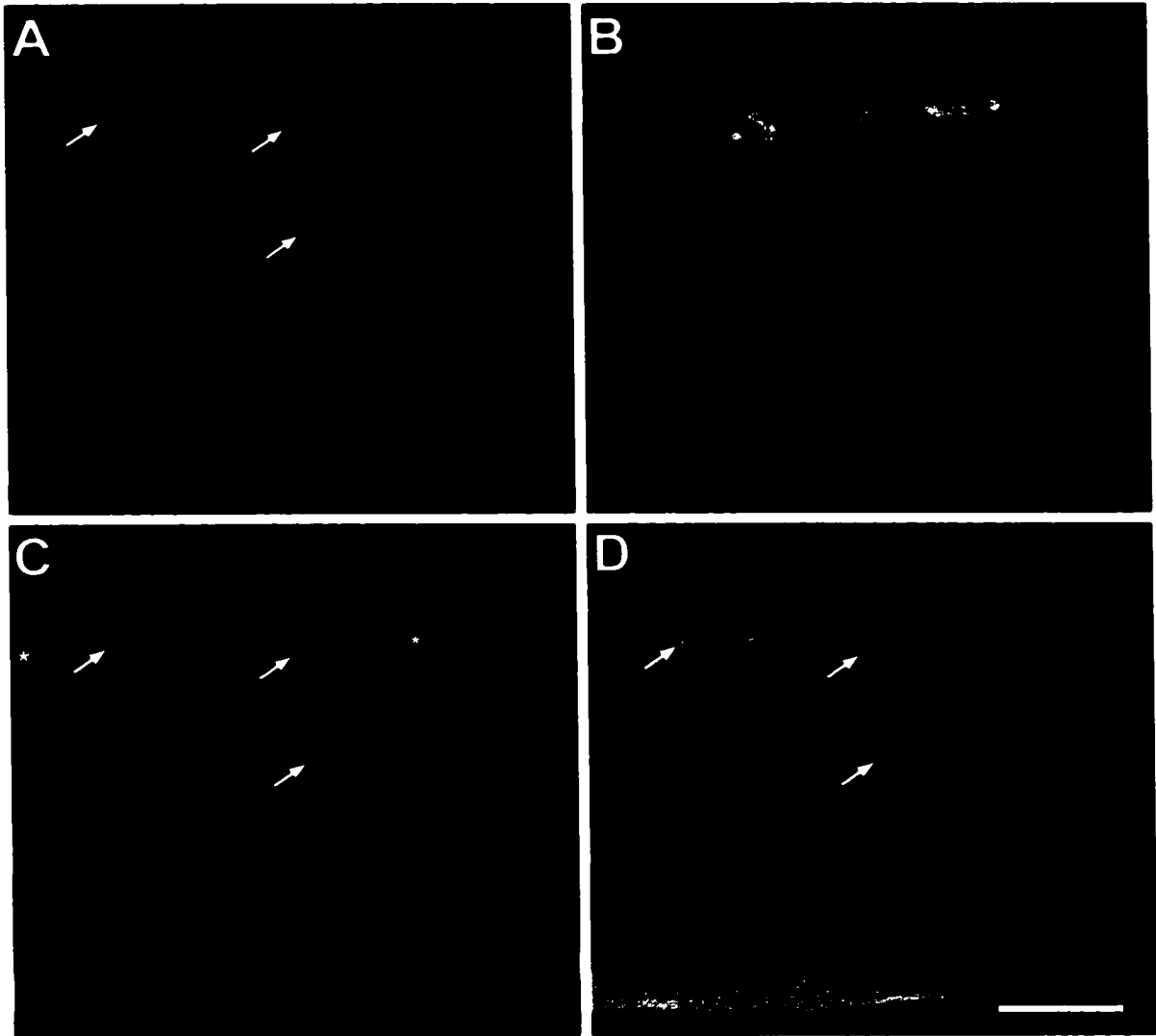


Figure 4.7

## **Discussion**

**This work examined the relationship between the effect of administration of BDNF and the injury-induced expression of Hsp27 in RGCs following axotomy. BDNF and Hsp27 have been shown individually to be protective in neuronal cells following injury (Mansour-Robaey et al., 1994; Yokoyama et al., 2001). Here, a single intravitreal injection of BDNF, administered at the time of optic nerve transection, enhances the survival of RGCs to 14 days following injury compared with vehicle-injection. These results are similar to other published reports (Mansour-Robaey et al., 1994; Peinado-Ramón et al., 1996; Sawai et al., 1996). Hsp27-immunofluorescent staining is detected in a subset of RGCs following axotomy in both BDNF and vehicle-treated retinas. However, with BDNF treatment, there are significantly fewer Hsp27-positive RGC profiles compared to vehicle-treated retinas from 4 to 14 days following axotomy. By 28 days, there is no significant difference in the number of Hsp27-positive RGCs between BDNF and vehicle-treated retinas. After optic nerve transection and vehicle treatment, retinas show a general increase in the percentage of Hsp27-positive RGCs over time indicating that the Hsp27-positive cell population dies at a slower rate than the general FG population. These results suggest that BDNF suppresses the expression of Hsp27 in axotomized RGCs to 14 days following injury. Past 14 days, the small population of Hsp27-positive cells is decreasing at a slower rate compared to the general FG population with both BDNF and vehicle treatment.**

***BDNF: short-term protection versus long-term survival of neurons***

BDNF treatment of injured RGCs enhances their survival but leads to a reduction in the number of Hsp27-positive RGCs compared to vehicle-treated retinas. This result suggests that the short term protection afforded to RGCs by a single administration of BDNF is not due to the stress response induction of Hsp27 in the surviving cells. In fact, the number of Hsp27-positive RGC profiles in both the BDNF-injected axotomized (Figure 4.4) and non-axotomized retinas is less (Figure 4.6) compared to vehicle injection indicating a general suppression of Hsp27 expression by BDNF.

Prolonged intravitreal administration of neurotrophin following optic nerve transection, either by repeated intravitreal injections (Mansour-Robaey et al., 1994) or by release from an osmotic mini-pump (Clarke et al., 1998) fails to maintain long-term survival of RGCs. It was proposed that long-term administration of various neurotrophins may actually down-regulate receptor function, consistent with the observation BDNF down-regulates its own receptor (TrkB) following ligand binding (Frank et al., 1996; Sommerfeld et al., 2000). The reduction in the number of Hsp27-positive RGCs following BDNF treatment between 4 and 14 days indicates that the use of an exogenously applied protective molecule such as BDNF not only down-regulates its own signaling response but suppresses alternate, endogenous cell survival pathways which are important for the long-term survival of these neurons. It is interesting that as the efficacy of BDNF wanes, there is an increase in the percentage of RGCs that are Hsp27-positive (see Figure 4.5).

***Hsp27 and neuroprotection***

By 28 days following axotomy, in both BDNF and vehicle-treated groups, there is a significant increase in the percentage of Hsp27-positive RGCs. The increased percentage of Hsp27-positive RGCs suggests that Hsp27 plays a role in promoting long-term sensory neuron survival. It was proposed in Chapter 3 that the expression of Hsp27 in a subset of RGCs may help to sustain these cells in the long-term. Indeed, a small population of RGCs survives in the retina up to 3 months following transection (Villegés-Pérez et al., 1993).

Several studies have reported a correlation between neuron survival and Hsp27 expression. In the adult rat, Hsp27 is constitutively expressed in dorsal root ganglion (DRG) cells (Plumier et al., 1997c; Costigan et al., 1998). Following peripheral nerve axotomy in the adult rat, Hsp27 expression is further elevated in these cells and most neurons survive for months following the injury (Hopkins et al., 1998; Costigan et al., 1998; Lewis et al., 1999). However, at postnatal day 0, little or no Hsp27 is detected in DRG neurons and few of these cells survive axotomy. It is interesting to note, however, that of the few DRG cells that survive axotomy at postnatal day 0, all are Hsp27-positive (Lewis et al., 1999). In addition, cultured neurons that express Hsp27 or over-express human Hsp27 preferentially survive nerve growth factor withdrawal (Lewis et al., 1999). Our study suggests a correlation between Hsp27 expression and the long-term survival of a small population of RGCs.

***Cell signaling and the inhibition of apoptosis: Hsp27 and BDNF***

In addition to microfilament binding (Lavoie., 1993a, b, 1995; Hout et al., 1996, 1997) and preventing protein aggregation (Jakob et al., 1993; Merck et al., 1993), Hsp27 interferes with receptor/ligand-mediated and mitochondrial-mediated programmed cell death. Hsp27 over-expression inhibits apoptosis induced by Fas/APO-1 receptor activation (Mehlen et al., 1996; Samali and Cotter, 1996) and Daxx-Ask1-dependent Fas-induced cell death (Charette et al., 2000). Recent studies suggest that Hsp27 interferes with the mitochondrial pathway of caspase-dependent cell death including the blockade of cytochrome-C-mediated activation of caspase-3 and -9 (Garrido et al., 1999; Bruey et al., 2000; Pandey et al., 2000) and negatively regulating cytochrome C release from the mitochondria (Paul et al., 2002). In the retina, inhibition of caspase-3 and caspase-9 activation has significantly improved the survival of RGCs following optic nerve transection (Chaudhary et al., 1999; Garrido et al., 1999; Kermer et al., 2000; Pandey et al., 2000). Activation of caspase-3 is reported to be the crucial step leading to neuronal apoptotic death (Nicholson and Thornberry, 1997) and caspase-9 functions as the upstream activator of caspase-3 (Kermer et al., 2000). The specificity with which Hsp27 inhibits these caspases via negative regulation of cytochrome C may indicate an important therapeutic role for this protein in preventing apoptosis of RGCs following optic nerve transection. Recently, the introduction of Hsp27 protein into RGCs by electroporation has been shown to rescue RGCs from apoptosis associated with ischemia-reperfusion injury (Yokoyama et al., 2001). In the glaucomatous retina, antibodies to Hsp27 induce RGC apoptosis (Tezel and Wax, 2000). Induction of endogenous Hsp27 as

well as the introduction of exogenous protein prior to injury may prove beneficial for RGC survival following injury.

BDNF appears to activate the Ras (GTP binding protein)/ mitogen-activated protein kinase (MAPK) pathway (reviewed in Heumann, 1994; Klöcker et al., 2000). As with Hsp27, BDNF inhibits caspase-3 activity (Klöcker et al., 2000).

In this study, the administration of BDNF rescues RGCs in the short-term, but at the same time, suppresses the stress-induced expression of Hsp27. It is possible that BDNF does not directly interfere with the expression of Hsp27 but that its presence signals an “all is well”. The expression of Hsp27 in some cells may indicate the incomplete diffusion or insufficient exposure of all RGCs to the neuroprotectant, BDNF.

#### ***Cell type-specific and stress-dependent expression of Hsp27***

Optic nerve transection induces the expression of Hsp27 in a limited population of RGCs (Figure 4.4; Chapter 3, Figure 3.5). The detection of Hsp27 in a few RGCs in our injection-only control retinas (see Figure 4.6) suggests that the injection procedure also causes the expression of Hsp27 in a limited number of RGCs.

Hsp27 was detected in some Müller cells (the primary glial cells of the retina) in both optic nerve transected BDNF or vehicle-injected groups and in the non-transected injection controls. The expression of Hsp27 in these Müller cells was not detected in the retina following optic nerve transection only (Chapter 3). Glial expression of Hsp27 appears to be specifically related to the injection itself and may be a retinal response to local damage caused by the posterior route of injection that takes the needle through the

layers of the retina and into the vitreous chamber. This route of injection is thought to minimize the release of endogenous retinal trophic response (Mansour-Robaey et al., 1994).

Our results indicate that various types of damage cause cell-type specific expression of Hsp27. Axotomy induces Hsp27 expression only in RGCs, while direct mechanical damage or perturbation of the retina by injection of BDNF or vehicle causes the expression of Hsp27 in some Müller cells and in a small number of RGCs.

The stress-dependent expression of Hsp27 in different cell types has been widely reported. In the visual system, optic nerve transection causes only some RGCs to become Hsp27-positive whereas widespread expression of Hsp27 is detected in astrocytes in the retinorecipient layers of the superior colliculus (Chapter 3, Figure 3.8). In the PNS, sciatic nerve injury and vagotomy have resulted in the induction and prolonged expression of Hsp27 in the affected neurons (Hopkins et al., 1998; Costigan et al. 1998, Lewis et al., 1999). Hyperthermia causes the induction of Hsp27 primarily in astroglia throughout the CNS; in addition, hyperthermia results in Hsp27 expression in specific nuclei associated with fluid homeostasis and thermoregulation (Chapter 2). With excitotoxic injury such as kainic acid-induced status epilepticus (Plumier et al., 1996), and potassium chloride-induced cortical spreading depression (Plumier et al., 1997b), Hsp27 is detected primarily in astrocytes. Our data provide further evidence for the cell-type specific, stress-dependent expression of Hsp27 in the CNS.

## **Conclusions**

**This work provides the first evidence that BDNF suppresses the expression of Hsp27 following injury. These results indicate that neurotrophin treatment may interfere with various cell survival pathways, including the stress-induced expression of Hsp27, and may help to explain the inability of prolonged neurotrophin administration to enhance the long-term survival of injured RGCs. Our study also shows that, by 28 days, the percentage of Hsp27-positive RGCs increases relative to the FG-labeled RGC population studied, suggesting that Hsp27 may be important for some of the few injured sensory neurons that survive long-term. Finally, it is apparent that different injuries cause the induction of Hsp27 in specific cell types, further supporting the notion of the cell-type specific and stress-dependent nature of Hsp27 expression in the CNS.**



**CHAPTER 5:**

**GENERAL DISCUSSION**

## **Summary**

There are three major findings of this thesis work: Hsp27 is transiently induced in the rat CNS in a cell type-specific and stress-dependent manner; RGCs that express Hsp27 appear to die at a slower rate following nerve injury; and neurotrophin treatment at the time of optic nerve transection suppresses RGC Hsp27 expression.

Following hyperthermic treatment in the rat (Chapter 2), Hsp27 is detected primarily in astrocytes throughout the forebrain and spinal cord, and in Bergmann glia and astrocytes of the cerebellum. Hsp27 is also detected in specific neuronal populations important for temperature regulation and fluid homeostasis (hypothalamus, subfornical organ and area postrema). Damage to the optic nerve (Chapter 3) induces Hsp27 expression in a limited number of RGCs and in astroglia of the optic tract and the retinorecipient layers of the superior colliculus. In the retina, the RGCs that express Hsp27 appear to die at a slower rate than the general RGC population, suggesting that Hsp27 may be important for the long-term survival of a select population of RGCs following injury. In the optic tract and superior colliculus, it is proposed that the astrocytic expression of Hsp27 is related to the onset of Wallerian degeneration of the axons in the optic nerve, and aberrant release of neurotransmitter and nerve terminal degeneration, respectively.

The administration of the neurotrophin BDNF at the time of optic nerve transection (Chapter 4), delays RGC apoptosis and also suppresses RGC expression of Hsp27, indicating that exogenously applied protective molecules may interfere with other endogenous cell survival pathways important for the long-term survival of some neurons following injury.

From these results, there are several important areas of discussion that will now be considered. First, Hsp27 is expressed in a cell-type specific and stress-dependent manner and this expression may have important functional implications for astrocytes and neurons following the different stress paradigms. Second, the importance of cell signaling in the expression, modulation and phosphorylation of Hsp27 in neurons following heat stress, axotomy and BDNF administration will be discussed. Last, the results of Hsp27 expression following CNS injury warrant a comparison with the induction and expression of Hsp27 following PNS injury.

### **Cell Type-Specific and Stress-Dependent Expression of Hsp27**

Until recently, the consensus has been that the heat shock response is a universal phenomenon, such that all cells respond similarly to a particular stressor by the induction of Hsps. The work of this thesis demonstrates that, *in vivo*, Hsp27 is expressed in a cell type-specific and stressor-dependent manner. Thus, the expression of Hsp27 is not just part of a general stress response at the molecular level but may actually have functional significance in the specific cell types following the various stresses.

#### ***Astrocytic expression of Hsp27: astrocytic activation and protection from glutamate neurotoxicity***

Whole body hyperthermia results in the expression of Hsp27 in astrocytes throughout the brain, with limited neuronal expression in some systems related to thermoregulation and osmoregulation. Astrocytic expression of Hsp27 is also detected in

the forebrain following ischemic injury (Plumier et al., 1997a), kainic acid-induced status epilepticus (Plumier et al., 1996; Kato et al., 1999b; Akbar et al., 2001) or potassium chloride-induced cortical spreading depression (Plumier et al., 1997b). Following direct nerve injury, as with axotomy of the optic nerve (Chapter 3), the expression of Hsp27 is detected in astrocytes of the optic tract and superior colliculus and correlates temporally with the degeneration of nerve fibers in the optic tract and terminals in the retinorecipient layers of the superior colliculus. The general response of the retina to local damage caused by a control injection was the induction of Hsp27 in some Müller cells (the astrocytes of the retina, Chapter 4). In contrast, the expression of Hsp27 was not detected in Müller cells following optic nerve transection only (Chapter 3).

These injury-specific responses suggest an important role of Hsp27 expression in the process of astrocyte activation. Reactive or activated astrocytes have been associated with various forms of injury and cellular stress in the CNS including ischemia (Imura et al., 1999), seizure (Murabe et al., 1981; Dusart et al., 1991; Lenz et al., 1997), cortical spreading depression (Kawahara et al. 1999), and axotomy (Guénard et al., 1996; Anguelova and Smirnova, 2000). Characteristically, reactive gliosis includes hypertrophy of cell bodies and nuclei, thickening of processes and the elevated expression of GFAP (Norton et al., 1992). Indeed, Hsp27 expression may be a better marker for astrocyte activation than the commonly used GFAP in that Hsp27 is not constitutively expressed in astrocytes (Plumier et al., 1997c) and that induction of Hsp27 clearly indicates those regions of the brain that are more directly affected by various forms of stress or injury (Lenz et al., 1997). The work presented in this thesis supports this hypothesis. For example, Hsp27 expression was limited to reactive astrocytes in

those layers of the SC that received direct input from the retina (Chapter 3). GFAP expression, on the other hand was detected throughout all layers of the SC following optic nerve transection and this expression remained high even as Hsp27 expression declined by 28 days (Chapter 3, Figure 3.10). Hsp27 expression clearly defined the specific regions of the SC that were directly affected by transection of the optic nerve while GFAP expression appears to define a more general astrocytic response to injury.

The specific role that Hsp27 plays in reactive astrogliosis is currently unknown; however, studies have indicated the importance of Hsp27 in regulating actin dynamics (Miron et al., 1991; Landry and Huot, 1999), intermediate filament interactions (Perng et al., 1999; reviewed in Quinlan, 2002) and cytoskeletal changes (reviewed in Quinlan, 2002). Therefore, the possibility exists that Hsp27 may regulate astrocytic cytoskeletal changes in response to stress or damage.

#### ***Neuronal expression of Hsp27: activation of specific neural systems following heat shock***

With whole body hyperthermia, the induced neuronal expression of Hsp27 is transient and is limited to neurons in neuronal systems involved with the maintenance of body temperature and fluid homeostasis. This expression may indicate that activation of certain neurons within these systems is important for stabilizing temperature and maintaining fluid balance during heat stress. Alternatively, this expression may identify those cells within these homeostatic systems that are more sensitive to hyperthermic stress. Hsp70, another inducible Hsp, has been localized to neural networks associated with the neuroendocrine response to stress including the hypothalamus and the

hippocampus (Blake et al., 1990b; Krueger et al., 1999) following hyperthermic treatment. The expression of the immediate early gene *c-fos* is associated with the activation of various neural circuits (reviewed in Herrera and Robertson, 1996). *c-fos* expression is induced in the rat hypothalamus (paraventricular nucleus, supraoptic nucleus, preoptic nucleus) with heatstroke (Tsay et al., 1999) and in the paraventricular nucleus of the hypothalamus and the subfornical organ following dehydration (Morien et al., 1999). *c-fos* expression in these areas following heat exposure and dehydration indicates activation of these neurons in response to stress. Therefore, the neuronal induction of Hsps following hyperthermic stress occurs in many of the same brain regions that are assumed to be activated to respond to the stress. Double-labeling of cells in these areas for both Fos and Hsp27 following heat shock would indicate the activity of the Hsp27 positive cells and may indicate a protective role for Hsps in these neurons during hyperthermic stress.

Exposure of HeLa cells to hypotonic and hypertonic solutions induces the activation of HSF1 (Huang et al, 1995; Caruccio et al., 1997). This activation does not result in the accumulation of Hsp70, and Hsp27 levels were not studied. It is apparent from the work presented here that Hsp27 may be a more suitable candidate for increased Hsp expression following osmoregulatory stress (Chapter 2). Interestingly, Liang et al. (1997) have shown that the expression of an Hsp27-like protein, p26, in encysted (desiccated) *Artemia* (brine shrimp) embryos, has the potential to protect macromolecular components of these embryos as they encyst or upon exposure to extreme environmental stress (Liang et al., 1997). This expression of p26 in *Artemia* could be interpreted as

being analogous to that of Hsp27 in the adult rat nervous system following heat shock and may represent a highly conserved response for the maintenance of fluid homeostasis.

***Neuronal expression of Hsp27: a possible role in protection from axotomy-induced apoptosis***

Following optic nerve transection, Hsp27 is expressed in a subset of RGCs from 4 to 28 days with a time-dependent increase in the percentage of RGCs that are Hsp27-positive (Chapter 3, Figure 3.6; Chapter 4, Figure 4.5). The majority of RGCs die by apoptosis from 4 to 14 days following axotomy (Villegas-Pérez et al., 1993; Berkelaar et al., 1994) and the loss of trophic support is thought to be an important factor contributing to apoptotic RGC death following optic nerve transection (reviewed in Sofroniew, 1999). Similarly, following lesioning of the sciatic nerve at p0, most of the axotomized p0 DRG neurons undergo apoptosis, with only 5-10% of the injured cells surviving by one week (Lewis et al., 1999). Interestingly, all surviving DRG neurons are Hsp27 positive (Lewis et al., 1999). Uninjured DRG neurons differ from uninjured RGCs in that approximately 7% of p0 DRG neurons constitutively express Hsp27 (Lewis et al., 1999) while there is little or no detection of Hsp27 in RGCs. These results support the hypothesis that those RGCs that express Hsp27 do not undergo apoptosis and may be marked for long-term survival following optic nerve transection. There is a small population of RGCs that survive for months following axotomy (Villegas-Pérez et al., 1993) and the detection of Hsp27 in a subset of RGCs following optic nerve transection may be an early indicator of a small population of cells that do not undergo apoptosis and continue to survive following this injury.

In PNS neurons in culture, those neurons that express Hsp27 survive nerve growth factor withdrawal (Lewis et al., 1999). In addition, transfection of cultured dorsal root ganglion neonatal neurons with an adenoviral vector expressing human Hsp27 rescued these neurons from death induced by NGF withdrawal (Lewis et al., 1999). The expression of Hsp27 in a subset of RGCs following optic nerve transection may be important for the longterm survival of a small population of RGCs by inhibiting apoptosis resulting from neurotrophin withdrawal.

Investigating other methods that may increase the endogenous expression of Hsp27 in RGCs prior to axotomy would demonstrate the relationship between Hsp27 expression and RGCs survival following axotomy. For example, Yokoyama et al. (2001) have shown that exogenous Hsp27 protein electroporated into RGCs *in vivo* can protect these cells from ischemia-reperfusion induced apoptosis. Whether exogenous Hsp27 applied in this manner would enhance RGC survival following axotomy remains to be determined.

In summary, the expression of Hsp27 in different cell types following various stressors appears to be related to the specific activation of either glial cells or particular populations of neurons in response to the stress. It is interesting to hypothesize that Hsp27 may perform different functions in these specific cell types depending on the stressor.

### **Cell Signaling: MAPK pathways**

The mitogen-activated protein kinase (MAPK) signaling pathways in eukaryotic cells transduce external stress stimuli internally allowing for the coordination of an



appropriate molecular response to the specific stimuli including the activation of gene transcription, protein synthesis and cell death (reviewed in Kyriakis and Avruch, 2001). There are three main mammalian MAPK pathways that are activated in response to various stimuli: the extracellular signal-regulated kinase (ERK) pathway, the stress activated protein kinase/c-Jun NH<sub>2</sub>-terminal kinase pathway (SAPK/JNK), and the p38 MAPK pathway (reviewed in Kyriakis and Avruch, 2001). All MAPK pathways include three levels of core signaling molecules. The MAPKs (ERK, SAPK/JNK, p38) are activated by a family of MAPK/ERK kinases called MEKs or MKK (MAPK kinases). The MEKs/MKKs are regulated by MAPK kinase kinases (MAP3Ks or MAPKKK). The MAP3Ks are activated or inhibited by various adapter proteins and the Ras superfamily of GTPases associated with various transmembrane receptors (reviewed in Kyriakis and Avruch, 2001). The varied stimuli that result in the activation of MAPK pathways include environmental stress, growth factors that signal through tyrosine receptor kinases (Trks) such as BDNF, neuropeptides and hormones acting through G protein-coupled seven-transmembrane receptors such as angiotensin II.

***Signaling in astrocytes: expression of Hsp27 following heat shock***

The cellular signaling responsible for induction of Hsp27 in these reactive astrocytes is not well understood. Interestingly, glutamate receptor antagonist application to the brain following cortical spreading depression suppressed the astrocytic expression of Hsp27 (Plumier et al., 1997b). In addition, the activation of  $\beta$ -adrenergic receptor may be important for Hsp27 expression and astrocyte activation following focal ischemia

(Imura et al., 1999) indicating that different stresses or injuries may induce the expression of Hsp27 through different cell surface receptors.

The aberrant release of glutamate from the degenerating nerve terminals in the SC following optic nerve transection may have caused the expression of Hsp27 in reactive astrocytes (Chapter 3) and it would be interesting to determine whether the glutamate receptor antagonist, MK-801, suppresses the expression of Hsp27 in the SC. The astrocytic expression reported throughout the CNS with heat shock may be related to the aberrant release of neurotransmitter and subsequent astrocyte activation to buffer neurons from the effects of hyperthermia. *In vitro*, hyperthermia (42°C) depletes adenosine triphosphate in hippocampal neurons and this depletion has been correlated with release of endogenous glutamate and the disruption of neuronal glutamate uptake (Madl and Allen, 1995). Knock out of astroglial glutamate transporters, both *in vivo* and *in vitro*, has indicated the importance of functional glial glutamate transporters for the maintenance of low extracellular glutamate and for the prevention of glutamate neurotoxicity (Rothstein et al., 1996; Tanaka et al., 1997). Whether this uptake of excess glutamate by astrocytes is important for neuron survival following hyperthermia or axotomy remains to be determined. It would be interesting to block the astrocytic transport of glutamate to investigate whether this action is important for the induction of Hsp27 and for the protection of neurons during hyperthermic stress or axonal injury. An investigation of the expression of Hsp27 in glial glutamate transporter knockout mice (Tanaka et al., 1997) following various stressors including kainic acid-induced seizure, CSD and hyperthermia would help to determine the relationship between Hsp27

induction and expression and glutamate signaling. If Hsp27 expression is blocked in these astrocytes, are the neurons more susceptible to injury?

Regardless of the factors that induce reactive gliosis, astrocytic expression of Hsp27 appears to be the result of, and defines the extent of, the primary reactive astrogliosis following various stressors and injuries in the CNS. The expression of Hsp27 appears to correlate with the activation of certain astrocytes to respond to increased levels of excitotoxic neurotransmitter.

***Signaling in neurons: a possible role for angiotensin II in expression of Hsp27 following heat shock***

The neuronal expression of Hsp27 following heat shock is not widespread and is limited to specific nuclei known to be involved with temperature regulation and fluid balance (Chapter 2). In the CNS, the neuropeptide angiotensin II is involved in the activation of central pathways involved in blood pressure regulation and body fluid homeostasis (reviewed in McKinley et al., 1996; Potts et al., 1999). Specifically, angiotensin II induces drinking, vasopressin and oxytocin release, increased sodium intake and vasoconstriction (Culman et al., 1995; Richards et al., 1999). Angiotensin II exerts its actions through its interaction with the G-protein linked angiotensin II seven-transmembrane receptor AT<sub>1</sub> (See Figure 5.1; reviewed in Aguilera et al., 1995). The production of active angiotensin II following stress is well defined and known as the renin-angiotensin system. Renin cleaves angiotensinogen to produce angiotensin I which is converted to angiotensin II by the angiotensin converting enzyme (reviewed in Aguilera et al., 1995). In rats, hyperthermia causes an increase in plasma renin (Groza et

**Figure 5.1: Schema summarizing several signaling cascades that may modulate the expression and phosphorylation of Hsp27 with the various protective functions of Hsp27 indicated. Activation of the TrkB receptor by brain derived neurotrophic factor (BDNF) leads to the activation of the MAPK pathway and to the inhibition of heat shock factor 1 (HSF1), thereby inhibiting Hsp27 expression. The activation of the angiotensin II receptor, AT<sub>1</sub>, by angiotensin II, leads to the induction, expression and phosphorylation of Hsp27 through the activation of HSF1 and p38. Heat shock activates HSF1 leading to the induction and expression of Hsp27. The phosphorylation state of Hsp27 is modulated by mitogen activated protein kinase activated protein kinase-2 (MAPKAP-2). Unphosphorylated Hsp27 can bind to actin to inhibit polymerization and can multimerize to perform chaperone functions. Dimers of Hsp27 can inhibit death associated protein (Daxx) mediated apoptosis. Hsp27 can also inhibit mitochondrial mediated apoptosis by inhibiting the actions of cytochrome c and the activation of procaspase-3. (Adapted from Heumann, 1994; Currie and Plumier, 1998; Murasawa et al., 2000; Beere, 2001; Yamauchi et al., 2001; Arrigo et al., 2002).**

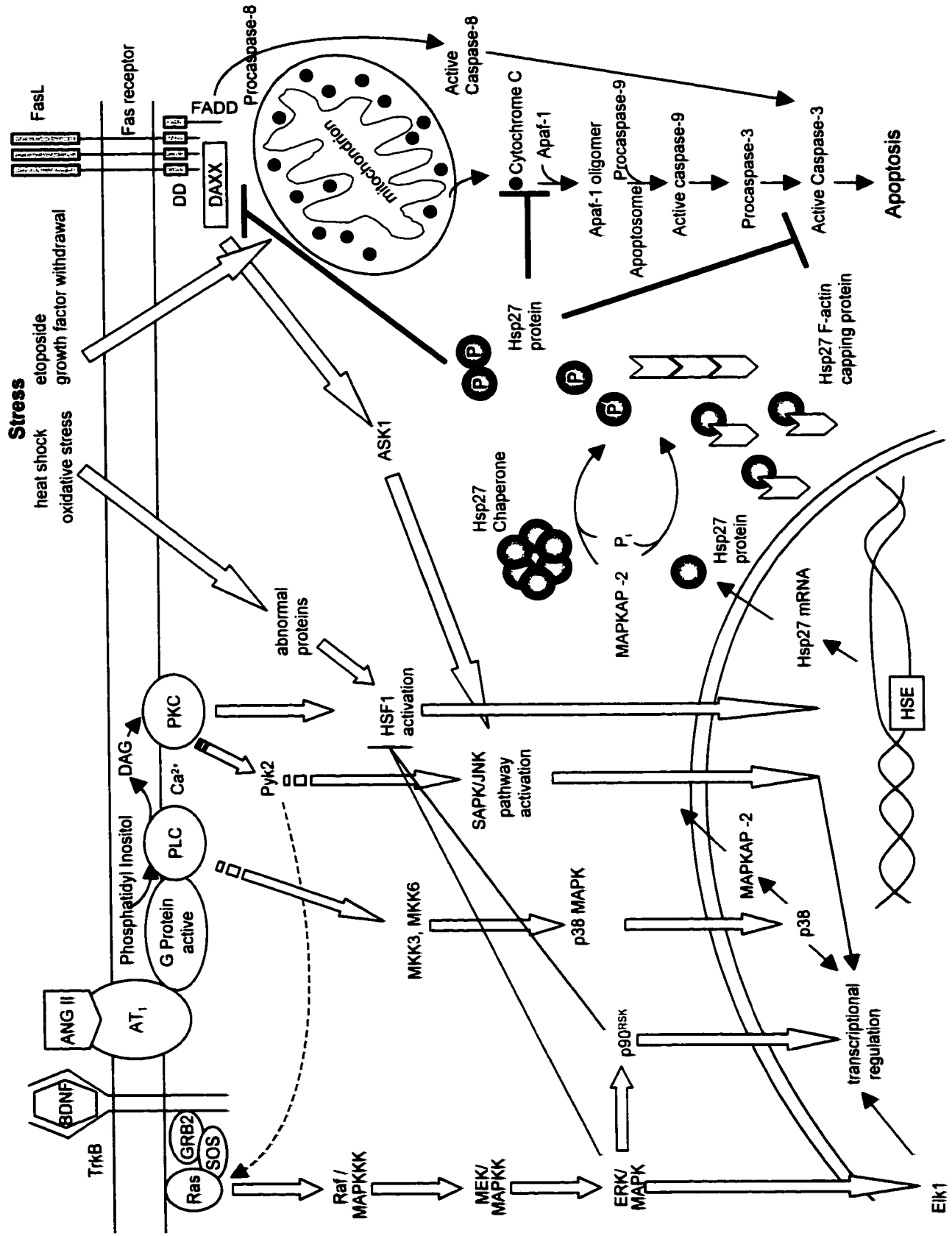


Figure 5.1

al., 1981) and an increase in angiotensin II in plasma and in the brain (reviewed in Watanabe et al., 1998). Interestingly, work in the kidney and the aorta has indicated that angiotensin II/AT<sub>1</sub> signaling mediates the induction and phosphorylation of Hsp27 (Ishizaka et al., 2001; Meier et al., 2001). In addition, high densities of AT<sub>1</sub> have been localized to many circumventricular organs including the subfornical organ, area postrema and the paraventricular nucleus of the hypothalamus (Mendelsohn et al., 1984; Tsutsumi and Saavedra 1991). The interaction of angiotensin II with the AT<sub>1</sub> receptor is important in mediating autonomic nervous system responses to heat stress (Kregel et al., 1994); therefore, angiotensin II may be important for the specific induction and expression of Hsp27 in certain circumventricular organs and other homeostatic nuclei. This response may be mediated through the angiotensin II/AT<sub>1</sub> pathway as described in the kidney and aorta (see Figure 5.1; Ishizaka et al., 2001; Meier et al., 2001).

Angiotensin II/AT<sub>1</sub> binding induces a wide array of intracellular signal transduction pathways in cultured CNS neurons (see Figure 5.1) including MAPK (Huang et al., 1996; Yang et al., 1996) and SAPK/JNK (Huang et al., 1998) pathways. In cardiac fibroblasts, angiotensin II signals through the SAPK and the MAPK pathway via G-protein mediated activation of a Ca<sup>++</sup> sensitive proline-rich tyrosine kinase-2 (Pyk2) (see Figure 5.1; Murasawa et al., 2000). In smooth muscle cells in the aorta, one of the major results of angiotensin II signaling is the phosphorylation of Hsp27 (Meier et al., 2001) indicating p38 MAPK pathway activation. It is now known that G protein-coupled seven transmembrane receptors like AT<sub>1</sub> induce p38 MAPK activation mediated by mitogen activated protein kinase kinase 3 (MKK3) and MKK6 (Yamauchi et al., 2001). AngiotensinII/AT<sub>1</sub> also stimulates phosphatidylinositol hydrolysis and

subsequent activation of protein kinase C (PKC) (Sumners et al., 1994) and calcium signaling (Gebke et al., 1998) in CNS neurons.

It is proposed that activation of PKC is responsible for the increased expression of Hsp27 in the kidney following angiotensin II administration (Ishizaka et al., 2001). Different isoforms of PKC activate HSF1 regulated transcription following stress (Baek et al., 2001). In addition, the activation of PKC is important in the development of thermotolerance in some cell lines (Shibuya et al., 2001). PKC induces the expression of Hsp27 directly following growth factor stimulation in osteoblasts and vasopressin stimulation in smooth muscle cells (Kaida et al., 1999; Kozawa et al., 2001). Therefore, the induction and expression of Hsp27 in neurons following hyperthermic treatment may be mediated through the intracellular signaling elicited by angiotensin II/AT<sub>1</sub> binding and PKC activation.

Losartan, an AT<sub>1</sub> receptor antagonist, blocks angiotensin II/AT<sub>1</sub> intracellular signaling (Gebke et al., 1998) and may thus block the induction of Hsp27 in this specific population of neurons following hyperthermic treatment. Inhibition of Hsp27 expression by AT<sub>1</sub> receptor antagonists will provide further information regarding the signaling pathways leading to the induction of Hsp27 in different cell types following various stresses.

### ***Signaling in RGCs: anti-apoptotic action of Hsp27***

Axotomy of the optic nerve causes the majority of RGCs to undergo apoptosis by 14 days following injury (Villegas-Pérez et al., 1993; Berkelaar et al., 1994). In this

model of CNS sensory neuronal injury, Hsp27 is induced and expressed in a limited number of RGCs by 4 days following injury (Chapter 3). Several recent studies suggest that Hsp27 interferes with mitochondrial-mediated, caspase-dependent cell death (see Figure 5.1). Hsp27 blocks cytochrome C-mediated activation of caspase-3 and -9 (Garrido et al., 1999; Bruey et al., 2000; Pandey et al., 2000) by sequestering cytochrome C and procaspase-3 (Concannon et al., 2001; Paul et al., 2002). Caspase-9 functions as the upstream activator of caspase-3 (Kermer et al., 2000) and activation of caspase-3 is reported to be the crucial step leading to neuronal apoptotic death (Nicholson and Thornberry, 1997).

Hsp27 may play a direct role in the blockade of caspase activation in the subset of Hsp27-positive RGCs following optic nerve transection and may lead to the long-term protection of this small population of neurons. The specificity with which Hsp27 inhibits caspase activation may indicate an important therapeutic role for this protein in preventing apoptosis of RGCs following optic nerve transection.

### ***Signaling in RGCs: BDNF modulates Hsp27 expression***

Binding of BDNF to the TrkB receptor causes autophosphorylation at several sites along the cytosolic portion of the receptor (see Figure 5.1). A series of adaptor proteins transduces a signal to activate the G-protein Ras (active in the GTP bound state). Ras, in turn, phosphorylates and activates the MAP3K, Raf, that phosphorylates and activates the MAPKK, MEK, that leads to the phosphorylation and activation of the MAPK, ERK that in turn activates various other kinases including p90RSK2 and the transcription factor ELK1 to induce the transcription of various mitogen responsive genes



(see figure 5.1 for overview; reviewed in Heumann, 1994; Bonni et al., 1999). In this thesis, Hsp27 expression was suppressed in RGCs following administration of BDNF. Heat shock genes including *hsp27* are regulated at the transcriptional level by the binding of activated HSF1 to heat shock elements (HSE) in the promotor region of the gene (Chapter 1). Activated MAPK (ERK) represses HSF1 function by phosphorylation, blocking its ability to bind to the HSE and initiate heat shock gene transcription (Chu et al., 1996). Also, p90RSK2, which is activated by ERK represses HSF1 function during stress (Wang et al., 2000). In neurons, the activation of the ERK pathway is primarily related to stimuli involved in developmental neuronal survival and neurite outgrowth growth (reviewed in Heumann, 1994). Chu et al. (1996) hypothesize that activation of the stress response, which blocks the transcription of normal genes and enhances the transcription of the heat shock genes (Ritossa et al., 1962) would be deleterious to cell survival during growth and development. The activation of the ERK pathway effectively represses HSF1 regulated expression of heat shock genes during growth and development. *In vitro*, the artificial activation of ERK and p90RSK2 have also been shown to repress the activation of HSF1 following heat shock (Chu et al., 1996, 1998; Wang et al., 2000).

The administration of BDNF suppressed the expression of Hsp27 following optic nerve transection (Chapter 4). This suppression of Hsp27 expression may be related to the repression of HSF1 following BDNF activation of the ERK pathway. Although the suppression of HSF1 is important during development to ensure the proper expression of developmentally regulated genes (Chu et al., 1996), its suppression following injury in the adult rat CNS may be detrimental to some neurons. The results presented here

indicate that the activation of neurotrophin-specific cell survival pathways may suppress other endogenous pathways important for the long-term survival of injured neurons. Understanding the intricate interactions of the many cell survival pathways will be useful for the development of proper strategies for the treatment of injured neurons.

***Hsp27 function: the importance of p38 MAPK and phosphorylation***

The three main functions of Hsp27 at the cellular level include chaperoning (Jakob et al., 1993; Merck et al., 1993), regulating actin and intermediate filament dynamics (Guay et al., 1997; Perng et al., 1999) and inhibiting apoptosis (Mehlen et al., 1996a,b; Garrido et al., 1999; Concannon et al., 2001). The ability of Hsp27 to perform these functions is related to the phosphorylation/oligomerization state of the protein (Guay et al., 1997; Prévaille et al., 1998; Lambert et al., 1999; Rogalla et al., 1999). As stated in the introduction, rodent Hsp27 can be phosphorylated at two sites, serine residues 15 and 86 (Gaestel et al., 1991). Hsp27 appears to dimerize via the  $\alpha$ -crystallin domain and forms larger molecular multimers through the interactions mediated by the phosphorylation-sensitive serine residues at the N-terminal domain (Lambert et al., 1999). The phosphorylation of Hsp27 impedes the formation of large Hsp27 oligomers that are important for chaperoning and cellular protection, and for the disaggregation of large Hsp27 protein oligomers (Kato et al., 1994b; Prévaille et al., 1998; Lambert et al., 1999). Small oligomers and monomers are important for modulating actin dynamics (Benndorf et al., 1994).

Hsp27 phosphorylation is modulated by the p38 mitogen activated protein kinase (p38/MAPK) family (reviewed in Tibbles and Woodgett, 1999; Kyriakis and Avruch,

2001). One of the actions of activated p38 MAPK is to phosphorylate MAPK activated protein kinase-2 (MAPKAP-2) (Rouse et al., 1994). MAPKAP-2 is responsible for the phosphorylation of Hsp27 (Rouse et al., 1994). In culture, the induction of Hsp27 in response to various stressors is linked to p38 activation, indicating that the activation of p38 is not only important for Hsp27 phosphorylation but is also involved in the pathway leading to its transcription under certain stressful stimuli (Kato et al., 1999a). Although the phosphorylation of Hsp27 was not studied in this thesis, one important next step would be to investigate the phosphorylation state and level of oligomerization of Hsp27 in the different cell types following hyperthermia and axotomy.

In solution, purified monomers of Hsp27 act as F-actin cap binding proteins that inhibit actin polymerization (Benndorf et al., 1991; Miron et al., 1991). During stress, phosphorylation of Hsp27 regulates actin reorganization (Lavoie et al., 1993a,b, 1995; Guay et al., 1997) and it has been proposed that phosphorylation of Hsp27 monomers causes conformational changes that allow for the polymerization and reorganization of actin (Arrigo and Landry, 1994). Large Hsp27 oligomers prevent the aggregation of heat-denatured proteins and stabilize the proteins for refolding (Ehrnsperger et al., 1997). The phosphorylation of Hsp27 appears to regulate both the oligomeric and monomeric pools of Hsp27 protein during stressful stimuli to modulate Hsp27 function (Kato et al., 1994; Prévaille et al., 1998).

In addition to modulating actin dynamics and molecular chaperoning, Hsp27 inhibits apoptosis (Mehlen et al., 1996a,b; Wagstaff et al., 1999; Concannon et al., 2001; see Chapter 4, Discussion). The form of Hsp27 that inhibits cytochrome c dependent apoptosis is not currently known (Arrigo et al., 2002).

The phosphorylation and oligomerization of Hsp27 appears to be important for regulating the different functions of Hsp27. Phosphorylation of Hsp27 can be determined and confirmed using two-dimensional gel electrophoresis in that non-phosphorylated, mono, and di-phosphorylated proteins have different isoelectric points. Studies of Hsp27 phosphorylation also employ the p38 MAPK-specific inhibitor SB203580 (Guay et al., 1997; Prévaille et al., 1998). Antibodies specific for the phosphorylated and non-phosphorylated form of p38 MAPK are also commercially available (New England BioLabs). Despite the availability of these compounds and antibodies, no studies have investigated the activation of p38 MAPK in the CNS following hyperthermic treatment. Activation of p38 MAPK in the Hsp27-positive astrocytic and specific neuronal populations would give further insight into the phosphorylation state and thus the function of Hsp27 in these different cell types *in vivo* following hyperthermic stress.

Following transection of the optic nerve, Hsp27 is detected in a limited population of RGCs (Chapter 3) and the phosphorylation state of Hsp27 is unknown. Activated p38 MAPK has been localized to the nuclei of RGCs by 1 day and expression levels remain high to 7 days following optic nerve transection (Kikuchi et al., 2000). The detection of activated p38 MAPK following axotomy makes it reasonable to hypothesize that any long-term protective effects of Hsp27 expression in RGCs may be mediated through its phosphorylation. Two dimensional gel electrophoresis of the isolated RGC layer would confirm Hsp27 phosphorylation and the change in phosphorylation with time following optic nerve transection. Determining Hsp27 phosphorylation along with its expression in different cell types following various injuries is important for understanding Hsp27 function.

### **Neuronal expression of Hsp27: PNS versus CNS**

The neuronal response to axonal injury in the CNS is markedly different from the response of PNS neurons. PNS neurons retain a significantly greater ability to regenerate following axotomy whereas the regeneration of CNS neurons is severely limited and in many cases nonexistent. The underlying molecular mechanisms responsible for this difference are not fully defined; however, evidence indicate that differences in the induction of several important genes may contribute to the ability of PNS neurons to survive and regrow following axonal injury, including *hsp27*. The neuronal induction and expression of Hsp27 in the CNS is markedly reduced when compared to the neuronal expression of Hsp27 following PNS injury. For example, with axotomy of the optic nerve, the expression of Hsp27 is limited to a small number of RGCs (Chapter 3, Figure 3.6) from 4 to 28 days following injury with widespread expression of Hsp27 in astrocytes of the retinorecipient layers of the SC (Chapter 3, Figure 3.8). Following fimbria-formix lesion Hsp27 is detected predominately in astrocytes in the hippocampus and in the septum from three to ten days following the lesion with the occasional Hsp27-positive septal neuron (Anguelova and Smirnova, 2000). These results are similar to those reported here for the optic nerve transection in that the target area for the cholinergic septal neurons (the hippocampus) contains Hsp27-positive astrocytes (similar to the SC following optic nerve transection) and that only a few of the injured neurons of the septum express Hsp27 (similar to RGCs). Both RGCs and septal neurons die by apoptosis following lesioning - RGC apoptosis begins at 4 days (Villegas-Pérez et al.,

1993; Berkelaar et al., 1994) while septal neuron apoptosis is delayed and begins at 16 days (Wilcox et al., 1995).

In the adult rat PNS, Hsp27 is constitutively expressed in DRG neurons (Plumier et al., 1997c) and nodose ganglion neurons (Hopkins et al., 1998). Sciatic nerve injury, which damages the peripheral axons of DRG neurons, does not result in apoptosis of DRG neurons and results in the induction and prolonged expression of Hsp27 in many DRG neuron cell bodies (Costigan et al., 1998). The constitutive, as well as the inducible, expression of Hsp27 in these neurons may be protective and important for neuron survival and regrowth. Axotomy of the vagus nerve, which has both motor and sensory components, causes the increased and prolonged up-regulation of Hsp27 expression in many of the motor neurons of the dorsal motor nucleus of the vagus and the nucleus ambiguus and an increased expression in the sensory neurons in the nodose ganglion (Hopkins et al., 1998). These neurons do not appear to die following cervical transection of the vagus nerve and the expression of Hsp27 correlates with this survival and also with sprouting observed in the rostral nucleus of the tractus solitarius and in the contralateral dorsal motor nucleus of the vagus nerve, regions of the dorsal vagal complex that do not normally receive vagal afferents (Hopkins et al., 1998). Interestingly, rhizotomy, which injures the central axons of DRG neurons, does not induce Hsp27 expression in these neurons and regrowth of these axons is limited (Costigan et al., 1998).

There are several lines of evidence that indicate that central axotomy does not induce the same changes in DRG gene expression as with peripheral axotomy. For example, c-Jun expression is quite robust in DRG neurons following peripheral (sciatic nerve) lesion, while, following central axotomy, there is minimal expression of c-Jun in

DRG neurons (Broude et al., 1997). In the nodose ganglion, peripheral axotomy (vagotomy) results in axonal outgrowth and induced changes in neuropeptide synthesis whereas central axotomy failed to induce these changes (Reimer and Kanje, 1999). The disruption of contact with peripheral targets increases the expression of survival and regenerative genes to a greater extent than disruption of contact with central targets.

Up-regulation of Hsp27 expression following peripheral nerve injury may be related to the molecular mechanisms involved in neuronal regeneration and axonal outgrowth following disruption of peripheral connections. That injured PNS neurons do not undergo apoptosis may be the result of constitutive Hsp27 expression in these PNS neurons. The diminished induction of Hsp27 in CNS neurons following axotomy is yet another example of the molecular differences that exist between the PNS and the CNS, and may be important for apoptosis and the lack of regeneration in the CNS.

## **Conclusions**

The expression of Hsp27 is inducible in the CNS in both neurons and astroglia. The results presented here provide further evidence for the cell type-specific stress-dependent expression of Hsp27 and point to various receptor-mediated cell signaling pathways that may be important for this differential expression including glutamate receptor signaling in astrocytes and AT<sub>1</sub> receptor signaling in neurons. BDNF administration at the time of optic nerve transection suppresses Hsp27 expression and provides *in vivo* evidence that the activation of the ERK/MAPK pathway may be important in modulating Hsp27 expression. The down-regulation of alternative cell

**survival pathways by known neuroprotective molecules raises a new challenge in developing strategies for the long-term treatment of injured neurons.**

**A more complete understanding of how neurons and astrocytes respond to injury will ultimately lead to the development of novel therapeutic strategies to enhance neuronal survival and recovery from various forms of CNS injury.**



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