THE EFFECT OF HYPERThERMIa ON THE INDUCTION
OF CELL DEATH AND HSP70 IN NEURAL AND
NON-NEURAL TISSUES OF THE RAT

By

Vania Rhea Khan

A thesis submitted in conformity with the requirements for the degree of

Master of Science

Department of Zoology, University of Toronto

© Copyright by Vania Rhea Khan (2001)
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
The effect of hyperthermia on the induction of cell death and hsp70 in neural and non-neural tissues of the rat

An abstract of a thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Zoology
University of Toronto

Vania Rhea Khan 2001

This thesis examines the effect of hyperthermia on the induction of cell death via apoptosis, assayed by TUNEL and active caspase 3 cytochemistry, in the adult rat brain, testis and thymus. A fever-like increase in temperature triggered apoptosis in dividing cell populations of the testis and thymus, but not in mature, postmitotic cells of the adult cerebellum. Western blot and immunocytochemical analysis of these tissues demonstrated that the induction patterns of hsp70 did not correlate with the induction of apoptosis in all three tissues. Hyperthermia-induced cell death was also examined in proliferative regions of the developing brain. The embryonic neuroepithelium and the external granule layer of the postnatal cerebellum, were susceptible to heat-induced apoptosis. The heat-sensitivity of the developing brain suggests that dividing neural cells are more prone to hyperthermia-induced cell death than mature, post-mitotic neural cells, which need to be protected because they are irreplaceable.
ACKNOWLEDGEMENTS

I wish to sincerely thank my supervisor, Ian Brown for his support, advice and constructive criticism concerning the direction of this project and the writing of the thesis. I thank also Sheila Rush for her thorough and patient instruction, technical assistance and proofreading this manuscript. I would like to thank Doug Bors for his time and help with the statistical analysis. I am especially grateful for past and present lab mates, Andrea Mothe, Fedra Molaie, Andrew Morrison, Sandra D’Souza, Cheryl D’Souza, David Bechtold and Hiwote Belay, who offered sound advice, criticism, encouragement and friendship. Thanks also to a few friends on the fifth floor who made my stay here more enjoyable in their own special way, Shelley Brun, Herman Cheung, Raffi Moussa, Adrienne Kong, Chai Chen, Richard Storey, Kate Hadden, and Cathy Pickett. My heartfelt thanks to close friends who have supported me outside the lab with their encouragement and prayers, Malaika Babb, Nikki Robinson and Flora Alevras. Thanks also to my brother Rob, who I admire and respect, for his kind, carefree spirit. I wish to express my love and gratitude to my parents, Bob and Cheryl, for their continuous love, and physical, emotional and spiritual support throughout my life. To my husband, Darryl, for his patience and loving care for as long as we have known each other and for reminding me that there is much more to life. I thank him. To the giver of life Himself, and for all spiritual intervention, thank you Infant Jesus, St. Joseph, and Mary, my heavenly mother.
## TABLE OF CONTENTS

**ABSTRACT** .................................................................................................................. ii

**ACKNOWLEDGEMENTS** ............................................................................................... iii

**LIST OF FIGURES** ....................................................................................................... vii

**LIST OF ABBREVIATIONS** ............................................................................................ ix

**INTRODUCTION** ........................................................................................................... 1

1.1. General Introduction ............................................................................................... 1

1.2. The Heat Shock Response ....................................................................................... 2

   1.2.1. Heat Shock Proteins ......................................................................................... 3

       Hsp70/Hsc70 ......................................................................................................... 4

       Hsp90 .................................................................................................................. 7

       Hsp60 .................................................................................................................. 8

1.2.2. Regulation of heat shock gene expression ......................................................... 8

1.2.3. Expression of hsps in the developing and adult mammalian brain ..................... 10

1.3. Apoptosis – a cellular response to stress ............................................................... 12

   1.3.1. Apoptosis versus necrosis ............................................................................... 13

   1.3.2. Apoptosis – a form of programmed cell death .............................................. 14

1.4. The induction of apoptosis by heat shock ............................................................... 16

1.5. Thesis objectives ..................................................................................................... 19
MATERIALS AND METHODS .......................................................... 21

2.1. Induction of hyperthermia .................................................. 21
2.2. Western blot analysis ....................................................... 22
2.3. Tissue preparation for TUNEL and immunocytochemistry ........... 23
2.4. Cell death detection by TUNEL .......................................... 24
2.5. Quantitative analysis ....................................................... 25
2.6. Immunocytochemistry ..................................................... 26

RESULTS .................................................................................. 28

3.1. The effect of hyperthermia on the induction of cell death in the adult brain, testis and thymus .................................................. 28

3.1.1. Induction of cell death in neural cells of the adult cerebellum .......................................................... 28

3.1.2. Analysis of hyperthermia-induced cell death in the adult rat testis ....................................................... 31

3.1.3. Analysis of hyperthermia-induced cell death in the adult thymus ......................................................... 37

3.2. Is the level of hyperthermic stress extreme enough to cause necrosis? .......................................................... 37

3.2.1. Specificity of the CM-1 antibody for active caspase 3 ....... 42

3.2.2. Immunocytochemical analysis of active caspase 3 in the adult testis and thymus ........................................ 45

3.3. Do the patterns of heat shock-induced apoptosis relate to the patterns of hsp70 induction in the adult cerebellum, testis and thymus? ........ 48
3.4. Do the levels of constitutive hsp90, hsc70 and hsp60 in the unstressed adult rat correlate to heat-induced apoptosis? .............................. 59

3.5. Are the embryonic and postnatally developing brain susceptible to hyperthermia-induced apoptosis? ................................................................. 59

3.5.1. Analysis of hyperthermia-induced apoptosis in the external granule cell layer of the cerebellum at postnatal day 7 .............. 62

3.5.2. The effect of hyperthermia on dividing cells in the cortical neuroepithelium and tectal neuroepithelium at embryonic day 17 ......................................................... 65

DISCUSSION ........................................................................................................ 74

4.1. Effect of hyperthermia on cell death in the adult brain, testis and thymus ............................................................ 75

4.2. Does this hyperthermic insult induce cell death via apoptosis or necrosis? ........................................................................ 77

4.3. Examination of the patterns of induction of cell death and hsp70 following hyperthermia .................................................. 78

4.4. The sensitivity of dividing cells to hyperthermia ......................... 82

4.5. The effect of hyperthermia on cell death in the developing brain .................................................................................. 86

4.6. Summary .................................................................................................. 90

REFERENCES .................................................................................................. 93
LIST OF FIGURES

Figure 1: The effect of hyperthermia on the induction of cell death in the adult rat cerebellum ................................................................. 29
Figure 2: Hyperthermia induces cell death in the adult rat testis ........... 32
Figure 3: Identification of heat-sensitive cell types in the adult testis .......... 35
Figure 4: Hyperthermia induces cell death in the adult rat thymus .......... 38
Figure 5: Quantitative analysis of the effect of hyperthermia on adult cerebellum, testis and thymus ...................................................... 40
Figure 6: Specificity of the CM-1 antibody for active caspase 3 .............. 43
Figure 7: Analysis of caspase 3 immunoreactivity in the adult testis and thymus ...................................................................................... 46
Figure 8: Time course analysis of hsp70 induction in adult rat tissues following hyperthermia ................................................................. 49
Figure 9: Analysis of hsp70 immunoreactivity in the control and hyperthermic adult cerebellum .............................................................. 51
Figure 10: Induction of cell death and hsp70 in rat testis following hyperthermia ....................................................................................... 54
Figure 11: Hyperthermia-induced cell death and hsp70 immunoreactivity in thymus ................................................................................... 57
Figure 12: Hsp90, hsc70 and hsp60 levels in adult tissues of the unstressed rat ......................................................................................... 60
Figure 13: Hyperthermia induces cell death in the external granule cell layer of the cerebellum at postnatal day 7 ...................................... 63
Figure 14: The effect of hyperthermia on dividing cells in the neocortex at embryonic day 17 ........................................ 66

Figure 15: Hyperthermia induces apoptosis in the tectal neuroepithelium at embryonic day 17 ........................................ 69

Figure 16: Quantitative time course analysis of the effect of hyperthermia on cell death in the developing brain at postnatal day 7 and embryonic day 17 ............................................... 72
LIST OF ABBREVIATIONS

aq  aq  aqueduct  adenosine triphosphate
ATP  BSA  bovine serum albumin  blood vessel
BSA  BV  C  calcium  cortex
Ca++  CB  cce-3  C. elegans death gene-3
CD  CP  OC  differentiation field
co  D  DF  DNA fragmentation factor
dNTP  DNA  deoxyribonucleic acid
dUTP  deoxyuridine triphosphate
dwm  deep white matter
day  DAB  3,3' -diaminobenzidine
day  df  differentiating field
DFF  DNA  3.3' -diaminobenzidine
DFF  DNA fragmentation factor
dNA  DF  DNA fragmentation factor
dUTP  DNA  deoxyribonucleic acid
dUTP  deoxyuridine triphosphate
dU  deep white matter
day  DAB  3,3' -diaminobenzidine
day  df  differentiating field
E  ECL  enhanced chemiluminescence
EDTA  EDTA  ethylenediaminetetra-acetic acid
eg  ecl  external granule cell layer
e  egl  external granule cell layer
g  g  granule cell layer
GD  gestational day
 grp  glucose regulated protein
H2O2  hydrogen peroxide
hr  hour
hsc  heat shock cognate
HSE  heat shock element
HSF  heat shock factor
hsp  heat shock protein
ICE  Interleukin-converting enzymes
IgG  immunoglobulin G
igl  internal granule cell layer
iz  intermediate zone
kDa  kilodalton
m  ml  molecular layer
m  mM  molar
min  millimolar
mz  mitotic zone
ug  micrograms
um  micrometer
ne  neuroepithelium
purkinje cellular layer
polyacrylamide gel electrophoresis
poly (ADP-ribose) polymerase
phosphate buffered saline
phosphate buffered saline (0.2% Triton X-100, 0.1% BSA)
proliferating cell nuclear antigen
postnatal day
spermatids
spermatozoa
stress-activated protein kinase/c-Jun N-terminal kinase
sodium dodecyl sulphate
subventricular zone
synthetic zone
Tris buffered saline Tween-20
terminal deoxynucleotidyl transferase
testis
thymus
Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
ultraviolet
white matter
INTRODUCTION

1.1. General Introduction

Studies in tissue culture systems have revealed two types of cellular responses to stress, the heat shock (stress) response and programmed cell death. In the heat shock response, there is a transient increase in the expression of a set of genes encoding heat shock proteins (hsp). While ongoing gene expression is downregulated (Lindquist and Craig, 1988; Pardue et al., 1992). These induced proteins play roles in cellular repair and protective mechanisms facilitating cell recovery (Parsell and Lindquist, 1993; Georgopoulos and Welch, 1993; Mailhos et al., 1993; 1994). In the cell death program, signalling pathways are induced, involving the coordinate action of multiple kinases and cysteine proteases, known as caspases, which cleave target substrates, bringing about the cell's own demise (Dorstyn et al., 1998; Wolf and Green, 1999; Earnshaw et al., 1999). This mode of programmed cell death is termed apoptosis. Many factors influence a cell's decision regarding which pathway to choose in response to stress, namely the stress response leading to repair and protection or apoptosis, resulting in cell death.

Previous studies in this laboratory have established that a physiologically relevant increase in body temperature is sufficient to activate the cellular stress response in the mammalian brain (Brown, 1994; Brown and Sharp, 1999; Bechtold and Brown, 2000). The objective of this study is to investigate whether a fever-like temperature increase activates the cell death program in neural cells of the adult rat brain, in vivo, compared to non-neural tissues, such as testis and thymus and how this correlates with the levels of inducible hsp70 within the tissues. Cells in the adult brain are postmitotic and fully differentiated (Altman, 1997) whereas cells in the testis and thymus are undergoing cell
division and differentiation processes throughout life (Westermann et al., 1989; Russell et al., 1990; Alam et al., 1997). The effect of hyperthermia on cell death in the developing brain is also explored, to determine if dividing neural cells are more prone to hyperthermia-induced cell death than mature, post-mitotic neural cells.

1.2. The Heat Shock Response

Organisms as diverse as bacteria, yeasts, plants and animals have developed an evolutionary conserved defense mechanism for protection against environmental insults, which is referred to as the cellular stress response. It was first discovered by Ritossa (1962) who observed a new pattern of chromosomal puffing in isolated *Drosophila* salivary glands that were exposed to a higher temperature. Changes in the polytene chromosome puffing pattern correlated with changes in protein synthesis after heat shock (Tissieres, 1974). Investigators later found that a range of stimuli induced the increased expression of hsps, including uncouplers of ATPase, anoxia, amino acid analogs, ethanol, heavy metal ions, ischemia, trauma and brain injury (Welch, 1992; Ritossa, 1996). Since these unrelated stimuli and pathological stresses caused denaturation of preexisting or newly made proteins (Hightower, 1980), it was suggested that the accumulation of abnormally folded proteins in a cell is a trigger that initiates the stress response (Goff and Goldberg, 1985; Ananthan et al., 1986).

This response is characterized by the induction of a set of genes encoding heat shock proteins and the simultaneous reduction in ongoing protein synthesis (Lindquist and Craig, 1988; Schlesinger, 1994). Although splicing mechanisms are compromised after heat shock treatment (Yost and Lindquist, 1986), stress-inducible heat shock genes
are not affected since they do not contain introns. The stress response is also marked by changes in cell physiology and morphology. Following heat shock, mammalian cells experience a rapid drop in intracellular pH, decreased levels of ATP and an increase in cytosolic calcium levels (reviewed by Welch, 1992). Morphological changes include an accumulation of perichromatin granules which probably represent unprocessed forms of RNA (Yost and Lindquist, 1986), collapse of cytoskeleton around the nucleus (Coss and Linnemans, 1996), and changes in the nucleolus, such as the aggregation of maturing preribosomes (Welch and Suhan, 1985).

1.2.1. Heat Shock Proteins

Apart from the induction of hsps under stressful conditions, many heat shock proteins are constitutively expressed, suggesting an important role for hsps in the physiology of the unstressed cell (Lindquist and Craig, 1988). One of the most widely characterized functions of hsps is their ability to act as molecular chaperones. Molecular chaperones are proteins which mediate protein transport and folding processes in a transient and ATP-dependent interaction, but are not components of the functional assembled structures themselves (Ellis and Hemmingsen, 1989; Hendrick and Hartl, 1995). In the unstressed cell, hsps are involved in protein folding, targeting proteins to organelles, maintaining them in a translocation competent state until they reach their intracellular destination, or directing proteins to degradation (Gething and Sambrook, 1992; Brodsky, 1996; Hartl, 1996). Heat shock proteins can be classified into major families based on their molecular weights (Welch, 1992). The major families include the HSP100s, HSP90s, HSP70s, HSP60s, HSP40s and HSP20s. The main focus of this study
is the stress-inducible hsp70, but constitutively expressed members, including hsp90, hsc70 and hsp60 are briefly explored.

**Hsp70/Hsc70**

This multigene family of highly conserved proteins contains constitutive and stress-inducible members, which play critical roles in normal cellular physiology and in cellular repair and protection after stress. All hsp70 related proteins share the ability to bind ATP with high affinity (Welch and Feramisco, 1985). The hsp70 gene was first isolated in *Drosophila* (Bardwell and Craig, 1984) and it exhibits 50% homology with the *E. coli* dna K, which is essential for replication of bacteriophage lambda (Lindquist, 1986).

The mammalian hsp70 family includes the constitutively expressed heat shock cognate, hsc70, and the stress-inducible hsp70. These proteins, which are present in the cytosol and nucleus, exhibit very high sequence homology (~95%) and similar biochemical properties and functions (reviewed by Welch, 1992). Other members include the glucose-regulated protein (grp78) in the endoplasmic reticulum and grp75 in the mitochondria (Kiang and Tsokos, 1998), which are involved in chaperoning events in their cellular compartments (reviewed by Welch, 1992). Special features of stress-inducible hsp70 genes include the absence of introns and the presence of 5' sequences in hsp70 mRNA, which render these transcripts insensitive to heat-induced inhibition of translation (Lindquist, 1986; Lindquist and Craig, 1988). Hsp70 protein consists of three functional domains, an N-terminal ATP binding domain (Chappell et al., 1986), a substrate binding domain (Wang et al., 1993) and a carboxy-terminal domain
(Bhattacharyya et al., 1995), which is important in the regulation of ATPase activity (Freeman et al., 1995).

Hsp70 and hsc70 function as molecular chaperones. Hsc70 also functions as a clathrin uncoating ATPase (Ungewickell, 1985; Chappell et al., 1986), mediating the recycling of clathrin coated vesicles. It is abundant in the brain where it comprises 1% of soluble protein content (Schlossman et al., 1984). These proteins are also synthesized in a cell-cycle dependent manner, with highest concentrations within the S-phase (Milarski and Morimoto, 1986; Milarski et al., 1989; Hang and Fox, 1995; 1996; Zeise et al., 1998).

In the stressed cell, hsp70, together with its cofactor hsp40, form a chaperone complex, which has been shown to protect luciferase from heat-induced aggregation and promote the renaturation of thermally denatured luciferase, using the energy of ATP (Minami et al., 1996; Michels et al., 1997; Ohtsuka and Hata, 2000). Cytosolic hsp70 and hsc70 exist mainly in an oligomer-monomer equilibrium, which is dependent on environmental temperature, the levels of ATP and the presence of denatured proteins (Angelidis et al., 1999). In their oligomeric form, hsp70 and hsc70 are unable to exert chaperone activity. Addition of denatured luciferase changes both proteins to their non-oligomeric forms, allowing them to resume chaperone functions.

The protective abilities of hsp70 have been examined in a number of studies. Inhibition of hsp70 expression in cultured cells, resulted in cell death after heat shock (Riabowol et al., 1988). The neuroprotective effects of whole body hyperthermia and the involvement of hsp70 have also been demonstrated in vivo (Barbe et al., 1988; Tytell et al., 1993). Constitutive overexpression of hsp70 via transfection has been shown to
protect neuronal cells from thermal stress, but not from stimuli that induce apoptosis (Mailhos et al., 1994). Upregulation of hsp70 in the brain or heart of transgenic mice, protects these organs against ischemic injury (Marber et al., 1995; Plumier et al., 1995; 1997). Specifically, hippocampal neurons were protected against ischemic injury in transgenic mice overexpressing human hsp70 (Plumier et al., 1997). Hsp70 has also been shown to protect cells by inhibiting specific steps of the cell death pathway (Samali and Cotter, 1996; Mosser et al., 1997). Recently, the stress-inducible hsp70 was reported to associate with synaptic elements, where it may facilitate the repair of stress-induced damage and contribute to neuroprotective events at the synapse (Bechtold et al., 2000).

Although hsp70 plays a significant role in protective mechanisms, it cannot be assumed that it is the key protective agent under all stressful conditions. For example, overexpression of hsp70 was shown to contribute to the stress-tolerant state by increasing chaperone activity in the cytoplasm of hamster fibroblasts (Nollen et al., 1999). However, hsp70 was insufficient to yield the level of recovery of luciferase activity in the nucleus of these cells, compared to thermotolerant cells expressing all hsps. In another study, when cortical neuronal cultures were exposed to heat stress, they did not induce hsp70, but their vulnerability to glutamate-induced cell death was reduced (Snider and Choi, 1996). Thus, mechanisms outside of the hsp70 pathway must be operational including other hsps such as hsp27 (Landry et al., 1989; Arrigo, 1998; Gorman et al., 1999b), and hsp110 (Oh et al., 1997), or other neuroprotective genes such as neuronal apoptosis inhibitory protein NAIP (Xu et al., 1997).
Hsp90

Members of the hsp90 family encode proteins, which are abundant in unstressed cells and are also induced by stress (Lindquist and Craig, 1988). Hsp90 is highly conserved, with over 40% amino acid sequence homology between E. coli and eukaryotes (Bardwell and Craig, 1987). Mammalian hsp90 consists of two isoforms, α and β, which are encoded by separate genes (Moore et al., 1987; 1989). Purified hsp90 exists as dimers of α-α and β-β (Minami et al., 1991; Welch, 1992) and comprises 1-2% of total cellular protein (Lai et al., 1984). It is found in abundant levels in the unstressed adult mammalian brain, localized to neurons (Quraishi and Brown, 1995). The molecular chaperone function of hsp90 is dependent on its ATP binding and hydrolysis activity in vivo (Obermann et al., 1998). Specifically, hsp90 associates with a range of kinases (Brugge, 1986; Whitelaw et al. 1991; Lewis et al., 2000) and cytoskeletal elements such as, actin (Nishida et al., 1986; Koyasu et al., 1986) and tubulin (Sanchez et al., 1988). Hsp90 is a component of steroid hormone receptor complexes, such as the glucocorticoid receptor, maintaining it in an inactive, non DNA-binding form, until the steroid hormone binds to the receptor, upon which there is an ATP-dependent release of hsp90 (Pratt, 1990; Georgopoulos and Welch, 1993; Kang et al., 1994). Hsp90 also functions cooperatively with hsp70 and DnaJ to renature damaged proteins, such as thermally denatured luciferase (Schumacher et al., 1996), and to form giant molecular chaperone complexes with hsp70 and hsp56 (Sanchez et al., 1990; Bucher, 1999).
Hsp60

Hsp60 belongs to a family of highly conserved proteins, known as chaperonins (Hartl, 1996) which also include hsp10 and a distant homologue of hsp60, the Tcp-1 proteins (Gupta, 1995). Hsp60 was first described in Tetrahymena, as a mitochondrial protein (McMullin and Hallberg, 1987). It is structurally homologous to the groEL protein of bacteria (Mizzen et al., 1989), which is essential for bacteriophage growth (Coppo et al., 1973) and equivalent to the Rubisco subunit-binding protein in the stroma of higher plant chloroplasts (Hemmingsen et al., 1988). Mammalian hsp60 is synthesized in the cytoplasm as a 60 kDa precursor protein and processed into a mature 58 kDa protein in the mitochondria, where it functions in the folding of monomeric proteins and proper assembly of oligomeric complexes (Mizzen et al., 1989). In eukaryotes, hsp60 together with its cofactor hsp10, binds to denatured proteins within the mitochondria, prevents aggregation and promotes folding of newly synthesized proteins (reviewed by Wynn et al., 1994; Martin, 1997) in an ATP-dependent process (Ostermann et al., 1989). The hsp60/hsp10 chaperone machine is thought to work in conjunction with the mitochondrial hsp70 (Hartl et al., 1994).

1.2.2. Regulation of heat shock gene expression

In eukaryotes, the stress response is regulated by the heat shock transcription factor HSF, which binds cooperatively to conserved, upstream response elements called heat shock elements (HSEs) in heat shock gene promoters (Xiao et al., 1991). First identified in Drosophila as a sequence required for heat inducibility of the hsp70 gene (Pelham, 1982), the HSE consists of multiple inverted repeats of a 5 base pair (bp)
sequence, 5'-nGAAn-3' (Xia and Lis, 1988). A family of HSFs exists in higher
eukaryotes (Morimoto et al., 1994). Although the members vary in size, with an overall
homology of less than 40%, all possess three highly conserved regions. These include an
amino-terminal DNA-binding domain, a trimerization domain containing "leucine
zipper" coiled-coil motifs and a carboxy-terminal domain, which maintains HSF in a non
DNA-binding state via hydrophobic interactions with the N-terminal domain (Morimoto
et al., 1994; Rabindran et al., 1993; Zuo et al., 1994).

In mammalian cells, HSF1 is constitutively synthesized and present as a non-
DNA binding monomer. In response to heat shock, HSF1 is activated by trimerization,
and acquires high-affinity DNA-binding activity (Baler et al., 1993). HSF1 also acquires
transcriptional competence (Zuo et al., 1995), and in some cases, is phosphorylated at
serine and threonine residues (Sorger, 1990; Jurivich et al., 1994). Hsp70 has been
proposed to participate in autoregulation of HSF1 activity by complexing with HSF1
monomers, which prevents trimerization (reviewed by Morimoto et al., 1994). In the
stressed cell, the accumulation of misfolded proteins competes with HSF for hsp70
binding, resulting in an increase in unbound monomeric HSF, which can trimerize and
activate hsp synthesis. Hsp70 in turn accumulates to a level where it is available to
complex with HSF1, which inhibits further activation of HSF1 (reviewed by Morimoto et
al., 1994; 1996). Consistent with this role for heat shock proteins in the negative
regulation of HSF1, are studies which show that overexpression of hsp70 or hdj-1/hsp40
in the absence of stress, blocks heat activation of HSF in HeLa cells (Abravaya et al.,
1992; Baler et al., 1996) and prevents the stress-inducible transcription of heat shock
genes (Mosser et al., 1993; Shi et al., 1998). Furthermore, complexes of hsp70 and HSF
trimers have been detected during attenuation of the heat shock transcriptional response (Shi et al., 1998). Besides activation of the heat shock response, HSF1 is implicated in the regulation of other important genes or signalling pathways as demonstrated in hsf1 (-/-) knockout mice (McMillan et al., 1998; Xiao et al., 1999).

1.2.3. Expression of hsp90 in the developing and adult mammalian brain

During mammalian embryogenesis, the capacity for induction of heat shock genes varies with the developmental stage. For example, during early development in the mouse and rabbit, hsp90 cannot be induced until after cleavage stages (Heikkila et al., 1986). In cultured whole rat embryos at embryonic day 9.5 (E9.5), control embryos exhibit high levels of hsp90 and hsc70, while hsp70 is undetectable. Exposure to mild hyperthermia (42°C for 10 min) induces hsp70 to a level sufficient for acquisition of thermotolerance, such that embryos are protected against a second, otherwise lethal heat shock. While hsp70 expression is observed in the mesoderm and regions of the neuroectoderm, following mild hyperthermia (Walsh et al., 1991), exposure to a higher temperature (43°C) or for a longer duration (42°C for 20-40 min), resulted in cell death. At this early embryonic stage, other studies also demonstrate a transient synthesis of hsp70, hsp90 and hsp27 following heat shock, with accumulation of hsp70 in neuroectodermal tissues, such as the optic vesicle (Fisher et al., 1995; Mirkes et al., 1996). Thus, the embryo is capable of mounting a protective heat shock response at critical stages of neural development, however this is dependent on the thermal dose.

During early postnatal development (P2 and P7), past studies revealed a developmental increase in the temperature required to induce synthesis of hsp70 (Brown,
In the unstressed rat, hsc70 and hsp90 proteins are abundant through postnatal neural development and exhibit a neuronal pattern of expression (D'Souza and Brown, 1998). Following temperature elevation to 40°C, activation of HSF1 and induction of hsp70, not hsc70 was detected in the brain of 2-day old rats (Morrison et al., 2000). Thus the rat brain is also capable of inducing hsp70 at early postnatal stages.

The constitutive and stress-inducible expression of hsp70 in the adult mammalian brain differs according to cell type. Hsc70 and hsp90 mRNA and protein are localized primarily to neuronal cell populations in the unstressed rabbit and rat, and are not detected in glial cell populations (Manzerra and Brown, 1992a; Manzerra et al., 1993; Foster et al., 1995; Quraishi and Brown, 1995; 1996; D'Souza and Brown, 1998). Rapid induction of hsp70 was observed in the brain following either drug induced-hyperthermia (Brown et al., 1982; Brown, 1985), amphetamine-induced hyperthermia (Nowak, 1988; Miller et al., 1991) or whole body hyperthermia (Currie and White, 1981; Brown, 1990). With the exception of small cerebellar granule cell neurons, the pattern of hsp70 induction following a fever-like temperature shock is predominantly glial, as demonstrated in Bergmann glia, oligodendrocytes of the deep white matter, and glial-enriched fibre tracts of spinal cord (Manzerra and Brown, 1992a,b; Manzerra et al., 1993).

Large neuronal populations fail to induce hsp70 protein following hyperthermia, however a delayed accumulation of hsp70 mRNA was detected in Purkinje neurons after the initial glial induction events (Manzerra et al., 1993). Tissue culture studies report that neuronal hsp70 mRNA induction may occur in response to higher temperatures or longer time intervals of hyperthermic stress (Blake et al., 1990; Pardue et al., 1992).
induction of hsp90 mRNA and protein was also not seen in glial cells, suggesting a more prominent role for hsp70 in the glial response to hyperthermia (Quraishi and Brown, 1995: 1996). Glial-specific induction of hsp70 after heat shock was also detected in vitro, in cultured astrocytes (Marini et al., 1990) and rat glial cells (Nishimura et al., 1988: 1991: Marcuccilli et al., 1996), as opposed to cultured neurons. The high levels of constitutive hsps (hsc70 and hsp90) in neurons have been proposed to buffer them against the effects of the heat shock (Manzerra et al., 1993).

Recently, it has been demonstrated that hyperthermia induces a robust expression of hsp27 and hsp32 in glial cells such as the Bergmann glia, which transport these hsps into their radial fibers, which project into the synapse-rich molecular layer of the cerebellum. Furthermore, these inducible hsps (hsp70, 32 and 27) associate with synaptic elements where they may facilitate the repair of stress-induced damage and contribute to neuroprotective events at the synapse (Bechtold et al., 2000; Bechtold and Brown, 2000).

1.3. Apoptosis – a cellular response to stress

The previous discussion provides an overview of heat shock proteins, their roles in the unstressed and stressed cell, and their patterns of induction in the mammalian brain following hyperthermia. Hsps are known to be constitutively expressed in unstressed cells during cell differentiation and development, and some family members are induced in response to pathological (ie. ischemia) and environmental (ie. heat shock) stress (reviewed by Morimoto, 1998). Apoptosis, a form of programmed cell death is also activated during cell differentiation and development, and induced by similar stressful stimuli. Studies have shown an overlap of signals that can induce both a stress response,
which can confer protection, and cell death via apoptosis, for example, the accumulation of damaged proteins (Samali and Cotter, 1996). Although a number of signals are known to induce both responses, the final outcome is different for individual cells within the same tissue or cell population. In response to stress, the first form of defense at the cellular level is probably to refold the damaged proteins. However if the stress is increased to a level where the stress proteins cannot protect the proteins and organelles, the cell will activate programmed cell death. In cases of extreme stress, cells will die of necrosis, a mode of cell death that differs from apoptosis (reviewed by Samali and Cotter, 1996; Punyiczki and Fesüs, 1998).

1.3.1. Apoptosis versus necrosis

Cell death has been defined as the irreversible loss of vital cellular structure and function (Buja et al., 1993). There are two fundamental types of cell death, which are morphologically and biochemically distinct, namely apoptosis and necrosis. Necrosis has been described as a pathological form of cell death to which cells passively respond, when the level of damage to a cell overwhelms its capacity to initiate and execute the apoptotic program (Buja et al., 1993). Necrosis often occurs in response to an overwhelming insult such as intracellular ATP depletion (Leist et al., 1997). Morphological alterations include cell swelling, degeneration of organelles, membrane disruption and cell lysis. There is random breakdown of DNA, which can be detected as a smear on DNA electrophoresis gels (Kerr and Harmon, 1991).

Apoptosis is a form of programmed cell death that occurs in the unstressed adult to regulate cell numbers (Buja et al., 1993). It is essential during embryogenesis,
maturation of the immune system (Bodey et al., 1998) and development of the nervous system (Blaschke et al., 1996; Thomaidou et al., 1997; Tanaka and Marunouchi, 1998). Physiological stress such as exposure to mild hyperthermia, anoxia, UV irradiation, and pathological conditions such as neurodegenerative diseases and stroke, all induce apoptosis (reviewed by Buja et al., 1993). Its morphological features are distinct from necrosis, and are a result of the activation of a cascade of proteases, lipases and nucleases.

Characteristic morphological changes include cytoplasmic shrinkage (versus swelling, seen in necrosis), convolutions or blebbing of the plasma membrane, chromatin condensation and nuclear disintegration. Organelles remain intact during apoptosis, whereas they degenerate in necrotic cells (Allen et al., 1997). Nuclear collapse is associated with the activation of endogenous Ca$^{2+}$-dependent endonucleases which cleaves double-stranded DNA into fragments of approximately 200 base pairs in length, which can be visualized as an electrophoretic ladder pattern of DNA fragments. DNA cleavage during apoptosis can also be detected in situ by the TUNEL method (Gavrieli et al., 1992). In the final phase, the cell fragments into apoptotic bodies which are rapidly phagocytized by macrophages (reviewed by Allen et al., 1997).

1.3.2. Apoptosis – a form of programmed cell death

Programmed cell death is governed by several “death genes” whose inactive proenzymes are constitutively expressed in all cells (Steller, 1995). Studies in enucleated cells suggest that programmed cell death does not require gene transcription (Jacobson et al., 1994). In mammalian cells, a family of proteins exhibiting strong sequence homology
to ced-3, the *C. elegans* death gene-3, has been discovered, and referred to as interleukin-converting enzymes (ICEs). One member of the ICE protease family, caspase 3, exhibits the highest similarity to ced-3 in both sequence homology and substrate specificity (Chopp et al., 1996). Although several apoptotic pathways exist, which are stimulated by different inducers, most of these pathways result in the activation of caspases, the most important effector molecules in apoptosis (Earnshaw et al., 1999).

Caspases are synthesized as proenzymes, which are activated by cleavage at specific tetrapeptide recognition sequences containing aspartate residues. This yields two subunits, which reassemble to form the active enzyme (Marks and Berg, 1999). The processing of caspases from their inactive precursor form to active subunits is evident in cells undergoing apoptosis, but not in cells resistant to apoptosis (Harvey et al., 1997). These activated proteases cleave their target substrates at specific aspartic acid residues. Targets of activated caspases (such as caspase 3), include PARP, poly (ADP-ribose) polymerase, and DNA-dependent protein kinase, both of which are normally involved in sensing DNA damage and repair (Earnshaw et al., 1999). Caspase 3 is also essential for activation of DNA fragmentation factor (DFF) which is directly linked to the induction of DNA cleavage by endonucleases (Liu et al., 1997). The importance of caspase 3 in regulating neural cell numbers during development was demonstrated in caspase 3 knockout mice (Kuida et al., 1996), in which the brains were enlarged due to superfluous cells within the germinal layers that were unable to execute apoptosis.
1.4. The induction of apoptosis by heat shock

Efforts have been made to elucidate the steps in the heat-shock induced apoptotic pathway. Heat shock activates sphingomyelinase, which hydrolyzes sphingomyelin to generate ceramide (Hannun, 1994; Verheij et al., 1996). Ceramide, which has recently been shown to increase in parallel with heat shock-induced apoptosis (Kondo et al., 2000b), mediates apoptosis by activating \textsuperscript{12} stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signalling pathway (reviewed by Kyriakis and Avruch, 1996; Verheij et al., 1996). This kinase subfamily responds to stresses such as UV light, oxidative stress and heat shock. Activation of JNK leads to the phosphorylation of c-Jun, which triggers the caspase cascade (Mosser et al., 1997). Active caspase 3 then targets specific proteins and brings about the morphological changes associated with apoptosis such as DNA fragmentation (Mosser et al., 1997; Kondo et al., 2000a). In vitro studies on heat-shock induced apoptosis in mouse fibroblasts and carcinoma cells (Chan et al., 1998) and in mouse embryos at gestational day 9 (Mirkes and Little, 2000), reveal activation of caspase-3, leading to cleavage of PARP and DNA fragmentation. Heat has also been reported to induce changes such as a drop in mitochondrial membrane potential and release of cytochrome c., which activates caspase 9 and in turn caspase 3 (Mirkes and Little, 2000).

Transfection studies have demonstrated that hspgs protect against apoptosis by inhibiting the activation of signal transduction pathways leading to programmed cell death. For example, overexpression of hsp70 blocked activation of the SAPK/JNK pathway, resulting in resistance of the cells to apoptosis and acquisition of thermotolerance (Gabai et al., 1997; Mosser et al., 1997; Buzzard et al., 1998). This
inhibition of apoptosis upstream of SAPK/JNK activation does not require the ATPase activity of hsp70 (Volloch et al., 1999). The protective effect of hsp70 is demonstrated in aged cells where hsp72 inducibility is greatly diminished, and subsequently hsp72-mediated control of the JNK signalling pathway is compromised. This results in increased rate of apoptotic cell death following heat shock (Volloch et al., 1998). Hsp70 can also inhibit apoptosis downstream of JNK activation, before activation of caspase 3 (Mosser et al., 1997). Hsp27 has also been shown to inhibit the activation of caspase 3 in vitro and in vivo, by binding to caspase 3 and blocking its activation by caspase 9 mediated proteolysis. This is the first evidence that a heat shock protein can repress caspase 3 activation (Pandey et al., 2000). In general, researchers believe that once caspase 3 is activated, cells can no longer be protected by anti-apoptotic proteins such as hsp70. One study on tumour cell lines overexpressing hsp70 demonstrated that cells respond to apoptotic stimuli by activating SAPKs and caspase-3-like proteases, but late caspase-dependent events such as changes in nuclear morphology were inhibited (Jäätelä et al., 1998). Thus hsp70 is unable to inhibit the activity of active caspase 3. Recently studies report that the chaperoning activity of hsp70 (ie. functional ATPase domain and C-terminal tetrapeptide sequence, that is essential for peptide binding) is required to prevent both the processing of procaspases 9 and 3, and release of cytochrome c from mitochondria, resulting in inhibition of apoptosis (Mosser et al., 2000).

Studies have shown that heat shock, which causes an accumulation of damaged proteins and triggers hsp induction (Ananthan et al., 1986), can also induce apoptosis. Harmon et al. (1990) heated cultures of a murine mastocytoma at temperatures ranging from 42-47°C for 30 minutes. While heating at 43-44.5°C led to marked enhancement of
apoptosis. exposure to 45°C enhanced both apoptosis and necrosis. Furthermore cell
death was mainly necrotic at 46-47°C indicating, that above a critical heat stress, the
mode of cell death changes from apoptosis to necrosis. The phenomenon of heat-
induced apoptosis has been demonstrated to be dependent on the temperature and
duration of the heat stress, in rat cell lines (Li et al., 1996) and human glioblastoma cells
(Fuse et al., 1998). Heat-induced apoptosis is associated with increased caspase activity
as shown in HL-60 myelocytic cells (Gorman et al., 1999a), and correlates with Ca\(^{2+}\)-
dependent regulation of endonucleases, as demonstrated in tobacco cells (Chen et al.,
1999).

In vivo studies of heat-induced apoptosis have been reported in non-neural tissues
including the rat small intestine (43°C for 30 min) (Allan and Harmon, 1986) and murine
and human tumors (Harmon et al., 1991). Whole body hyperthermia induced apoptosis
in rat thymus, spleen and small intestine, peaking at 4-8 h following heat (41.5°C for 2
h), but other tissues such as the heart, lung, kidney, liver and pancreas were resistant
(Sakaguchi et al., 1995). It has been suggested that cells in tissues with a high-turnover
state such as the thymus and intestine are programmed to undergo apoptosis and thus
induce apoptosis in response to lethal stimuli (Wyllie, 1992). Exposure to hyperthermia
in vivo or in vitro also results in abundant cell death in rats at embryonic day 10, causing
delayed or abnormal CNS, optic cup, somite and limb development (Edwards et al.,
1974; Walsh et al., 1994; Mirkes et al., 1997; Breen et al., 1999).
1.5. Thesis Objectives

Very few studies have examined the effect of hyperthermia on the mammalian brain with respect to the induction of cell death. Hyperthermia is a physiologically relevant phenomenon, since clinical studies have demonstrated the deleterious effects of fever in young children and teratogenic effects of hyperthermia during early embryonic development (Graham et al., 1998). Even slight temperature elevations at crucial developmental stages can cause neural defects resulting in brain malformations (Edwards et al., 1974; Walsh et al., 1994; Mirkes et al., 1997; Breen et al., 1999). The adult (30d), postnatal day 7 (P7) and embryonic day 17 (E17) rat brains have been selected for this study, as representative stages of development, to assess the impact of a fever-like temperature shock on neural cell death in vivo.

The specific objectives of this thesis are:

1) To investigate whether a physiologically relevant increase in body temperature activates the cell death program in the adult mammalian brain, where cells are postmitotic and fully differentiated, compared to the non-neural tissues, testis and thymus, which are undergoing cell division and differentiation processes throughout life. Furthermore, if the level of hyperthermic stress used in this study induces cell death, is it via apoptosis?

2) To examine if the pattern of induction of cell death in these adult tissues relates to the pattern of induction of hsp70 following hyperthermia, in vivo.

3) To determine if the effect of hyperthermic treatment on cell death in the brain, changes during development. That is, are dividing neural cells at earlier stages of
development, more prone to hyperthermia-induced cell death than fully differentiated, post-mitotic neural cells in the adult brain?
MATERIALS AND METHODS

2.1. Induction of hyperthermia

The body temperature of 30 day male Wistar rats was elevated 3.5 ± 0.8 °C above normal body temperature (-37.8 ± 0.6 °C) by placement in a dry air incubator preheated to 42 °C. Body temperature was monitored using a rat rectal thermistor probe. Once peak rectal temperature was reached (usually 30 min after placement in the incubator), the elevated temperature was maintained for 1 hr. Rats were then removed from the incubator, given water ad libitum and allowed to recover at room temperature for varying lengths of time. Animals were sacrificed at 2.5, 5, 10, 15 and 24 hr after the onset of heating.

Postnatal day 7 (PD7) rat pups were removed from their mothers and their body temperatures were elevated from 33.8 ± 0.8 °C to 41.3 ± 0.8 °C within 20 min of heating in a dry air incubator preheated to 42 °C. Body temperature was monitored with a needle thermistor probe placed under the forelimb. This elevated temperature was maintained for 1 hr, at which time the pups were removed from the incubator, allowed to cool to normal temperature and then returned to their mothers. The pups were sacrificed at the following time points after the onset of heating: 5, 10, 15 and 24 hr. Three animals were used per time point.

The body temperature of pregnant Wistar rats was elevated 3.5 ± 0.8 °C above normal body temperature (-37.3 ± 1.0 °C) by placement in a dry air incubator preheated to 42 °C. Body temperature was monitored using a rat rectal thermistor probe. Maximal rectal temperature was reached 45 min after placement in the incubator (due to the size of the mothers) and this temperature was maintained for 1 hr. The pregnant rats were then
transferred to room temperature and were sacrificed at 2.5, 5 and 10 hr from the onset of heating.

2.2. Western blot analysis

Control and heated Wistar rats at 5, 10, 15 and 24 hr post-heat were sacrificed by decapitation. Tissue was dissected from brain regions (cerebellum, cerebral hemispheres and core), thymus and testis and homogenized in 0.32 M sucrose. Protein concentrations were determined using the BioRad protein assay. All tissue homogenates were stored at -20 °C. Protein samples were solubilized by boiling for 5 min with an equal volume of dissociation buffer (8 M urea, 2% SDS, 2% β-mercaptoethanol, 20% glycerol). SDS-polyacrylamide gel electrophoresis was performed on 10% gels with a 5% stacking gel, using the discontinuous buffer system of Laemmli (1970). Aliquots of 50 µg of protein were loaded per lane. Gels were stained with Coomassie Blue in order to test for equal protein loading in each lane.

Proteins were electrophoretically transferred onto a nitrocellulose membrane for 16 – 18 hr in a solution of 50 mM boric acid, 4 mM β-mercaptoethanol and 2 mM EDTA at 400 mA. Blots were stained briefly with Ponceau S in order to verify equal loading and efficient transfer of protein. The blots were then washed 4 x 5 min in TBST buffer (10 mM Tris, 0.25 M NaCl, 0.5% Tween-20, pH 7.5) and blocked for 2 hr at room temperature in 5% Carnation milk powder in TBST buffer. Blots were subsequently incubated overnight in primary antibody, diluted in 1% purified BSA in TBST with 0.02% sodium azide. The primary antibodies and their respective dilutions include the following: 'C92' mouse monoclonal anti-human hsp70 (StressGen, SPA-810) at 1:5000;
‘1B5’ rat monoclonal anti-hsc70 at 1:100,000 (Stressgen, SPA-815); ‘29A’ mouse monoclonal anti-rat hsp90 (gift from A.C. Wikström) at 1:5,000; mouse monoclonal anti-hsp60 (gift from R. Gupta) at 1:20,000; and ‘CM-1’ rabbit anti-human active caspase 3 (donated by IDUN Pharmaceuticals) at 1:1500. Following incubation with primary antibody, blots were washed 4 x 10 min in 1% BSA (Sigma Grade) in TBST and incubated with horseradish-peroxidase conjugated secondary antibody diluted to 1:5000 in 1% BSA in TBST for 2 hr at room temperature. Secondary antibodies included anti-mouse IgG (Sigma) for hsp70, hsp60 and hsp90, anti-rat IgG (Sigma) for hsc70, and anti-rabbit IgG (Sigma) for active caspase 3. Blots were subsequently washed 6 x 5 min in TBST. Immunoreactive bands were visualized using ECL Western blotting detection reagents (Amersham RPN 2106). Data shown for the heat shock time course are representative of independent experiments carried out on three sets of animals.

To ensure specificity of the CM-1 antibody for the p18 subunit of the active caspase 3 enzyme, Western blotting analysis was performed. Aliquots of 20 µg of protein from Jurkat total cell extract, induced with camptothecin to undergo apoptosis (BioVision), was loaded on a 15% SDS-PAGE gel with a 5% stacking gel in a tris/tricine buffer system. Protein was transferred to a nitrocellulose membrane and processed as above.

2.3. Tissue preparation for TUNEL and immunocytochemistry

Wistar rats (30d) and P7 pups were anesthetized with sodium pentobarbital (50mg/kg body weight) and perfused intracardially with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brain, thymus and testes were removed from the 30d rats, while only the
brain was removed from P7 pups. All tissues were placed in 4% paraformaldehyde overnight at 4 °C. Pregnant rats were not perfusion-fixed but rather sacrificed by decapitation. Embryos were removed, rinsed in 0.1 M PBS and also placed in 4% paraformaldehyde overnight at 4 °C. For cryoprotection, tissue was then equilibrated through a sucrose gradient series (5, 10 and 20% sucrose in 0.1 M PBS). The tissues were mounted in OCT embedding compound (Miles Inc.) and stored at -70 °C until use.

Glass microscope slides coated with a solution of 1% gelatin and 0.05% chromium potassium sulphate were used to collect 20 μm thick cryostat sections floating on water (for adult thymus and brain, and P7 brain). Cross-sections of testis and embryo were collected directly on the slide. Sections were air-dried for at least 2 hr before processing for TUNEL, immunocytochemistry and staining with a 1% solution of cresyl violet.

2.4. Cell Death Detection by TUNEL

For in situ cell death detection, the TUNEL (terminal deoxynucleotidyl transferase - mediated dUTP nick end labelling) method, based on Gavrieli et al. (1992) was performed, with slight modifications according to Sgonc et al. (1994). The in situ cell death detection kit (Roche Diagnostics) was used and its contents were diluted to half strength for optimum use. Cryostat cut tissue sections were fixed with 4% paraformaldehyde for 5 min and washed with 1X PBS (0.1 M) for 30 min. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol for 30 min, followed by a 5 min rinse with PBS. For cell permeabilisation, slides were immersed in solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice (4 °C) and then rinsed 2 x 5 min in PBS. Slides were incubated for 1 hr at 37 °C in a humid chamber, in TUNEL reaction
mixture containing a 1:10 ratio of Tdt (terminal deoxynucleotidyl transferase) and fluorescein conjugated dUTP. Slides were then rinsed 3 x 5 min in 1% BSA in PBS. Incorporated fluorescein was detected by incubation with a sheep anti-fluorescein antibody conjugated with horse-radish peroxidase for 30 min at 37 °C. After rinsing slides for 3 x 5 min in 1% BSA in PBS, TUNEL labelling was visualized with a 10 min Diaminobenzidine colorimetric reaction with Nickel Chloride (DAB substrate kit, Vector Labs. Burlingame, CA) and light microscopy. Images were captured using Northern Eclipse Software (Empix Inc.).

2.5. Quantitative analysis

TUNEL positive cells were quantified for the tissues examined. The number of TUNEL positive cells in the granule cell layer, molecular layer and deep white matter of the 30 d cerebellum were counted for 10 randomly selected areas of 12,000 μm² for each of three animals. For the thymus, the number of TUNEL positive cells within each of 10 randomly selected areas of 6,000 μm² within the cortex and medulla, were counted. This smaller area was selected in order to avoid overlapping of the cortex and medulla, so that cells could be counted within each region exclusively. For the testis, the number of TUNEL positive cells within 60 seminiferous tubules (approximately 170 μm in diameter) were counted for each of 3 animals per time point and the average number of TUNEL positive cells per seminiferous tubule was determined and plotted against time. Counts of TUNEL positive cells in the external granule layer (egl) of the P7 cerebellum were made within 10 randomly selected areas of 12,000 μm² for each of three animals per time point. The number of TUNEL positive cells within the embryonic neocortex and
developing tectum were counted within several regions, each spanning 800 μm in length of the entire neuroepithelium. For each set of animals this experiment was performed in triplicate. The data plotted are representative of the mean number of TUNEL positive cells within the designated area for each time point after heat shock. Error bars indicate the standard error of the mean. ANOVA statistical analysis was performed and data were considered significant when \( p < .05 \).

2.6. Immunocytochemistry

Tissue sections were rehydrated for 20 min at room temperature in PBS-G buffer (0.1M PBS, pH 7.4, 0.2% Triton-X 100, 0.1% BSA). Sections were then blocked in PBS-G buffer with 10% normal goat serum for 1 hr for CM-1 (IDUN Pharmaceuticals), and with 5% horse serum for 2 hr for PCNA (Pharmingen, 32551A), and C92 (Stressgen, SPA-810) immunocytochemistry. Sections were incubated for 1 hr at room temperature in primary antisera CM-1, diluted 1:3000 in buffer (PBS-G buffer with 0.02% sodium azide) or overnight in mouse anti-PCNA or C92 mouse monoclonal anti-hsp70 both diluted 1:600 in the same buffer. After washing 2 x 5 min in PBS-G buffer, sections were incubated in biotinylated goat-anti-rabbit IgG diluted 1:1000 for 45 min at RT (for CM-1) or for 1.5 hr in biotinylated anti-mouse IgG (for PCNA and C92), diluted at 1:400. Following another 2 x 5 min wash in PBS-G buffer, endogenous peroxidase activity was blocked by immersing sections in 0.3% \( \text{H}_2\text{O}_2 \) in methanol for 30 min. The tissue sections were subsequently washed for 20 min in PBS-G buffer and then processed with the Vectastain Elite ABC kit (Vector Labs). Diaminobenzidine (DAB) was used as the chromogen for hsp70 and active caspase 3 immunocytochemistry, resulting in a brown
immunoreactive product. For PCNA immunocytochemistry, DAB was used with nickel chloride, to give a black immunoreactive product. Following dehydration in ethanol, the sections were cleared in xylene, then coverslipped with a 50/50 xylene-permount mixture. The immunoreactive signal was detected with light microscopy. When either the primary or secondary antibodies were omitted, no immunoreaction was detected. Data shown are representative of independent experiments carried out on three animals per time-point. For each group, immunocytochemical studies were performed in triplicate.
RESULTS

3.1. Effect of hyperthermia on the induction of cell death in the adult brain, testis and thymus

The effect of a fever-like increase in body temperature on the induction of cell death in neural cells of the adult rat brain, which are post-mitotic and fully differentiated, was examined. Hyperthermia-induced cell death was also investigated in the same animals, in the testis and thymus, two non-neural tissues, which unlike the brain, contain dividing and differentiating cells at the adult stage. The TUNEL method was used throughout the study for cytochemical detection of DNA fragmentation, a hallmark of apoptosis (Gavrieli et al., 1992).

3.1.1. Induction of cell death in neural cells of the adult cerebellum

The response of the adult brain to hyperthermia was investigated in the cerebellum. This region was focused on because it is a multi-layered structure where neuronal and glial cell populations are distinct and easily identifiable within the layers. In the control cerebellum and during the heat shock time course which was examined (0 to 24 hr post heat), a few scattered cells stained positively with the TUNEL method as indicated by arrows in Fig. 1, Panels A-E (visualized as a black precipitate). Whole-body hyperthermia (3.5 ± 0.8 °C increase above normal body temperature) did not however, induce cell death in any layer of the adult cerebellum. This was determined by the lack of an increase in TUNEL labelling in the neuronal-enriched granule cell layer (g) and in the glial-enriched deep white matter (dwm) as shown in Fig. 1 at 5, 10, 15 and 24 hr post
Fig. 1. The effect of hyperthermia on the induction of cell death in the adult rat cerebellum

Sagittal sections of 30 d cerebellum from control, 5, 10, 15 and 24 hours after hyperthermia (Panels A-E respectively), were processed with the TUNEL method. Panels A-E: There was no evidence of an increase in TUNEL positive cells in any layer of the cerebellum, from control to 24 hours post heat shock. In the granule cell layer (g) and deep white matter (dwm) of both control and hyperthermic brain, a few scattered TUNEL positive cells were observed (indicated by arrows). At 15 hours (panel D), the inset shows no cell death in the large Purkinje neurons (arrowheads). Panel F: A cerebellar section stained with cresyl violet to show morphology of the cellular layers. dwm, deep white matter; g, granule cell layer; m, molecular layer; p, Purkinje cellular layer; Bar = 55 μm in A-F and in Panel D inset, bar = 13.8 μm.
heat (panels B-E), compared to controls (panel A). Similarly, no increase in cell death was observed in cells of the cerebellar molecular layer (m). Higher magnification of the Purkinje cellular layer (p) (Fig.1D, inset) revealed the same result i.e. no induction of cell death after heat shock in large Purkinje neurons (arrowheads). Panel F shows a cresyl violet-stained sagittal section of the adult cerebellum, demonstrating the morphology of the cellular layers discussed above.

A quantitative analysis of the number of TUNEL positive cells per designated area in each of the cerebellar layers, was performed at specific time-points following hyperthermia. Fig. 5A shows no significant increase (p>.05) in the number of TUNEL positive cells in the granule cell layer, molecular layer or deep white matter of the cerebellum. Other regions of the adult brain examined, namely the cerebral cortex and hippocampus did not show an increase in TUNEL positive cells after hyperthermia (results not shown).

3.1.2. Analysis of hyperthermia-induced cell death in the adult rat testis

The effect of hyperthermia on cell death in the adult testis was examined next. A time course analysis of hyperthermia-induced cell death in the testis is shown at the cellular level in Figs. 2 and 3, and at the quantitative level in Fig. 5. In a cross-section of the seminiferous tubules in the control testis (Fig. 2, panel A), a few TUNEL positive cells were detected which were localized at the periphery of the tubule (indicated by arrows). Based on the histology of the tubules, these TUNEL positive cells are type A spermatogonia, found in the basal layer of the seminiferous epithelium where they divide.
Fig. 2. Hyperthermia induces cell death in the adult rat testis

Time course analysis of heat-induced cell death in cross-sections of the rat seminiferous tubules. TUNEL staining was detected in a few peripheral cells of the seminiferous tubules in the unstressed control rat (Panel A, arrows). The number of TUNEL positive cells increased slightly at 2.5 hours (Panel B, arrows) and a further increase, both in the number of tubules being affected and the number of TUNEL positive cells per tubule, was observed at 5 hours post hyperthermia (Panel C). Asterisks (*) indicate tubules which did not appear to undergo heat-induced cell death. The number of TUNEL positive cells continued to increase at 10 hours (Panel D), and up to 15 hours post hyperthermia (Panel E), which was the time-point of maximal cell death, followed by a decline at 24 hours (Panel F). Bar = 55 μm.
by mitosis, giving rise to further stem cells (Burkitt et al., 1993). Following hyperthermia, there was an increase in TUNEL positive testis cells at 2.5, 5, 10 and 15 hours (panels B-E). The incidence of cell death was most prevalent at 15 hours (Fig. 2, panel E) and had greatly subsided by 24 hours post-hyperthermia (panel F). It was evident by the lack of TUNEL staining in certain cross-sections, that not all testis tubules undergo cell death to the same extent. These ‘resistant’ tubules are indicated by asterisks in Fig. 2, panels C through E. The average number of TUNEL positive cells per tubule (approximately 170μm in diameter) was plotted against time and the effect of heat on the induction of cell death is represented quantitatively in Fig. 5B. This quantitative analysis confirmed that a significant increase in cell death was observed following hyperthermia (p<0.001) and that maximal cell death occurred at 15 hr.

To identify the cell types in the testis that were sensitive to the heat, adjacent sections of a 15 hr post heat shock tubule (the time of maximal cell death), were processed for TUNEL (Fig. 3, panel A), PCNA (proliferating cell nuclear antigen) immunocytochemistry, to identify dividing cells (Fig.3, panel B) (Bravo et al., 1987; Latropoulos and Williams, 1996), or stained with cresyl violet, to morphologically identify cell types, as shown in Fig. 3, panel C. The PCNA method identified mitotically active cells at the periphery of the tubule, which were type A spermatogonia (Chandra et al., 1997). A comparison between TUNEL-labelled tubules and PCNA-stained tubules revealed that the mitotically active type A spermatogonia were heat-sensitive (arrows). The next layer of cells beneath the spermatogonia, proceeding towards the central lumen, consists of primary spermatocytes, characterized by their large size and abundant cytoplasm. These cells, which are undergoing the first round of meiosis were also heat-
Fig. 3. Identification of heat-sensitive cell types in the adult testis

Adjacent sections of a 15 hour testis tubule at high magnification, processed with TUNEL (panel A), PCNA immunocytochemistry (panel B) and cresyl violet stain (panel C), respectively. TUNEL labelling (A) shows that certain cell types were more sensitive to the heat than others. No cell death was observed in cells near the lumen. To aid in the identification of these cells, immunocytochemical analysis of PCNA, which selectively labels mitotically active spermatogonia (B), was performed. Comparative analysis shows that type A spermatogonia (indicated by arrows) were susceptible to heat-induced cell death. The TUNEL positive cells located in the layer beneath the spermatogonia, proceeding towards the central lumen (indicated by large arrows) corresponded to primary spermatocytes, as was evident by cresyl violet staining (C). It was evident by the lack of TUNEL labelling in the lumen (Panel A) that spermatids (s1) and spermatozoa (s2) in Panel C, located near the lumen did not undergo cell death. Bar = 13.8 μm.
sensitive as demonstrated by the TUNEL labelling (indicated by large arrows). The absence of TUNEL staining in spermatids (s1) and spermatozoa (s2), located near the central lumen, suggests that these testis cell types are not heat-sensitive (refer to the cresyl violet stained section, Fig. 3C for the location of the spermatids and spermatozoa).

3.1.3. Analysis of hyperthermia-induced cell death in the adult thymus

The thymus is another tissue, which undergoes cell division and differentiation even in the adult. The tissue was isolated at time-points following hyperthermia and examined for the presence of TUNEL positive cells. As shown in Fig. 4 panel A, scattered TUNEL positive cells were present in the cortex (c) and medulla (m) of control rat thymus (indicated by arrows). A major transient increase in TUNEL positive cells was observed at 10 hr post heat (Fig. 4, panel D), followed by a decline at 15 hr (Fig. 5, panel E). The cortex and medulla are clearly distinguished by cresyl violet staining in Panel F. A quantitative time course analysis (Fig. 5C) revealed that the number of TUNEL positive cells in the thymus peaked at 10 hr post-hyperthermia and was greater in the cortex of the thymus compared to the medulla. The increase in cell death was statistically significant (p<0.001).

3.2. Is the level of hyperthermic stress extreme enough to cause necrosis?

Studies in tissue culture have shown that the mechanism of cell death changes from apoptosis to necrosis above a critical heat stress (Harmon et al., 1991). It was of interest to determine whether the level of whole body hyperthermia (3.5 ± 0.8 °C increase in body temperature) used in the present study, triggered apoptosis or necrosis in tissues
Fig. 4. Hyperthermia induces cell death in the adult rat thymus

Panels A-E: TUNEL method performed on sections of control (A), 2.5 hour (B), 5 hour (C), 10 hour (D), and 15 hour (E) thymus, post hyperthermia. A few scattered TUNEL positive cells were present in the control thymus, indicated by the arrows. After hyperthermia, there was a gradual increase up to 5 hours post-heat, peaking at 10 hours (Panel D) and declining by 15 hours. TUNEL positive cells (indicated by arrows) were evident in both the cortex (c) and medulla (m), however the majority were present in the cortex. Panel F: A cresyl violet-stained thymus section showing the morphology of the cortex and medulla. c, cortex; m, medulla. Bar = 55 μm.
Fig. 5. Quantitative analysis of the effect of hyperthermia on adult cerebellum, testis thymus

The average number of TUNEL positive cells are shown for each tissue over the heat shock time course. Error bars indicate the standard error of the mean (sem). Asterisks (*) indicate the time-point of maximal cell death. Statistical analysis was performed using ANOVA and data were considered significant when $p<.001$. Panel A: The mean number of TUNEL positive cells per 12,000 $\mu m^2$ area, were plotted for each layer of the cerebellum over the heat shock time course. The cerebellar layers which were examined, included the granule cell layer, molecular layer and deep white matter. No significant change in the number of TUNEL positive cells was observed in any layer ($p>.05$), in accordance with analysis at the cellular level, shown in Fig. 1. TUNEL counts for the Purkinje cellular layer are not shown because none of these cells were observed to be TUNEL positive under control or hyperthermic conditions. Panel B: The average number of TUNEL positive cells per cross-section of tubule (average diameter 170 $\mu m$) was determined for each hyperthermic time-point and plotted. A significant increase in TUNEL positive cells per tubule was observed ($p<.001$), peaking at 15 hours post hyperthermia (asterisk) and subsequently declining at 24 hours. Panel C: The number of TUNEL positive cells per 6000 $\mu m^2$ area of cortex and medulla in the thymus was plotted against time. An increase in TUNEL positive cells was seen in both the cortex and medulla over the heat shock time course, peaking at 10 hours post heat (asterisk) and declining almost to basal levels by 24 hours, with the greater incidence of cell death in the cortex.
which were examined. In Figures 1 to 4, cell death was assayed by the TUNEL method which detects DNA fragmentation in situ, one of the hallmarks of apoptosis (Gavrieli et al., 1992). Recent data suggests, however, that the TUNEL assay does not exclusively label apoptotic cells, and may label cells in the latter stages of necrosis, which also undergo breakdown of DNA (Gold et al., 1994). These limitations warranted the use of an additional biochemical marker of apoptosis at the single-cell level, in order to confirm that the mode of hyperthermia-induced cell death in our study was indeed apoptosis.

Caspase 3, one of the effectors of apoptosis which targets many cellular substrates to bring about apoptotic cell death, is activated by cleavage of its proenzyme (32 kDa form) to yield two active subunits, p18 and p12 which together form the active enzyme (Raff, 1998). Caspase 3 has been shown to be activated in heat-induced cell death in vitro (Chan et al., 1998) and is specific for apoptosis, not necrosis (Armstrong et al., 1997). Thus the activation of caspase 3 was used as an improved index of apoptosis.

3.2.1. Specificity of the CM-1 antibody for active caspase 3

The CM-1 antibody was characterized by Srinivasan et al. (1998) and demonstrated to be useful for in situ detection of activated caspase 3. To ensure that the CM-1 antibody recognized only the p18 subunit of active caspase 3 (molecular weight of 18 kDa) and not the 32 kDa proenzyme (unprocessed form), Western blotting was performed using a protein extract from Jurkat cells, in which apoptosis had been induced with camptothecin (Johnson et al., 1997). Fig. 6 shows that the CM-1 antibody recognized the large p18 active subunit of caspase 3, not the unprocessed form (32 kDa).
Fig. 6. Specificity of the CM-1 antibody for active caspase 3

Western blot analysis of Jurkat total cell extract (20 μg protein), induced to undergo apoptosis with camptothecin, demonstrated the specificity of the CM-1 antibody for the large p18 subunit of the active enzyme caspase 3. The unprocessed form (32 kDa) was not detected by the CM-1 antibody. The molecular weight markers, 45, 34 and 17 kDa are indicated by arrows. Although the identity of the other CM-1 cross-reactive band is unknown, it did not interfere with the specificity of the antibody for active caspase 3 in immunocytochemistry. This was demonstrated by the absence of CM1 immunoreactivity in caspase 3 (-/-) knockout mice (Srinivasan et al., 1998).
JURKAT CELL EXTRACT

45 kDa →
34 kDa → ▶ 32 kDa
17 kDa → ▶ P18
3.2.2. Immunocytochemical analysis of active caspase 3 in the adult testis and thymus

To confirm that the mode of cell death in the adult rat testis and thymus was indeed apoptosis, active caspase 3 immunocytochemical analysis was performed. Cross sections of control (Fig. 7, panel A) and 15 hr heat shock testis (Fig. 7B) were processed for active caspase 3 immunocytochemistry. In the control testis (Fig. 7A), some peripherally located spermatogonia were positive for caspase 3 immunoreactivity (arrows) similar to the position of TUNEL labelling of control testis (Fig. 2A). At 15 hr post-hyperthermia, the time-point of maximal cell death in the testis, active caspase 3 immunoreactivity was observed in several cells near the tubule periphery (indicated by arrowheads in Fig. 7B). The activation of caspase 3 is a cytoplasmic event (Earnshaw et al., 1999) and thus immunopositive staining was localized to the cytoplasm of the large primary spermatocytes (Panel B, inset). Studies of experimental cryptorchidism (Socher et al., 1997) have shown that mild exposure of the testis to abdominal temperatures (~37°C) is sufficient to induce apoptotic pathways. Analysis of caspase 3 activation was also performed on sections of control (Fig.7C) and 10 hr hyperthermic thymus (Fig.7D). Caspase 3 immunoreactivity was detectable in a few scattered cells of the control thymus as indicated by arrows. At 10 hr post-hyperthermia, the time-point of maximal cell death in the thymus, a significant increase in active caspase 3 immunoreactivity was detected predominantly in the cortex (c)(arrows) as opposed to the medulla (m), similar to TUNEL.
Fig. 7. Analysis of caspase 3 immunoreactivity in the adult testis and thymus

Cross-sections of control (Panel A) and 15 hour hyperthermic testis (Panels B) were processed for active caspase 3 immunocytochemistry. Caspase 3 immunoreactivity was detected in a few peripherally located spermatogonia (Panel A, arrows). At 15 hours post-heat, a significant increase in active caspase 3 immunoreactivity, was detected in primary spermatocytes of several tubules (Panel B, arrowheads). Panel B, inset reveals the cytoplasmic localization of active caspase 3 enzyme within the cell. Sections from control (panel C) and 10 hour hyperthermic (panel D) thymus were also processed with active caspase 3 immunocytochemistry. Scattered cells within the control thymus demonstrated active caspase 3 immunoreactivity (C). At 10 hours post hyperthermia, active caspase 3 immunoreactivity was detected in the thymus cortex (panel D), where TUNEL positive labelling was previously observed (Panel D).

c. cortex; m. medulla. Bar = 27.5 μm for Panels A and B; Bar = 5.5 μm for Panel B inset; Bar = 55 μm for Panels C and D.
staining. Thus, the mode of hyperthermia-induced cell death in the thymus was determined to be apoptotic.

3.3. Do the patterns of heat shock-induced apoptosis relate to the patterns of hsp70 induction in the adult cerebellum, testis and thymus?

Figures 1 to 7 demonstrate that the adult cerebellum was not as prone to heat-induced apoptosis as the testis and thymus. To correlate the heat sensitivities of the adult cerebellum, testis and thymus to the pattern of hsp70 induction after hyperthermia, a Western blot analysis was performed. Basal hsp70 levels were highest in the thymus (THY) (Fig. 8), and not detectable in the cerebellum (CB) or testis (TE). After hyperthermia, these tissues induced hsp70 but to different extents. A prominent induction of hsp70 in the thymus and cerebellum was evident by 5 hr and levels were maintained up to 15 hr post-hyperthermia, declining slightly at 24 hr in the cerebellum. The testis displayed a less robust and delayed induction of hsp70, which did not peak until 15 hr post-hyperthermia. Hsp70 inducibility correlated with resistance to hyperthermia-induced cell death in the cerebellum, but not in the thymus which shows high levels of cell death, yet a robust induction of hsp70.

The induction of hsp70 was also examined immunocytochemically in the cerebellum, testis and thymus, to determine if any differences in the patterns of induction of cell death and hsp70 exist at the cellular level. Hsp70 immunoreactivity was not detected in the control cerebellum as demonstrated in Fig. 9A. This was supported by the Western analysis in Fig. 8, in which basal levels of hsp70 in the cerebellum were not detected. Accumulation of hsp70 protein was detected by 5 hr (Fig. 9, panel B) post
Fig. 8. Time course analysis of hsp70 induction in adult rat tissues following hyperthermia

The induction of hsp70 was analyzed in control (C) animals and at 5, 10, 15, and 24 hours following hyperthermia. Western blot analysis of cerebellum (CB), thymus (THY) and testis (TE) showed high basal levels of hsp70 in the control thymus, but not in cerebellum or testis. Following hyperthermia, an accumulation of stress-inducible hsp70 was observed in the cerebellum and thymus at 5 hours and levels are maintained up to 15 hours, after which they decline slightly in cerebellum. A delayed and less robust induction of hsp70 was seen in the testis, peaking at 15 hours post heat and declining thereafter.
Fig. 9. Analysis of hsp70 immunoreactivity in the control and hyperthermic adult cerebellum

Hsp70 was not detected in any layer of the control cerebellum (Panel A). Immunocytochemical analysis of hsp70 following hyperthermia revealed a strong induction at 5 and 10 hours (Panels B and C, respectively) in the granule cell layer (g), blood vessels (BV) (indicated by arrows), and glial cells of the deep white matter (dwm). Hsp70 was not induced in large Purkinje neurons (indicated by asterisks in Panel C, inset). At 15 hours post-hyperthermia (Panel D), the hsp70 signal decreased. BV, blood vessels; dwm, deep white matter; g, granule cell layer; m, molecular layer; p, Purkinje cellular layer. Bar = 55 µm for Panels A through D; Bar = 13.8 µm for Panel C, inset.
hyperthermia and localized to the granule cell layer (g), scattered cells in the molecular layer and within the vasculature (BV). Hsp70 immunoreactivity was strongest in the cerebellum at 10 hr (Panel C), where it was localized to the neurons in the granule cell layer (g), blood vessels (BV), deep white matter (dwm) and scattered glia throughout the molecular layer (m). The signal was not detected in the Purkinje neurons (indicated by asterisks) (Fig. 9C, inset). The signal decreased by 15 hr post hyperthermia (Fig. 9D). It was evident that, regardless of their ability to induce hsp70 following hyperthermia, adult neural cells in the cerebellum did not undergo heat-induced apoptosis. There was no evidence of an increase in TUNEL positive cells in any cerebellar layer following hyperthermia (Fig.5A).

The induction of cell death and hsp70 was analyzed in adjacent sections of the adult testis. In the control testis (Fig. 10, panel A), scattered TUNEL positive cells were evident and previously identified as type A spermatogonia, based on cellular location. An adjacent section of the control testis revealed no hsp70 immunoreactivity in these cells (Fig. 10, panel B). At 10 hr post-heat (Fig. 10, panel C) cell death was observed by TUNEL in one of the tubules shown (indicated by asterisk). The other tubule (arrow) was clearly resistant to the effects of the heat, based on the absence of TUNEL labelling. In an adjacent testis section at 10 hr post heat (Panel D), processed for hsp70 immunoreactivity, it was evident that the tubule which did not undergo cell death, induced hsp70, and this signal was primarily localized to cells near the periphery. Hsp70 immunoreactivity was also detected in the interstitial Leydig cells (arrowheads), which were not observed to be TUNEL positive. At 15 hr post-hyperthermia, the time-point of maximal cell death according to quantitative data (Fig. 2B) and maximal hsp70 induction
Fig. 10. Induction of cell death and hsp70 in rat testis following hyperthermia

TUNEL positive cells were identified in the control testis (Panel A) in Type A spermatogonia localized at the periphery of the tubule (arrows). Hsp70 immunoreactivity was not detected in the control testis (Panel B). Following hyperthermia, a correlation was observed between induction of hsp70 and sensitivity to cell death. At 10 and 15 hours post heat (Panels C and E) respectively, some tubules were undergoing cell death (arrows), while others were resistant to heat-induced cell death (asterisks). A comparison of adjacent sections labelled with TUNEL (Panels C and E) and processed for hsp70 immunocytochemistry (Panels D and F) revealed that tubules which strongly label with TUNEL did not induce hsp70 (arrows). In contrast, hsp70 immunoreactivity was detected in tubules that were resistant to heat-induced cell death (asterisks), specifically in the peripherally located type A spermatogonia. Bar = 27.5 μm.
(Fig. 8), the same phenomenon seen at 10 hr was observed in adjacent testis sections (Fig. 10, panels E and F). That is, hsp70 was induced to a greater extent in tubules not undergoing cell death (Panel F, arrow), than in those tubules with many TUNEL labelled cells (asterisks). Thus a correlation existed between sensitivity to heat-induced cell death and lack of hsp70 induction in this tissue.

The induction of hsp70 and cell death following hyperthermia in the adult thymus was examined next. Western blot analysis demonstrated high basal levels of hsp70 in the control thymus and robust induction of hsp70 after hyperthermia (Fig. 8). The thymus was also sensitive to heat-induced cell death, where maximal cell death occurred at 10 hr (Fig. 4D and 5C). Fig. 11, panel A shows that under control conditions there is a low level of cell death as evidenced by TUNEL staining in the cortex (c). Basal levels of hsp70 were detected primarily in the cortex (Fig. 11B). TUNEL positive cells and hsp70 immunoreactive cells are indicated by arrows in Fig. 11. At 10 hr post hyperthermia, the time-point of maximal cell death, TUNEL positive cells were detected mainly in the cortex (Panel C), but also in the medulla. Hsp70 immunoreactivity was strongly detected in the medullary regions of the thymus (Fig. 11D). At 15 hr post-hyperthermia, when cell death began to decline (Fig. 11E), hsp70 immunoreactivity was observed primarily in the cortex (Panel F), similar to the control thymus (Panel B). Thus it appears that cortical cells were prone to heat-induced cell death despite high basal hsp70 levels and cells in the medulla were also heat sensitive, despite the induction of hsp70 in this region. Since the patterns of hsp70 induction and cell death do not correlate in all adult tissues examined, this suggests that the induction of hsp70 is not the only factor determining whether cells will be sensitive or resistant to hyperthermia-induced cell death.
Fig. 11. Hyperthermia-induced cell death and hsp70 immunoreactivity in thymus

Adjacent sections of control (Panel A), 10 hour (Panel C), and 15 hour post heat shock (Panel E) thymus were processed for TUNEL labelling and hsp70 immunoreactivity (Panels B, D, and F, respectively). Cell death was evident in the cortex (c) of control thymus (A), not in the medulla (m). Basal levels of hsp70 were also localized to the cortical areas of the thymus (Panel B). At the time-point of maximal cell death (10 hours, Panel C), cell death was observed in the cortex (c) and medulla (m), but more predominant in the cortex. Hsp70 immunoreactivity was detected in the thymus medulla, and to a lesser extent in the cortex (Panel D) at the same time-point. By 15 hours, the TUNEL signal (Panel E) and hsp70 immunoreactivity (Panel F) both decreased, and hsp70 was localized primarily to the cortex as it was in the control (Panel B). c, cortex; m, medulla. Bar = 55 μm.
3.4. Do the levels of constitutive hsp90, hsc70 and hsp60 in the unstressed adult rat correlate to heat-induced apoptosis?

The possibility that differences in the levels of constitutively expressed hsps could account for the differential sensitivities to heat-induced cell death in the adult cerebellum, testis and thymus was next investigated. The levels of hsp90, hsc70 and hsp60 in the unstressed cerebellum (CB), thymus (THY) and testis (TE) were analyzed by Western blotting. Hsp90 protein levels were highest in non-neural tissues, particularly the testis (Fig. 12). An analysis of the hsc70 levels among the unstressed tissues revealed comparatively lower levels in the testis, compared to the thymus and cerebellum. Hsp60 protein was detected in all three tissues of the unstressed animal, with negligible variation in the levels between tissues. Although low levels of hsc70 in the testis may account for heat-induced apoptosis in this tissue, the thymus showed high levels of all three constitutive hsps and was still prone to hyperthermia-induced cell death.

3.5. Is the developing brain at late embryonic and early postnatal stages susceptible to hyperthermia-induced apoptosis?

Dividing and differentiating cells in the adult rat testis and thymus were demonstrated to induce apoptosis in response to hyperthermia, as assayed by increased TUNEL-labelling and active caspase 3 immunoreactivity. In contrast, cells of the adult cerebellum, which are neither dividing nor differentiating, but post-mitotic, were resistant to hyperthermia-induced cell death. The induction of hsp70 and cell death did not correlate in all tissues examined. The response of dividing neural cells at earlier stages of development to hyperthermia was next investigated. Regions of active neurogenesis
Fig. 12. Hsp90, hsc70 and hsp60 levels in adult tissues of the unstressed rat

Western analysis of adult cerebellum (CB), thymus (THY) and testis (TE) demonstrated tissue-specific differences in the levels of hsp90, hsc70 and hsp60 in the control rat. Hsp90 levels were highest in the non-neural tissues, testis and thymus. Hsc70 was constitutively expressed in all tissues, however its level of expression was much less in the testis. Hsp60 protein was present in all tissues examined and levels did not vary between the unstressed cerebellum, thymus and testis.
were selected for examination, including the external granule layer of the cerebellum at postnatal day 7 (PD7) and the neuroepithelia of the developing cerebral cortex and colliculi at embryonic day 17 (E17).

3.5.1. Analysis of hyperthermia-induced apoptosis in the external granule cell layer of the cerebellum at postnatal day 7

The external granule cell layer (egl) of the cerebellum at postnatal day 7 (P7) consists of actively proliferating cells which migrate through the molecular layer (ml) and Purkinje cellular layer (pcl) to their final destination at the inner granule cell layer (igl) where they achieve terminal differentiation (Fig. 13, panel E) (Altman, 1997). The dividing cells of the egl in P7 rats (indicated by arrowheads) were susceptible to heat-induced cell death as evidenced by TUNEL positive staining (compare Fig. 13A (control) to C (10hr post heat)). These results were confirmed by quantitative analysis in Fig. 16A which indicates a significant increase in TUNEL positive cells, observed in the egl at 10hr post-hyperthermia, with a subsequent decline at 24 hr (p<.01). In addition to the egl, TUNEL positive cells were also detected in the deep white matter (dwm) in both control (Fig. 13A) and 10hr (Fig. 13B) cerebellar sections (indicated by arrows). This was not an effect of the heat since a previous study showed that in the unstressed P7 rat cerebellum, the deep white matter contains several TUNEL positive cells, which were determined to be astrocytes naturally undergoing cell death (Krueger et al., 1995).

Adjacent sections of the control and 10hr P7 cerebellum, labelled with TUNEL (Fig. 13, panels A and C), were immunocytochemically stained with the CM-1 antibody to detect the presence of activated caspase 3, and shown at higher magnification (Fig. 13,
Fig. 13. **Hyperthermia induces cell death in the external granule cell layer of the postnatal day 7 cerebellum**

Adjacent sections of control (A and B) and 10 hour (C and D) postnatal day 7 (PD7) cerebellum were processed for TUNEL (A and C) and active caspase 3 immunocytochemistry (B and D). Panels A and C reveal the increase in TUNEL positive cells (arrowheads) in the egl at 10 hours post hyperthermia (C) compared to the control (A). Arrows in panels A and C, indicate that cells undergoing cell death in the dwm in both the control and 10 hour cerebellum, were likely astrocytes. Panels B and D are higher magnifications of adjacent sections of the boxed areas in Panels A and C respectively. There was no evidence of caspase 3 activation in the control egl (B). At 10 hours post-heat, however, caspase 3 immunoreactive cells were observed in the egl (D), indicated by large arrows. Panel E: P7 cerebellar section stained with cresyl violet demonstrating morphology of the layers. Panel F: Adjacent section processed with PCNA immunocytochemistry to label actively dividing cells revealed that cells of the egl are actively dividing at P7. dwm, deep white matter; egl, external granule cell layer; igl, internal granule cell layer; ml, molecular layer; pcl, purkinje cellular layer. Bar = 27.5 μm for Panels A, C, E and F. Bar = 13.8 μm for Panels B and D.
panels B and D). It was evident by the presence of CM-1 immunoreactivity in cells of the egl that caspase 3 is activated after the hyperthermic treatment (indicated by arrows). Thus the mode of cell death induced by this level of hyperthermia was apoptosis. It is important to note that caspase 3 immunocytochemistry and TUNEL would not be expected to necessarily label the same cell, since activation of caspase 3 occurs upstream of DNA fragmentation, which is detected by TUNEL. To confirm that cells in the external granule cell layer were mitotically active at postnatal day 7, PCNA (proliferating cell nuclear antigen) immunocytochemistry was performed. This antigen selectively labelled cells in the external granule layer compared to other cerebellar layers (Fig. 13, panel F).

3.5.2. The effect of hyperthermia on dividing cells in the cortical neuroepithelium and tectal neuroepithelium at embryonic day 17

Cerebral cortical cells, which are postmitotic in the adult rat, did not demonstrate heat-induced cell death (data not shown). At embryonic day 17 (E17) these cells, which form the neocortex, are in a dividing state and their response to hyperthermia was next examined. Under control conditions (Fig. 14, panel A), TUNEL positive cells were not visible in the neocortex. Following hyperthermia, there was a major increase in TUNEL reactivity at the cellular level at 10 hr post heat, particularly in the neuroepithelium (ne) (Fig. 14C). This was confirmed by a quantitative analysis in Fig. 16B demonstrating a significant increasing trend of cell death following maternal hyperthermia (p<.001). Activation of caspase 3 was observed in cells of the same region, at 10 hr post heat,
Fig. 14. The effect of hyperthermia on dividing cells in the neocortex at embryonic day 17

Adjacent sections of control (A and B) and 10 hour (C and D) cortical neuroepithelium at embryonic day 17 (E17), were processed by the TUNEL method (A and C) and active caspase 3 immunocytochemistry (B and D). A major increase in TUNEL positive cells was detected in the neuroepithelium (ne) at 10 hour post-hyperthermia (C) compared to control (A), indicated by arrowheads. Fewer TUNEL positive cells were evident in the subventricular zone (sv) (Panel C, arrows). Active caspase 3 immunoreactivity demonstrated a similar induction in the neuroepithelium at 10 hour post heat (panel D, arrowheads), with a negligible signal in the control (B).

Scattered CM-1 positive cells were seen in the subventricular zone (panel D, arrows). Panel E: A cresyl violet stained section of the developing cortex demonstrating morphology of the cellular layers. Panel F: Adjacent section to (E) processed with PCNA immunocytochemistry showing actively dividing cells (black precipitate) in ne and sv regions. cp. cortical plate; iz. intermediate zone; lv. lateral ventricle; mz. mitotic zone; ne. neuroepithelium; sv. subventricular zone; sz. synthetic zone; wm, white matter.

Bar = 27.5μm.
(compare Fig. 14, panels C and D), which confirmed the mode of cell death to be apoptosis.

A histologically stained section of the developing cerebral cortex at E17 (Fig. 14, panel E) shows that the neuroepithelium consists of a spatially segregated mitotic zone (mz), where cells are predominantly in the mitotic phase, and a synthetic zone (sz) where most cells are duplicating their DNA. At this stage of cortical development (E17) the neuroepithelium is maximal in thickness and almost all proliferating cells are confined to this region (Bayer and Altman, 1991). PCNA immunoreactivity which identifies dividing cells (Fig. 14, panel F) was localized primarily to cells within the neuroepithelium (ne), while the intermediate zone (iz), white matter (wm) and the cortical plate (cp), which contains young postmitotic cells of future cortical layers V and VI, were not immunoreactive. Given the histology of the neocortex, it appeared that cells in both the mitotic (mz) and synthetic zones (sz) of the neuroepithelium were more susceptible to heat-induced apoptosis than the non-proliferating cells of the cortical plate and intermediate zone at E17 (Fig. 14, panels C and D). In addition to the neuroepithelium, some scattered cells in the subventricular zone also underwent apoptosis (indicated by arrows in Fig. 14C and D).

Examination of the E17 neocortex revealed another neuroepithelial layer in the developing midbrain, which labelled strongly with TUNEL. This region, identified as the tectal neuroepithelium, later becomes the inferior and superior colliculi of the brain. The cells in this neuroepithelial region are also mitotically active at E17 and undergo DNA synthesis as indicated by PCNA immunoreactivity (Fig. 15F), compared to cells in the differentiating field (df) which are not dividing, yet some of them are still susceptible to
Fig. 15. Hyperthermia induces apoptosis in the tectal neuroepithelium at embryonic day 17

Panels A-D: Adjacent sections of control (A,B) and 10 hour (C,D) tectal neuroepithelium were processed for TUNEL and active caspase 3 immunocytochemistry. Hyperthermia induced cell death in the neuroepithelium (ne) at 10 hours (C) compared to control (A) as shown by the presence of TUNEL positive cells (arrows). Similarly, active caspase 3 immunoreactivity was strongly detected at 10 hours post heat (D) and negligible in the control (B). The majority of TUNEL positive and active caspase 3 immunoreactive cells were present in the neuroepithelium, compared to the differentiating field (df), which primarily contains cells that have ceased dividing and are undergoing maturation processes. Panel E: A cresyl violet stained section of developing tectum showing the distinction between the neuroepithelium (ne) and the differentiating field (df). Panel F: Adjacent section to (E) processed for PCNA immunocytochemistry demonstrating that actively dividing cells are present in the ne. aq, aqueduct; df, differentiating field; ne, neuroepithelium. Bar = 27.5μm.
heat-induced cell death. A significant increase (p< 0.001) was observed in the number of TUNEL positive cells, parallel to that seen in the cortical neuroepithelium (Fig. 16B). Cellular observations demonstrate this increase in TUNEL positive (Fig. 15, panels A and C) and active caspase 3 immunoreactive cells (Fig. 15, panels B and D), from control to 10 hr post heat, within the same region of adjacent sections. Thus the tectal neuroepithelium provided another example of heat-induced apoptosis in mitotically active neural cells.
Fig. 16. Quantitative time course analysis of the effect of hyperthermia on cell death in the developing brain at postnatal day 7 and embryonic day 17

The mean number of TUNEL positive cells per 12,000 μm² area, were plotted for the external granule cell layer of the cerebellum at postnatal day 7 in Panel A. The mean number of TUNEL positive cells within several regions spanning 800 μm in length along the neuroepithelium at E17, are shown over the heat shock time course in Panel B. Error bars indicate standard error of the mean (SEM). The asterisk (*) indicates the time-point of maximal cell death. Statistical analysis was performed using ANOVA test and significant data yielded p < .05. Panel A: The external granule layer (egl) of the cerebellum at PD7 showed a significant increase in mean number of TUNEL positive cells (p < .01), peaking at 10 hours post hyperthermia and declining thereafter towards basal levels. Panel B: The developing cerebral cortex and tectal neuroepithelium at E17 were susceptible to heat-induced cell death as demonstrated by the significant increase in number of TUNEL positive cells up to 10 hours post heat (p < .001).
A

P7 Cerebellum

# Tunel positive cells (+/- sem) per 12,000 μm² area

Time (hrs)

B

E17 Cerebral Cortex and Tectal Neuroepithelium

# Tunel positive cells (+/- sem) per 800 μm length

Time (hrs)
DISCUSSION

The expression of heat shock genes in the mammalian brain has been extensively studied under normal and hyperthermic conditions (Brown, 1990; 1994; Brown and Sharp, 1999). Following hyperthermia, previous studies report a strong glial induction of hsp70, while large neurons, which do not induce hsp70, maintain a high constitutive expression of hsp90 and hsc70 (Manzerra et al., 1993; Brown, 1994; Manzerra and Brown, 1996; Bechtold et al., 2000). If the induction of hsp70 is an indicator of stress, these results would suggest that populations of large neurons are not sensitive to the hyperthermic insult. It has been proposed that these neurons are either buffered against the effects of heat stress by their high levels of constitutive hsp (Manzerra et al., 1993) or perhaps protected at their synapses by hsps localized to their pre- or post-synaptic faces (Bechtold et al., 2000).

Hyperthermia has also been demonstrated to induce the apoptotic response in cells, resulting in a loss of cell viability (Harmon et al., 1991; Sakaguchi et al., 1995). The effects of hyperthermia on the induction of cell death in the brain has been the focus of several studies on the use of hyperthermia as a treatment for malignant brain tumours. These studies reveal that the minimum temperature required for necrosis is 43.9°C, while lower temperatures permit reparative responses (Fike et al., 1991; Feyerabend et al., 1997). Apart from these studies on extreme temperatures, very few studies have actually examined the effect of a fever-like temperature shock on the induction of cell death in the adult mammalian brain in vivo.

This study was undertaken to investigate whether a fever-like increase in temperature, which is sufficient to activate the heat shock response, could induce cell
death in neural cells, which are fully differentiated and post-mitotic in the adult. This was compared to the non-neural tissues, testis and thymus which, contain populations of actively dividing and differentiating cells throughout life. To further assess the impact of a fever-like increase in temperature on neural cell death in vivo, the late embryonic and early postnatally developing brain, which contain populations of dividing neural cells, were also examined.

4.1. Effect of hyperthermia on cell death in the adult brain, testis and thymus

The incidence of cell death was assayed throughout this study at the cellular level, by the TUNEL method, which labels DNA fragmentation (Gavrieli et al., 1992). TUNEL staining revealed that hyperthermia induced cell death in the adult thymus and the testis, but not in any glial- or neuronal-enriched layer of the adult rat cerebellum. Although, neural cell death via apoptosis occurs developmentally, and in response to environmental and pathological stress (Krueger et al., 1995; Pettmann and Henderson, 1998; Gu et al., 1999), the level of hyperthermia employed in this study (3.5 ± 0.8°C for 1 hour) did not activate apoptotic pathways in the adult rat brain. In contrast, the adult thymus experienced heat-induced cell death in the cortex and to a lesser extent in the medulla. Although cell death was observed in cortical cells of the control thymus, it increased significantly following hyperthermia, peaking at 10 hr post-heat shock and subsequently declining by 15 hr. The adult testis was also sensitive to hyperthermia-induced cell death over the time course. In the unstressed rat, cell death was observed in type A spermatogonia. Following hyperthermia, the incidence of cell death increased in these spermatogonia as well as in primary spermatocytes, peaking at 15 hr post heat and
declining thereafter. It was interesting that specific cells of the testis and thymus would activate cell death pathways in response to hyperthermia, while neural cells of the adult cerebellum were resistant.

The few studies that have examined the effect of hyperthermia on cell death in the mammalian brain, have demonstrated negligible effects, unless very high temperatures are employed. It is important to note that the thermal dose is dependent on both the magnitude of increase above normal body temperature and duration of the exposure. One study showed that when dogs were exposed to hyperthermia (42°C for 1 hr) by extracorporeal heating of the blood, and assessed histologically one week after heating, they did not suffer any neurological deficits, nor damage to the spinal cord or any of brain regions examined (Takahashi et al., 1999). This represented a 5°C temperature increase from their normal body temperature of 37°C. Similarly, no cell death was observed in the rat brain (normally maintained at ~38°C) after a bacterial endotoxin, lipopolysaccharide-induced fever, (Mouihate and Pittman, 1998) or following a temperature shock of 41°C (McCabe & Simon, 1993). Mouihate and Pittman (1998) suggest that a protective system, yet to be defined, may be present in the CNS, blocking the deleterious effects of infectious agents and cytokines. In the mouse brain (normally maintained at ~37°C), an increased hyperthermic stress (43.5°C for 30 minutes) was reported to induce hsp70 and apoptosis which was detected by internucleosomal DNA fragmentation analysis (Leoni et al., 2000). The maximum induction of hsp70 coincided with maximal DNA damage, however no distinction is made between the responses of different regions and or cell-types to the heat shock. Overall, it appears that temperatures greater than 43°C can cause cell death in the mammalian brain which is normally
maintained at a temperature of 37-38°C (Fike et al., 1991; Leoni et al., 2000). This is in contrast to the thermal exposure used in this study (3.5 ± 0.8°C for 1 hour), which is not detrimental to adult neural cells, compared to certain thymus and testis cells of the same animals.

4.2. Does the level of hyperthermic stress induce cell death via apoptosis or necrosis?

Studies in tissue culture have demonstrated that the mechanism of cell death changes from apoptosis to necrosis above a critical heat load (Harmon et al., 1990; Samali et al., 1999). For example, heat shock at a mild temperature (42°C) induces hsp70, whereas higher temperatures (44-45°C) induce apoptosis, and extreme heat stress (46°C) results in necrosis (Harmon et al., 1990; Samali et al., 1999). In vitro studies suggest that cells fail to activate hsp70 at a higher temperature and this lack of hsp induction is the cause rather than the consequence of cell death (Samali et al., 1999). To confirm that the cells in the thymus and testis were undergoing heat-induced apoptosis in the present investigation, a biochemical assay of apoptosis was employed. Immunocytochemical analysis of the activation of caspase 3 confirmed that the mode of heat-induced cell death was indeed apoptosis in these tissues and that the hyperthermic exposure used in this study was not severe enough to cause necrosis. The exact pathway of heat shock-induced apoptosis is as yet undefined. However, it is known that it occurs via activation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway (reviewed by Kyriakis and Avruch, 1996; Verheij et al., 1996). This in turn triggers activation of the caspase cascade, which targets several proteins to bring about apoptotic cell death. Caspase 3 activation is involved in apoptosis stimulated by fluid percussion-induced traumatic brain
injury in rats (Yakovlev et al., 1997), potassium or serum deprivation (Armstrong et al., 1997), optic nerve transection (Kermer et al., 1999), cerebral ischemia (Namura et al., 1998), the negative selection of autoreactive thymocytes (Alam et al., 1997) and by heat shock (Chan et al., 1998). Thus, the activation of caspase 3 is an effective marker of apoptosis.

4.3. Examination of the patterns of induction of cell death and hsp70 following hyperthermia.

Besides activation of cell death pathways, hyperthermia is known to induce the heat shock response. One of the major hsp 70 induced after heat shock in mammalian tissues is hsp70. To correlate the effect of hyperthermia on cell death with the induction of hsp70 in the three tissues, a time course analysis of the hsp70 induction profile was performed at the tissue and cellular levels. Western analysis revealed a robust induction of hsp70 in the cerebellum, which correlated with the lack of heat-induced cell death observed in this tissue. Immunocytochemical analysis of hsp70 in the cerebellum revealed maximal induction at 10 hr post heat, in the granule cell layer, deep white matter, and the microvasculature, not in Purkinje neurons. The localization of hsp70 to glial-enriched regions and the granule cell layer, as opposed to large neuronal populations, have been reported in earlier studies (Brown, 1994; Brown and Sharp, 1999). Despite these cell type-specific differences in hsp70 induction patterns, neither fully differentiated glia nor post-mitotic neurons were sensitive to hyperthermia-induced cell death. Thus hsp70 induction of and by itself, does not determine whether neural cells will be sensitive to or protected from hyperthermia-induced cell death. Although studies
on thermoprotection suggest that hsp70 plays a major role, one cannot ignore the possible contribution of other stress-inducible hsps ie. hsp27 and hsp32, or non-hsp protective agents such as NAIP (Xu et al., 1997).

A delayed, less robust induction of hsp70 was observed in the testis by Western blotting and this correlated well with its sensitivity to hyperthermia-induced cell death. At the cellular level, cross sections of seminiferous tubules revealed that not all tubules activated cell death to the same extent. Adjacent cross-sections of tubules which stained intensely with TUNEL were not hsp70 immunopositive. In contrast, adjacent sections of tubules in which TUNEL staining was absent, were observed to induce hsp70 in cells near the periphery. It is possible that cells which induced hsp70 early on are protected against the effects of the heat, whereas once cells activated the cell death program, they were no longer able to induce hsp70. The possibility that other stress-inducible hsps may contribute to protection against thermal damage in the testis, cannot be ignored (Dix and Hong, 1998). Studies have also shown that the highest incidences of heat-induced germ cell death occur at specific stages of the rat spermatogenic cycle (Blanco-Rodriguez and Martinez-Garcia, 1998; Lue et al., 1999). Along the length of a seminiferous tubule, there is a distinct ordering of cell associations, such that different segments of a tubule consist of groups of germ cell types at particular phases of development (Russell et al., 1990). Perhaps, the tubule cross-sections in which TUNEL staining was negligible, were representative of stages that are not as sensitive to hyperthermia-induced cell death.

The expression of members of the hsp70 family in the unstressed and hyperthermic testis has been thoroughly investigated (reviewed by Dix and Hang, 1998). Following heat shock, hsp70-1 and hsp70-3 members exhibit stress-inducible expression
in the mouse testis, particularly in spermatocytes and Sertoli cells (Dix and Hang, 1998). Sarge et al. (1995) demonstrated that there is a male germ cell-specific temperature set-point of the heat stress response. Heat shock at 38°C activates HSF1 in germ cells which results in hsp70-1/hsp70-3 expression, while a 42°C heat shock is required to activate HSF1 in the somatic cells (i.e., interstitial Leydig cells and supportive Sertoli cells). The level of hyperthermia used in this study is thus sufficient to induce hsp70 in somatic cells (Sertoli and Leydig cells), which reach their set-point of activation of HSF1 and do not undergo cell death. However, this hyperthermic exposure may represent a more severe stress to spermatogenic cells at certain stages of differentiation, which are not capable of mounting a protective heat shock response, and instead are pushed into a cell death program, as indicated by heavily TUNEL-labelled tubules. The time of maximal apoptosis (15 hr) corresponded to the time of maximal hsp70 accumulation. Thus hyperthermia can induce both responses in the testis, however, cells in which apoptotic pathways have already been activated, do not induce hsp70. This phenomenon was observed in rats, following permanent middle cerebral artery occlusion, where cells that induced hsp70 did not show evidence of DNA fragmentation and vice-versa (States et al., 1996). Overall, the delayed induction of hsp70 in the testis could partially account for its increased susceptibility to heat-induced apoptosis. Mezquita et al., (1998) compared avian (normally maintained at 40-41°C in vivo) and mammalian testicular cells (maintained at 30°C) with respect to the induction of hsp70 following heat shock. When chicken testis cells were exposed to 46°C in vitro, they increased transcription of hsp70, whereas no such changes were observed in mouse testis cells that were exposed to 42°C.
They proposed that the lack of hsp70 induction in the mouse contributed to its lack of thermotolerance, compared to avian cells.

In the present investigation, the thymus was demonstrated to activate apoptosis, despite its high basal levels of hsp70 and robust hsp70 induction as indicated by Western blotting analysis. At the cellular level, it became evident that although basal hsp70 levels were high in the cortex, this region readily underwent heat-induced cell death. Following hyperthermia, hsp70 was induced to a greater extent in the medulla, which also underwent apoptosis. In fact, the timing of maximal induction of apoptosis (10 hr) corresponded to a time when hsp70 levels were high. Tissue culture studies have demonstrated the induction of apoptosis (DNA fragmentation) and hsp70 in mouse thymocytes following heat shock (43 °C for 1hr) (Sellins and Cohen, 1991; Mosser et al., 1993; Migliorati et al., 1992). However, the newly synthesized hsp70 protected the surviving thymocytes against other apoptotic stimuli, such as dexamethasone (Migliorati et al., 1992). In this investigation, inducible hsp70 was not sufficient to protect cells in the thymus from heat-induced apoptosis.

Since the differences in heat sensitivities of these tissues could not simply be attributed to the induction levels of hsp70, the role of constitutive hsp70 in the phenomenon of heat-induced apoptosis was examined. The rat testis displayed the lowest levels of hsc70, which has also been found in a range of mouse testes (Tanguay, 1993; Hunt et al., 1999). In contrast the levels of hsp90 in the unstressed testis was higher than that observed in the thymus or brain (Quraishi et al., 1995). Although the low levels of hsc70 may partially account for this tissue’s sensitivity to heat shock, this was not the case in the thymus, which showed comparatively higher levels of hsc70 (similar to that in
cerebellum) and almost equivalent levels of hsp90, yet was very sensitive to heat-induced apoptosis. Thus variations in the levels of constitutive hsp9s did not correlate with the observed differences in the heat sensitivities of these tissues in the adult rat.

4.4. The sensitivity of dividing cells to hyperthermia

Since the pattern of induction of hsp70 and cell death did not correlate in all three tissues, a more in depth analysis of the reasons for activation of cell death in the testis and thymus was undertaken. The objective of this analysis was to increase our understanding of the processes occurring in these non-neural tissues that may contribute to their increased sensitivity to hyperthermia, compared to the neural cells in the cerebellum which were resistant.

In the seminiferous tubules of the adult testis, a few TUNEL positive spermatogonia were detected in the control rat. Spontaneous spermatogenic cell death by apoptosis is common in mammals and is found most frequently in spermatogonia (Allan et al., 1992; Bartke, 1995; Mori et al., 1997). In fact only 25% of type A spermatogonia, actually become spermatocytes. The mouse testis for example, which is normally maintained at 30°C, a temperature below that of body tissues, is very sensitive to heat, which, even at low doses, can inhibit spermatogenic cell function causing infertility (Chowdhury and Steinberger, 1964; Mieusset et al., 1987). It has been proposed that the underlying mechanism for the heat-induced inhibition of spermatogenesis is the inhibitory effect of heat shock on certain steps of the cell cycle, resulting in apoptosis (Sarge and Cullen, 1997).
Following hyperthermia, cell death was observed in primary spermatocytes (undergoing meiosis) and spermatogonia (mitotically active), not in mature spermatozoa. It was confirmed that these cells were mitotically active by their PCNA immunoreactivity. Proliferating cell nuclear antigen is a 36 kD nonhistone nuclear protein which is required by DNA polymerase-δ for its catalytic activity (Bravo et al., 1987). Since it is an important component of the events leading to DNA replication, and its expression level peaks during S-phase, it is often used to label proliferating cells (Iatropoulos and Williams, 1996).

Early studies have shown that heat induces cell death in the testis, however only recently, has apoptosis been defined as the mode of cell death (Yin et al., 1997; Blanco-Rodriguez and Martinez-Garcia, 1998; Lue et al., 1999). The most heat-sensitive cells in these studies, were spermatogonia, primary spermatocytes and round spermatids, while advanced spermatids and Sertoli cells remained unaffected. Furthermore, the stages at which there was an increased incidence of germ cell death coincided with stages at which the greatest numbers of spontaneous germ cell deaths occurred in control animals. It is believed that testicular germ cell apoptosis occurs through a caspase-dependent mechanism (Pentikäinen et al., 1999). Thus the testis results indicate that cells actively undergoing mitosis (spermatogonia) and meiosis (primary spermatocytes) are prone to heat-induced apoptosis.

The thymus is a tissue which also engages in naturally occurring cell death, during the maturation process (von Bohemer et al., 1989). As the immune system matures, immature thymocytes undergo a process, whereby thymocytes with T-cell receptors that are capable of recognizing self-antigens, are selected and destroyed. This
process is termed negative selection and as a result, many immature thymocytes never exit the thymus as mature T-lymphocytes, but instead die within the thymus, via apoptosis (Blackman et al., 1990; Cohen, 1991). Specifically the activation of caspase 3 has been demonstrated as an early effector of the pathway leading to negative selection of autoreactive thymocytes (Alam et al., 1997). In addition to differentiation processes, vast proliferation also occurs in the adult thymus, providing lymphocytes for exit to the immune system (Westermann et al., 1989). Collectively, these properties render the thymus sensitive to heat-induced apoptosis, as demonstrated in both in vivo and in vitro studies. For example, heat shock was reported to induce extensive DNA fragmentation predominantly in immature, double positive thymocytes, undergoing negative selection (Sellins and Cohen, 1991; Mosser et al., 1993). Hyperthermia also accelerated the normal process of cellular differentiation in the adult mouse thymus, causing decreased numbers of immature (double positive) thymocytes (Mansoor et al., 1992). Mature thymocytes that survive, were found in the medulla (Sentman et al., 1991). Whole body hyperthermia induced apoptosis in the rat thymus, where cells which are in a high turnover state, are programmed for apoptosis, and thus easily activate this mode of cell death in response to lethal stimuli (Sakaguchi et al., 1995). In this study, other tissues containing rapidly renewing cell populations, such as the spleen, lining of the intestine and bone marrow also induced apoptosis following heat, as opposed to the kidney, heart, pancreas, and liver, which were resistant.

In the thymus and testis, although hsp70 is induced, cell death still occurs. Thus once cells are susceptible to apoptosis during the course of their development (ie. during negative selection in the thymus) or undergoing cell division (in the thymus and testis),
heat may simply activate and accelerate the apoptotic pathway, despite the presence of hsp70 protein at both 45 and 48°C. However, hsp70 only protected cells against apoptosis at 45°C by blocking activation of JNK, not at 48°C, where cells underwent sustained activation of c-jun N-terminal kinase pathways (Li et al., 1999).

Numerous studies on the effects of temperature on cultured cells in vitro have demonstrated that rapidly proliferating cells are more temperature-sensitive than slowly proliferating cells (Johnson and Pavlec, 1972). Heat causes mitotic delay and cell cycle arrest (Edwards et al., 1974; Upfold et al., 1989; Maldonado-Codina et al., 1993), damage to mitotic spindle apparatus (Debec and Marcaillou, 1997), chromosomal aberrations (Wong and Dewey, 1982), plasma membrane damage (Coss et al., 1979), decay of topoisomerase II (required for DNA replication) (Goswami et al., 1996) and cell death (Edwards et al., 1974; Breen et al., 1999). Moreover, mitosis and the S-phase have been determined to be the most sensitive phases of the cell cycle (Westra and Dewey, 1971; Bhuyan et al., 1977; reviewed by Kühl and Rensing, 2000). The constitutive and inducible expression of hsp70 have also been investigated throughout the cell cycle. Milarski and Morimoto, (1986) and Milarski et al., (1989) demonstrated that hsp70 protein becomes concentrated in the nucleus of HeLa cells, upon entry into S-phase, implying a distinct role for hsp70 in the nucleus during replication. Hang and Fox, (1995) found no variation in hsp70 levels throughout the cell cycle in unheated Chinese hamster ovary cells, however after heat shock, hsp70 was induced to a high level in early S-phase and this was dependent on DNA synthesis. This need for a high level of hsp70
coincides with the extreme sensitivity of S-phase cells to heat shock (Hang and Fox, 1996; Zeise et al., 1998).

4.5. The effect of hyperthermia on cell death in the developing brain

Since heat shock induces mitotic delay and cell death particularly in dividing cells, and heat-induced cell death was observed in testis and thymus, tissues which undergo cell division and differentiation processes in the adult, it was logical to explore the effect of hyperthermia on cell death in the developing brain, where neural cells are dividing. In order to determine whether neural resistance to heat-induced apoptosis in the adult was due to the cells' non-proliferative, post-mitotic nature, highly proliferative regions of the brain during late embryonic and early postnatal development were examined following hyperthermia.

Early studies on the effect of hyperthermia during embryonic development revealed that cell death occurred in regions consisting of rapidly proliferating cells (Edwards et al., 1974; Walsh et al., 1991). In the present investigation, proliferating cells in the external granule layer of the cerebellum at postnatal day 7 were sensitive to hyperthermia-induced cell death. The activation of caspase 3 in the dividing cells of the external granule layer provides evidence that the mode of cell death is apoptosis. Naturally occurring programmed cell death is a feature of postnatal cerebellar development (Lewis, 1975; Krueger et al., 1995; Tanaka and Marunouchi, 1998), however no studies to date have shown the effect of heat on these dividing cells. The susceptibility of the external granule cell layer to other apoptotic-inducing stimuli, including exposure to X-irradiation and the cytotoxic agent methylazoxymethanol.
(MAM) has been reported (Ferrer et al., 1993; Lafarga et al., 1997). In contrast, the results of this investigation revealed that neither cells in the Purkinje cell layer nor internal granule cell layer underwent heat-induced apoptosis. On postnatal day 7, the cells in these regions are no longer mitotically active (Altman, 1997).

The neuroepithelia of the embryonic neocortex and developing tectum were also susceptible to hyperthermia-induced cell death. There was a significant increase in TUNEL positive cells, concomitant with active caspase 3 labelling in the same regions of the rat brain at embryonic day 17, indicating apoptosis. The lethal effects of maternal hyperthermia on the developing embryo have been extensively studied in guinea pigs (Edwards et al., 1974), embryonic rats (GD10) and in mice (E9) (Walsh et al., 1987; 1991; Mirkes et al., 1997; Mirkes and Little, 1998; Breen et al., 1999). These studies have collectively shown that the embryo at the stage of neural tube closure (between embryonic day 9 and 10) is very sensitive to heat stress. The induction of cell death and mitotic delay in the neuroectoderm at this stage (which consists of rapidly proliferating cells), results in abnormal forebrain, optic cup and somite development.

The findings in the present investigation indicate that even latter stages of embryonic development (E17) are very sensitive to maternal hyperthermia, particularly in the neuroepithelium, where cells are actively dividing and replicating DNA. During neurogenesis, these regions (the proliferative ventricular and subventricular zones) that give rise to the neuronal and glial cell types of the rat and mouse cerebral cortex, show higher incidence of naturally occurring programmed cell death than other regions (Thomadiou et al., 1997; Blaschke et al., 1996). Thus, their increased susceptibility to heat-induced apoptosis may be due to an active cell cycle and natural disposition to cell
death (Ross, 1996). Evidence to support the theory that developing neurons are more susceptible to apoptotic-inducing stimuli, exists in studies demonstrating increased NMDA- or kainate-induced cell death in the neonatal versus adult rat brain (van Lookeren Campagne et al., 1995). In tissue culture, heat shock at 45°C for 30 minutes was stressful enough to cause apoptotic cell death of older neuronal cultures, but this hyperthermic exposure represented a more extreme stress and caused necrosis in young primary neuronal cultures (Vogel et al., 1997). Embryonic proliferating cortical neurons also have a high vulnerability to apoptotic stimuli due to their expression of Fas/Apo-1 receptors (Cheema et al., 1999). Fas, is a member of the tumor necrosis factor receptor family and plays a critical role in limiting cell proliferation by apoptosis (Nagata and Goldstein, 1995). It has been suggested that such pathways are absent or down regulated in post-mitotic neurons, in keeping with the need to preserve cells which are irreplaceable (Marks and Berg, 1999). Furthermore, although the induction of hsp70 could provide protection and resistance to surviving cells against further stressful insults, it may be more beneficial to induce apoptosis in proliferating cells so as not to perpetuate possible mutated DNA (Samali et al., 1999). Thus, triggering cell death pathways in response to stress, may be a good strategy for these dividing cells in order to avoid increasing numbers of cells with mutated DNA.

Studies on cultured cells reveal that the temperature and duration of the heat shock determines which pathway is activated, such that when the threshold for induction of the stress response has been surpassed, apoptotic pathways are triggered and cell death occurs. Furthermore, once apoptosis is activated (whether during the natural course of development or through apoptotic stimuli), there is no turning back. Subsequent hsp70
induction will not protect the cells, and the heat shock stimulus will instead produce enhanced apoptosis (Schett et al., 1999). The bulk of this knowledge has been acquired from in vitro studies, while less is known about the fine line between these two responses to hyperthermia, within a given cell in the intact organism. It is therefore of interest to determine in vivo, the difference in thresholds for induction of the heat shock response leading to cell recovery and protection, versus activation of apoptotic pathways resulting in cell death.

The level of hyperthermia employed in this study was shown to elicit an apoptotic response within dividing neural cells of the developing brain, but not in mature, postmitotic cells of the adult. At these stages of development, it is widely accepted that neurons in particular, are produced in excess, and compete for access to neurotrophic factors, produced by their cellular targets. Those neurons which are unsuccessful die via apoptosis (Oppenheim, 1991). Since the neural cells in the neuroepithelia and external granule cell layer of the cerebellum are continuously dividing during embryonic and early postnatal development (Altman, 1997; Bayer and Altman, 1991), these regions could tolerate the effects of apoptosis, induced by a single fever-like temperature shock. In contrast, adult neural cells are non-dividing and incapable of replacing themselves, thus it is imperative that they are protected at all costs. The fact that neurons, in particular, are terminally differentiated cells, incapable of cell division and replacement in the event of cell death (Burkitt et al., 1993), warrants the need for a local neuroprotective mechanism, perhaps by localization of hsp at the synapse, (Bechtold et al., 2000; Bechtold and Brown, 2000) or some form of inherent resistance to minor stressful stimuli. This is
especially important for processes such as neurotransmission, which are critical to the functioning of the organism.

In summary the present report demonstrates the phenomenon of hyperthermia-induced cell death in dividing cells of the adult rat testis and thymus, not in the adult cerebellar cells where cell division and differentiation processes have ceased. The differential apoptotic responses of these tissues to hyperthermia could not solely be attributed to varying induction levels of hsp70. Activation of apoptosis in the developing rat brain in response to hyperthermia was observed, specifically in neural regions undergoing high proliferative activity. This study stresses the need for resistance of adult neural cells to a fever-like temperature shock because they are irreplaceable and non-dividing, and thus cannot tolerate heat-induced cell death as well as the developing brain. Whether or not levels of hsp70 contribute to these protective properties, it is now clear that the level of cell maturity (ie. dividing versus post-mitotic) contributes to the decision of whether or not heat induces apoptosis in neural cells.

4.6. Summary

1. This study shows that in response to a fever-like temperature increase, sufficient to activate the heat shock response in the mammalian brain, adult neural cells which are post-mitotic and fully differentiated, do not activate the cell death program and are therefore resistant to heat-induced cell death. This was determined by the absence of an increase in TUNEL staining post heat. Non-neural tissues of the same animals, namely the testis and thymus, which engage in continuous cell division and differentiation processes even at the adult stage, were susceptible to hyperthermia-
induced cell death. The mechanism of heat induced cell death in these tissues was determined to be apoptosis, based on the activation of caspase 3 following hyperthermia.

2. Western blot analysis and immunocytochemistry revealed that the induction patterns of hsp70 correlated with the heat-induced cell death pattern in the brain and testis, but not the thymus. The robust induction of hsp70 seen in the cerebellum correlated with the lack of heat-induced cell death seen in this brain region. Likewise, the delayed, less robust induction in the testis correlated well with its sensitivity to hyperthermia-induced cell death. The thymus however, readily underwent apoptosis despite its high basal levels of hsp70 and robust hsp70 induction. Thus, the differences in heat sensitivities of these tissues did not correlate with the observed differences in varied induction levels of hsp70. The levels of constitutive hsps, hsp90, hsc70 and hsp60 also did not correlate with the patterns of heat-induced cell death in the adult tissues.

3. Since the level of thermal exposure did not induce cell death in post-mitotic, differentiated neural cells, compared to dividing and differentiating cell populations in thymus and testis of the same animals, it was hypothesized that adult neural cells were more resistant, because of their mature, post-mitotic state. This was investigated by examining the effects of hyperthermia on cell death in dividing cell populations of the developing brain. Cells in the external granule layer of the cerebellum at postnatal day 7, and the neuroepithelial layers of the developing cortex and tectum at embryonic day 17, were highly susceptible to heat-induced apoptosis.
as evidenced by TUNEL staining and caspase 3 immunoreactivity in adjacent sections. It was proposed that their increased sensitivity to heat-induced apoptosis may be a result of an active cell cycle and natural disposition to cell death, as opposed to adult neural cells which are non-dividing and irreplaceable. Thus, despite possible contribution of protection by hsp70, this study suggests that adult neural cells may be resistant to a fever-like increase in temperature because of their mature, post-mitotic nature.
REFERENCES

hsp70 interacts with HSF, the transcription factor that regulates heat shock gene
expression. *Genes Dev. 6*, 1153-64.

(1997) Specific activation of the cysteine protease CPP32 during the negative

Allan DJ, Harmon BV (1986) The morphologic categorization of cell death induced by
mild hyperthermia and comparison with death induced by ionizing radiation and

Oxford.


Altman J (1997) Development of the cerebellum: in relation to its evolution, structure,

Ananthan J, Goldberg AL, Voellmy R (1986) Abnormal proteins serve as eukaryotic

Angelidis CE, Lazaridis I, Pagoulatos GN (1999) Aggregation of hsp70 and hsc70 in vivo
is distinct and temperature-dependent and their chaperone function is directly related


Sanchez ER, Faber LE, Henzel WJ, Pratt WB (1990) The 56-59 kDa protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70 and 90 kDa heat shock proteins. Biochemistry 29, 5145-52.


Tissieres A, Mitchell THK, Tracy UM (1974) Protein synthesis in salivary glands of

Tytell M, Barbe MF, Brown IR (1993) Stress (heat shock) protein accumulation in the
central nervous system: its relationship to cell stress and damage. In: Advances in

Ungewickell E (1985) The 70-kDa mammalian heat shock proteins are structurally and
functionally related to the uncoating protein that releases clathrin triskelia from
coated vesicles. EMBO J. 4, 3385-91.

hyperthermia on cell death and proliferation in the guinea pig brain on day 21 of

and kainate induce internucleosomal DNA cleavage associated with both apoptotic

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI,
ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 380,
75-9.

Vogel P, Dux E, Wiessner C (1997) Evidence of apoptosis in primary neuronal cultures

Volloch V, Gabai VL, Rits S, Sherman MY (1999) ATPase activity of the heat shock
protein hsp72 is dispensable for its effects on dephosphorylation of stress kinase


