Oxygen Glucose Deprivation and Hyperthermia Induce Cellular Damage in Neural Precursor Cells and Immature Neurons

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OXYGEN GLUCOSE DEPRIVATION AND HYPERTERMIA INDUCE CELLULAR DAMAGE IN NEURAL PRECURSOR CELLS AND IMMATURE NEURONS

by
Luminita Luca

A DISSERTATION
Submitted to the
Faculty of the University of Miami
In partial fulfillment of the requirements for
The degree of Doctor of Philosophy

Coral Gables, Florida
December 2008
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
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OXYGEN GLUCOSE DEPRIVATION AND HYPERTHERMIA INDUCE
CELLULAR DAMAGE IN NEURAL PRECURSOR CELLS AND IMMATURE
NEURONS

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Hyperthermia damages both developing and adult brains, especially when it occurs after ischemia or stroke. Work presented in this dissertation used in vitro models of these stresses to investigate mechanisms underlying damage to young neurons and neural precursors cultured from embryonic rat brain.

Studies described in Chapter 2 investigated the effects of a brief, intense hyperthermic stress (30-45 min at 43°C). This stress produced a selective depletion of nestin-immunoreactive neural precursor cells, and reduced proliferation, as evidenced by reduced BrdU incorporation into young Tuj1-immunoreactive neurons. The stress activated caspase 3, and produced multiple signs of nuclear damage as well as early and persisting mitochondrial depolarization. Cycloheximide, an inhibitor of protein synthesis, reduced cell death. All these findings suggest an apoptotic death process.

Studies described in Chapter 3 used a combination of oxygen-glucose deprivation (OGD, 2 h) followed by mild 41°C hyperthermia for 90 min (T). The combined OGD T stress reduced both survival in monolayer cultures and colony-forming ability in neurospheres. Cell death occurred gradually over 2 days, and was accompanied by caspase activation that began within 6 h post-stress. Post-stress application of cycloheximide or a general caspase inhibitor (especially qVD-OPH) reduced cell death, but specific inhibitors of
caspases 2, 3, 8 or 9 were ineffective. OGDT led to upregulation of the pro-apoptotic protein Bim as well as redistribution of Bax from cytoplasm to mitochondria within 6 h. Persisting mitochondrial depolarization began within 3 h following the combined OGDT stress, but not following individual OGD or T stresses alone. These findings suggest that OGD sensitizes neural precursor cells to hyperthermia-induced damage, and that the combined OGDT stress kills neural precursors via apoptotic mechanisms that include activation of mitochondrial death pathways.

Results of these studies suggest that young neurons and neural precursors are especially vulnerable to hyperthermia-induced damage via apoptotic mechanisms. Pan-caspase inhibitors may be a promising therapeutic strategy to preserve viability of these cells following stroke with hyperthermia.
ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. John Barrett, for his dedication in guiding my work, for his enthusiasm, support and encouragement during the last 5 years. I was lucky to find not only a great mentor, but also a kind friend.

My appreciation also goes to my dissertation committee members, Dr. Jacqueline Sagen, Dr. Daniel Liebl, and Dr. Bingren Hu, for their guidance and insightful comments.

I would like to thank Dr. Ellen Barrett, for her feedback and for showing me how to write scientifically, and to Dr. Gavriel David, for his help with the confocal microscopy.

I am grateful to all my colleagues in Barrett labs: Doris Nonner, for helping me troubleshoot everyday problems in the lab, and Florence Chaverneff, for her expertise with westerns. Thank you, Michael White, Kiran Panickar, Sam Saleh, Khanh Nguyen, Janet Talbot, Luis Garcia-Chacon. I already miss the morning coffee.

My thanks go to the Neuroscience Program and to the Physiology Department, for creating a stimulating and warm environment.

I would like to thank to my friends, who helped me stay on track during this long journey. I could have not achieved this without my family. Thank you, Danut and Cornel.
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LIST OF ABBREVIATIONS

AIF, apoptosis inducing factor,
APAF-1, apoptotic protease–activating factor-1,
ATP, deoxyadenosine triphosphate,
Bax, Bcl-2 associated X protein,
Bad, Bcl-xl/Bcl-2 associated death promoter,
Bak, Bcl-2 homologous antagonist/killer protein
Bcl-2, B-cell lymphoma protein 2,
Bcl-xl, Bcl-2 class protein extra long,
BDNF, brain derived neurotrophic factor,
Bid, BH3 interacting domain death agonist,
Bim, Bcl-2 interacting mediator of cell death,
BrdU, bromodeoxyuridine,
CA1, cornu ammonis 1 region of hippocampus,
CNS, central nervous system,
CD133, cluster of differentiation 133 glycoprotein,
DCX, doublecortin
DIABLO, direct IAP binding protein with low pi, see Smac
DMNQ, 2,3-dimethoxy-1,4-naphthoquinone
DMSO, dimethyl sulfoxide
EGF, epidermal growth factor,
Emx2, encoding empty spiracles homeobox 2
ER, endoplasmic reticulum,
FADD, Fas-associated death domain protein,
FGF, fibroblast growth factor,
FLICA, fluorophore labeled inhibitor of caspase
FOXO 3A, class O forkhead box transcription factor-3A
h, hours
HI, hypoxia ischemia,
HB-EGF, heparin-binding EGF-like growth factor,
HSP-70, heat shock protein of molecular mass 70 kDa,
IAPs, inhibitors of apoptosis proteins,
JNK, c-Jun N-terminal Kinase,
MAP2, microtubule associated protein 2
MASH1, Mammalian achate schute Homolog 1
Musashi, marker of asymmetric cell division,
NS, neurospheres,
NMDA, N-methyl D-aspartate,
PARP, poly (ADP-ribose) polymerase
Pax6, Paired box homeotic gene 6,
PBS, phosphate-buffered saline,
PBST, 0.1% triton 100 in PBS,
PhiPhiLux, fluorogenic substrate for caspase 3/7,
PI, propidium iodide,
PSA-NCAM, Polysialic Acid-NCAM,
qVD-OPH, quinolyl-valyl-O-methylaspartyl-(2,6-difluorophenoxy)methyl ketone,
Smac, or Smac/DIABLO, second mitochondria-derived activator of caspase,
SR-VAD-FLICA, S-rhodamine valyl-alanyl-aspartyl fluorophore labeled inhibitor of caspase,
Sox-2, sex determining region Y-box 2 transcription factor,
SVZ, subventricular zone,
tBid, truncated Bid,
TMRM, Tetramethyl rhodamine methyl ester
TNF, Tumor necrosis factor,
TNFR1, TNF receptor 1
Tunel, Terminal Transferase dUTP Nick End Labeling,
VEGF, vascular endothelial growth factor,
Z-DEVD-fmk, Z-Asp-Glu-Val-Asp-fluoromethylketone, caspase 3 inhibitor,
Z-IETD-fmk, Z-Ile-Glu-Thr-Asp-fluoromethylketone, caspase 8 inhibitor,
Z-LEHD-fmk, Z-Leu-Glu-His-Asp-fluoromethylketone, caspase 9 inhibitor,
Z-VDVAD-fmk, Z-Val-Asp-Val-Ala-Asp-fluoromethylketone, caspase 2 inhibitor,
zVAD-fmk, z-Val-Ala-Asp(OMe)-fluoromethylketone, pan-caspase inhibitor,
XIAP, X-linked Inhibitor of Apoptosis Protein
Chapter 1

Introduction

Cells from certain regions in the brain are able to proliferate in culture and differentiate into neurons, astrocytes and oligodendrocytes. These proliferating cells, named neural precursor cells or NPCs, are studied here in culture to determine the effect of different stresses on their survival and death. The experiments described in this dissertation investigate mechanisms underlying the damage to NPCs and immature neurons by stresses involving hyperthermia. Specifically, I tested severe hyperthermia alone (Chapter 2) and mild hyperthermia following oxygen and glucose deprivation (OGD, Chapter 3). This Introduction will first describe the cells studied, namely neural stem cells, neural progenitors and precursors, and young neurons. The next section defines and describes effects of hyperthermia on the developing nervous system and on the adult nervous system following stroke. My results indicate that the cell death produced by both stresses (severe hyperthermia, and mild hyperthermia following OGD) involves apoptotic mechanisms, so the remainder of the Introduction discusses death mechanisms, focusing on apoptosis. Hopefully, understanding the mechanisms underlying cell damage produced by hyperthermia will help lead to treatments that might reduce death of neurons and neural precursors following hyperthermia during pregnancy and following stroke.

Neural stem cells and progenitor cells

In the adult mammalian brain, neurons were once considered to be irreplaceable. Proliferating neural cells were first identified in postnatal rat hippocampus (Altman and Das, 1965) and in specific regions of postnatal guinea pig brain (Altman and Das, 1967).
More recently neurogenesis has been shown to occur in adults primarily in two discrete regions, the subventricular zone (SVZ), (Eckenhoff and Rakic, 1988), and the dentate gyrus of the hippocampus (Gage et al., 1998). Neural cells in these regions proliferate and give rise to mature neurons. The human hippocampus retains its ability to generate neurons from dividing progenitor cells in the dentate gyrus throughout life (Eriksson et al., 1998).

In 1992, Reynolds and Weiss isolated cells from adult rodent striatum that were able to proliferate and give rise to neurons and astrocytes in culture (Reynolds and Weiss, 1992). They developed a method to maintain stem cells in culture (as neurospheres) and described neural stem cells functionally. Neural stem cells are specified in vivo as cells that can self-renew, i.e., give rise to other cells of the same type by cell division, and that can also generate other nervous system cells such as neurons and glia (Seaberg and van der Kooy, 2003). Long-term self-renewal can be demonstrated by isolation of stem cells from adult as well as from old animals, thus showing that the neural stem cell population has been maintained (Seaberg and van der Kooy, 2003). In-vivo neurogenesis may be underestimated, since quiescent, or non-actively dividing stem cells are not detected by proliferation assays.

Criteria that define a neural stem cell in culture include the ability (1) to self-renew over time and proliferate and (2) to give rise to all three cell lineages: neurons, astrocytes, oligodendrocytes (Reynolds and Rietze, 2005). By contrast, neural progenitors have a limited ability to proliferate and their potentiality is restricted to one or two phenotypes (Seaberg and van der Kooy, 2003). Reynolds and colleagues developed In vitro assays to characterize neural stem and progenitor cells (Reynolds and
Weiss, 1992; Reynolds and Rietze, 2005). Fig 1.1 shows a schematic of the assay used to maintain the stem cells and progenitors in culture. Theoretically, stem cells retain their ability to proliferate in vitro indefinitely under growth factor-enriched conditions, thus giving rise to colonies or neurospheres. Progenitor cells form smaller colonies that may be passaged two to three times, but are often committed to one phenotype. Reynolds and Rietze (2005) showed that only 2.4% of viable cells dissociated from a neurosphere population formed secondary spheres, and the frequency of stem cells was only 0.16%. Thus neural stem cells comprise only a small fraction of standard cultures. The term neural precursor cell, NPC, refers to a broadly defined proliferative cell including neural stem cells and all progenitor types (Bull and Bartlett, 2005).

Neural stem cells and progenitors have been characterized based on their immunoreactivity for nestin, an intermediary filament, CD133, a transmembrane protein, Sox-2, a nuclear transcription factor responsible for maintenance of “stemness” and Mushashi, a nuclear antigen (Oliver and Wechsler-Reya, 2004). However, these markers are not unique to stem cells. For example, nestin intermediary filament is present in young neurons that still retain some characteristics of progenitors as they differentiate into mature neurons. Improved histochemical markers for neural stem cells are being developed, but currently their characterization/identification is done using functional criteria, namely the ability to self-renew, proliferate and give rise to cells that become neurons, astrocytes and oligodendrocytes.

In this study, I use the term neural precursor cell, NPC, when referring to nestin (+) cells in culture. In my experiments, NPCs are thus identified as nestin immunoreactive cells. For the experiments where I specifically tested the proliferative
ability of the cultured neural cells, I used the terms neural stem cells and neural progenitor cells.

**Neural stem cells during development**

The mammalian nervous system develops from embryonic neural stem cells. These cells are found in a germinal zone, or a single neuroepithelial cell layer surrounding the neural tube. The brain further develops from this layer of cells enclosing the ventricular compartment (Conover et al, 2002). A schematic of the neural stem cells during development is shown in Fig. 1.2. Actively dividing stem cells are found in the ventricle wall, or ventricular zone, VZ. Later during development, a new actively dividing zone arises, named subventricular zone, SVZ, and both neurons and glia are formed. These two proliferative zones are reduced during postnatal development, and only a thin SVZ persists through adulthood, still responsible for active neuroproliferation.

The early stem cells are neuroepithelial cells: a single layer of columnar cells, touching both ventricle and pial surfaces. Then these cells undergo rapid proliferation and renewal during the expansion phase occurring at E8-E10 in mouse (Temple, 2001). Around E13 in mouse, a neurogenic phase takes place and the cortex thickens. Newly born neurons migrate toward the pial surface where they form the subplate and cortical plate. During this phase, a subpopulation of stem cells form radial glia, cells that span the CNS from the pial membrane to the ventricle, thus serving as a guide for neurons to migrate towards the pial surface.

After the neurogenic phase, the stem cells make predominantly astrocytes and oligodendrocytes (Temple 2001). This phase is called gliogenic phase and occurs around
E18 in mouse. Thus, the original population of stem cells undergo repeated divisions, generate first neurons, then glia. Only few stem cells persist postnatally in subventricular zone and become slowly dividing adult neural stem cells. In the first days of embryonic development, up to E11 in mice, the pool of stem cells is enhanced by symmetric division, resulting similar daughter cells with characteristics of neural stem cells. Subsequently, the stem cells undergo asymmetric division resulting in a committed neuronal precursor as well as a stem cell progeny (Lathia et al, 2007). Noctor et al (2008) found that radial glia undergo symmetrical division early during development, then switch from producing symmetrical progenies to asymmetrical daughter cells at the beginning of neurogenesis. Radial glia cells maintain contact with both pial and ventricular surfaces, undergo self renewal and are multipotent. These cells likely represent neural stem cells in dissociated cultures (Noctor et al., 2008).

The frequency of neural stem cells is high early in development, such that 10-20% of the cells in the E10 mouse telencephalon are stem cells (Qian et al., 2000). However, the number of stem cells declines rapidly during embryonic development (Temple, 2001; Kalyani et al., 1997).

**Embryonic versus adult stem cells**

Embryonic and adult stem cells are phenotypically different: first, embryonic stem cells are neuroepithelial cells, and later on, are described as radial glia, spanning the whole thickness of the cortical plate. Adult stem cells resemble astrocytic –like cells, but no radial glia or radial migration of neurons were found in the adult mammalian brain (Ricard and Liebl, 2004). Interestingly, stem/progenitor cells have cili protruding into the
ventricular space; these cili are immunopositive for Prominin/CD133, a cell surface protein with a proposed role in symmetrical division. (Dubreuil et al, 2007). The increased amount of Prominin/CD133 in the CSF correlated with the increased proliferative activity in subventricular zone during development (Marzesco et al., 2005).

Functionally, cells in the developing embryo undergo neural induction to become neuronal precursors. Embryonic neural stem cells exhibit “temporal specification” (Temple, 2001): they produce mostly neurons during neurogenic phase, then glia during gliogenic phase. The expression sequence may be determined by temporal gradients of signaling molecules, e.g. EGF and FGF (Qian et al, 2000).

Embryonic stem cells are actively dividing. As opposed to embryonic stem cells, adult neural stem cells are quiescent, and enter the cell cycle rarely. The adult stem cells proliferate slowly, giving rise to more actively dividing intermediate precursors: transit amplifying cells, then neuroblasts. Embryonic neuroblasts initiate differentiation prior to migration, while neuroblasts in the adult SVZ can divide even after initiating migration (Ricard and Liebl, 2004).

Differences between embryonic and adult neural stem cells have been found in vitro. Eucher and colleagues studied the differentiation profile of embryonic versus adult hippocampal neural precursor cells (NPCs) after 7 days in culture, and found that embryonic NPCs give rise to many more neuronal cells compared to adult NPCs (~30% vs 2%), while significantly less olygodendrocytes were formed: 5% from embryonic vs 50% from adult NPCs (Eucher et al., 2006). These differences may emerge from the different cell population cultured: while embryonic cells are enriched in stem cells, adult hippocampus contains neural precursors only and not true stem cells. However, exposure
to beta amyloid did not alter their intrinsic differentiation profile, suggesting that embryonic cell culture is still a suitable paradigm for adult brain stresses.

**Regulation of neurogenesis**

Stem cells often reside in environmental “niches” in proximity to blood vessels. This proximity suggests that trophic factors and mitogens released from endothelium (especially vascular endothelial growth factor, VEGF) may influence proliferation of stem cells (Fabel et al. 2003, Shen et al. 2004). Other nearby cells (astrocytes, oligodendrocytes and mature neurons) may also play a role in neurogenesis (Jordan et al., 2007).

The proliferative response of the NPCs is tightly regulated. Intracranial infusion of growth factors such as epidermal growth factor (EGF) and fibroblast growth factor2 (FGF2) induce neuroproliferation, by increasing the number of neural progenitors in SVZ after intracranial infusion (Kuhn et al, 1997). In the same study, FGF2 increased the number of neurons in the olfactory bulb, whereas EGF increased the generation of glia. FGF2 induces neuroproliferation in vitro (Murphy et al, 1990). FGF2, but not EGF alone, can promote the survival and proliferation of neurospheres in vitro, through insulin-like growth factor-1 (IGF-1) dependent mechanism (Arsenijevi et al., 2001). EGF inhibits differentiation of some precursor cells (Doetsch et al., 2002).

Brain-derived neurotrophic factor (BDNF) is a positive regulator of neurogenesis. Overexpression of BDNF enhances progenitor cell migration to the rostral migratory stream and to striatum (Henry et al., 2007), whereas depletion of p75, a BDNF/neurotrophin receptor, leads to decreased neurogenesis in the SVZ and decreased
proliferation in vitro (Young et al, 2007). Mechanisms underlying the proliferative response of neural progenitors include increased expression of other growth factors, such as heparin-binding EGF-like growth factor (HB-EGF), which stimulates neurogenesis by mechanisms triggered in hypoxic conditions (Jin et al. 2002). Several mechanisms responsible for regulation of neurogenesis are summarized in Fig. 1.3.

Other factors that modulate neurogenesis include transcription factors required for maintenance of NPCs during adulthood: B lymphoma Mo-MLV insertion region (Bmi-1) has a role in gene silencing, thus preventing NPCs differentiation and senescence (Molovski et al, 2005). Nuclear receptor tailless (TLX) is important in transcriptional repression of glia-specific markers (TLX) (Shi et al, 2004). Other transcription factors regulate neurogenesis by controlling cell cycle: mice deficient in p27kip1 (a cell cycle inhibitor) showed increased proliferation in SVZ (Doetsch et al, 2002).

Neurogenesis is modulated by cell-cell or cell-matrix interactions (Fig. 1.3). Eph/Ephrin pathways play important roles in migration of SVZ neuroblasts (Zhao, et al, 2008). EphB2 enhances the proliferation of NPCs in vitro (Katakovski et al, 2005), whereas ephrin B3 was found to be an inhibitor of cell proliferation in vivo and in vitro (Ricard et al, 2006).

Although many newly born neurons die after birth, some of these cells can be rescued by activity-dependent interventions. For example, survival of new olfactory neurons depends on sensory input (Petreanu and Alvarez-Buylla, 2002). Neurogenesis is responsive to environmental cues. Enriched environment, physical exercise and food deprivation enhance hippocampal proliferation (Kempermann et al. 1997, van Praag et al. 1999, Lee et al. 2002), while stress reduces neuroproliferation in primates (Gould et al.,
1998). The prevalence of stem cells appears to decline with age and with conditions that mimic accelerating aging. Decreased neurogenesis has been reported in transgenic models of Alzheimer’s disease (Wen et al., 2004). Stem cells are responsive to trophic cues, since strategies to restore neurogenesis in aged mice by delivery of growth factors such as fibroblast growth factor, FGF, have been successful (Jin et al., 2003).

Several pathological conditions have been shown to stimulate neurogenesis, including ischemia, epilepsy and trauma. Epileptic activity was correlated with increased neurogenesis in animal models (Parent et al., 1997).

**Evidence that neurons born in the adult are functional**

Newly generated neurons in adult mammalian dentate gyrus acquire morphological characteristics of granule cells over several months and display electrophysiological properties similar to those of mature granule neurons (e.g. resting and synaptic potentials, firing rate, van Praag et al., 2002), suggesting that these newborn neurons are functional. Following ischemia, examination of the CA1 region showed that some cells that labeled with bromodeoxyuridine (BrdU) were also immunoreactive for synaptophysin and microtubule associated protein 2 (MAP2), suggesting that these cells acquired characteristics of functional neurons (Nakatomi et al., 2002). BrdU-labeled neurons also show morphological characteristics of functional synapses in electron micrographs. Recently-generated neurons might be involved in acquisition of hippocampal-dependent memories, since experimentally reducing the number of newly generated neurons results in impairment of hippocampal-dependent associative
memories, and restoring hippocampal neurogenesis improved this type of memory (Shors et al., 2001).

**Role of neural progenitor cells in recovery after stroke**

There is growing evidence that stem cells and progenitors divide actively after ischemic injury, giving rise to new neurons. In gerbils exposed to global ischemia, BrdU uptake in hippocampus peaked at 11 days after the injury, and persisted at least 3 weeks (Liu et al., 1998). BrdU labeling was seen in the hippocampal dentate gyrus of mice (Takagi et al., 1999) and rats (Kee et al., 2001; Takasawa et al., 2002), with peak levels at 7 days following ischemia. Ischemic injury induced neural proliferation in the SVZ (Zhang et al., 2001), estimated as a 37% increase in the number of proliferating cells (Felling and Levison, 2003). One concern associated with using the BrdU labeling method was that it might also detect cells incorporating BrdU during DNA repair. However, pharmacologically blocking cell division with cytosine arabinoside abolished BrdU labeling (Arvidsson et al., 2001), suggesting that BrdU labeling was a reliable method to detect proliferation. Immunostaining for different markers of progenitor cells (Pax6, Mash1, Emx2) showed an abundance of these cells in hippocampus and the periventricular zone following transient ischemia (Nakatomi et al., 2002).

There is evidence that neuroblasts migrate away from the proliferative zones (Arvidsson et al., 2002). These investigators identified cells that co-stained for BrdU and doublecortin (DCX) in the striatum of post-ischemic rats. Presumably these cells were replacing damaged striatal neurons. Another group (Nakatomi et al., 2002) demonstrated that cells that had originated from the periventricular zone (labeled with a fluorescent dye) and expressed a progenitor cell marker (Pax6) had made their way into damaged
CA1 hippocampal region. Taken together, these data suggest that newly born neurons migrate to the site of injury (Arvidsson et al., 2002) become mature, and are probably functional. New astrocytes are also formed after stroke (Stoll et al., 1998). Oligodendrocyte proliferation was seen at the border of the infarcted area (Mandai et al., 1997), thus supporting the idea that stem cells and/or precursors for all three lineages proliferate after ischemia.

**Vulnerability of the developing nervous system to hyperthermia**

Hyperthermia during pregnancy is teratogenic, inducing a number of developmental defects including embryonic death, growth retardation, and mental retardation. Exposure to temperatures of 41ºC or above can damage the embryonic brain (Edwards, 1998; Paula-Lopes and Hansen, 2002). Developmental events thought to be especially sensitive to hyperthermia include neuronal migration and proliferation of neuronal progenitors. Mouse embryos exposed to hyperthermia exhibit neural tube defects including exencephaly (defect in the skull causing partial or total extrusion of the brain), anencephaly (absence of the brain), and cranial neural tube defects with facial cleft (Shiota et al., 1988). A 12 minute exposure to 43 ºC during embryonic development can induce neuronal apoptosis, reducing the thickness of the cortical gray matter (Hinoue et al. 2001). In guinea pigs, 1 h of hyperthermia during early neurogenesis increases the incidence of microencephaly (Edwards et al., 2003). Two teratogenic windows are described in guinea pigs exposed to maternal hyperthermia: embryonic day 13 (E13), which corresponds to closure of the neural groove and anterior neuropore, and E21, corresponding to formation of the cortical plate. Heat stress at E13 produces a high incidence of neural tube defects, including exencephaly, branchial arch defects,
microphthalmia, and scoliosis/kyphosis (Smith et al., 1992). Taken together, all these data support the deleterious effects of hyperthermia early during development of nervous system.

Epidemiological data strongly suggest an association of fever in pregnant women with neural tube defects in their offspring. Human embryos exposed to hyperthermia during the period of neural groove closure (E23–E25) show neural tube defects similar to those seen in the guinea pigs mentioned above (Smith et al., 1992). Mothers of children with spina bifida had a higher incidence of fever during pregnancy than mothers of unaffected children (Layde et al., 1980). Milunsky and colleagues found that exposure to hot tubs, saunas, or fever during the first trimester of pregnancy is correlated with increased risk of neural tube defects (Milunsky et al., 1992). In human embryos, exencephaly is considered a specific neural tube defect induced by fever during early pregnancy (Shiota, 1982).

These neural tube defects are produced by mechanisms including abnormal apoptosis. Fever increases apoptosis in neocortex and tectum of E17 rats as well as in rapidly dividing cells of the testis and thymus, suggesting that actively-dividing populations are highly susceptible to hyperthermia-induced apoptosis (Khan and Brown, 2002). Mitotic activity is inhibited in embryonic tissues for several hours after hyperthermia, and picnotic, damaged cells are increased after stress, suggesting that the neural tube defects seen in mouse embryos are the result of cessation of cell proliferation and cell death (Shiota et al., 1988).
Damaging effects of combined ischemia and hyperthermia

Following stroke, 30% of patients experience fever (more than 37.5 °C) within the first 24 hours, and up to two-thirds of patients experience fever within 72 hours, that have been correlated with an increase in mortality rate and neurological deficits (Reith et al., 1996; Castillo et al., 1998). Low body temperature (less than 37 °C) in patients admitted to the hospital for stroke was considered an independent predictor of a favorable prognosis (Kammersgaard et al., 2002).

Hyperthermia is often seen after experimentally–induced ischemia in rodents. In gerbils, hyperthermia occurring within the first hours of reperfusion after ischemia is associated with extensive hippocampal necrosis (Colbourne et al., 1993). Ischemia induction via the two vessel occlusion model produced damage to hippocampal CA1 region. Ischemia also abolished the circadian temperature fluctuations, and the mean body temperature was 39.2 degrees for the following 3 days. Although brief periods of hyperthermia are physiological in rodents, elevation of the body temperature for extended periods of time (e.g. 3 days) was seen after experimental stroke. Cooling procedures or antipyretic treatments reduced damage to the hippocampal CA1 region (Coimbra et al, 1996). Other studies on rats showed that whole body temperature elevation for 3 hours following ischemia resulted in a 2.6-fold increase in the number of damaged hippocampal neurons (Baena et al., 1997) and increased infarct volumes (Kim et al., 1996), suggesting that hyperthermia exacerbates ischemia-induced brain damage.

Hyperthermia during middle cerebral artery occlusion in rats results in production of spectrin fragments, suggesting activation of calpain-dependent mechanisms (Morimoto et al., 1997). Immunoreactivity for calpain-cleaved spectrin was not seen in animals
undergoing ischemia only, so it was concluded that calpain activation was the result of the combination of ischemia and hyperthermia. It is believed that glutamate toxicity further increase intracellular calcium and calpain activation.

**Apoptotic death pathways**

Apoptosis is a form of delayed cell death that requires energy, as distinct from necrosis, which is a passive form of death. Apoptosis is an evolutionarily conserved form of cell suicide (Thornberry and Lazebnik, 1998) that requires specialized machinery involving a cascade of caspases, specialized proteases that ultimately cleave structural proteins and thus disassemble the cell. Cells undergoing apoptosis exhibit cytoplasmic shrinkage, nuclear fragmentation and chromatin condensation (Kerr et al., 1972; Chang et al., 2003). Classically, two apoptotic pathways are described. In the intrinsic, or mitochondrial, pathway caspase activation is regulated mainly by the release of cytochrome c from mitochondria (Li et al., 1997). In the extrinsic pathway, the death signal is transduced from the cell surface via Fas ligand to activate caspase 8 and ultimately caspase 3 (Walczak and Krammer, 2000). These pathways are illustrated in Fig. 1.4.

Following activation of the mitochondrial pathway, release of cytochrome c from the mitochondrial intermembrane space initiates oligomerization of apoptotic protease–activating factor-1 (APAF-1). This allows binding of pro-caspase 9, in the presence of ATP, and formation of the apoptosome, a complex of cytochrome c, APAF-1 and caspase 9 (Li et al., 1997). The cascade proceeds further with activation of caspase 9 and caspase 3. Other mitochondrial proteins released into cytoplasm, such as Smac/DIABLO
(Sugawara et al., 2002) and apoptosis initiating factor AIF (Hong et al., 2004) are pro-apoptotic. Other factors, such as inhibitors of apoptosis proteins (IAPs such as X-linked IAP, XIAP) modulate this process and act as pro-survival proteins (Liston et al., 2003).

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

Bcl-2 family members play important roles in regulating cell death. Anti-apoptotic proteins such as Bcl-2 and Bcl-xl prevent the release of cytochrome c from mitochondria by counteracting the pro-apoptotic members. In particular, Bcl-xl blocks the insertion of the pro-apoptotic protein Bax into the outer mitochondrial membrane (Wang et al., 2004). Even after oligomerization, Bax can interact with Bcl-xl, thus preventing mitochondrial pore formation (Tan et al., 1999). Bcl-xl can inhibit caspase 8 cleavage and subsequently Bid cleavage by caspase 8. Upregulation of Bcl-xl occurs after ischemic preconditioning, suggesting that the protein may be protective during ischemic events (Rybnikova et al., 2006).

Based on the presence of Bcl-2 homology (BH) domains, there are two groups of pro-apoptotic proteins, the BH3-only sub-family that contain only one BH3 region (e.g., Bim, Bid, Noxa, PUMA, Bad) and BH multi-domain proteins (e.g., Bak, Bax).

**Pro-apoptotic BH3-only members Bim and Bid**

Bim binds to Bcl-2 and Bcl-xl, inhibiting their anti-apoptotic effects and thus contributing to cell death following insults (Terradillos et al., 2002). A more direct apoptotic mechanism has also been described: Bim can bind Bax to induce Bax conformational changes and formation of mitochondrial pores (Zimmermann et al., 2005). Thus, Bim potentiates activation of Bax, as well as the release of cytochrome c and Smac/DIABLO from mitochondria into the cytosol (Gillespie et al., 2006).
Bim is essential for apoptosis of different cell types, e.g. leukocytes (Bouillet et al., 1999) and neurons deprived of growth factors (Putcha et al., 2003). A Bim-dependent mechanism may be activated by hypoxia, since Bim L was induced after transient focal cerebral ischemia (Okuno et al., 2004) and neonatal mice lacking Bim displayed reduced brain damage after hypoxia compared to wild type mice (Ness et al., 2006).

It is not yet clear how specific insults induce Bim activation. Several reports suggest that Bim is induced by class O forkhead box transcription factor-3A (FOXO 3A) in response to deprivation of growth factors (Dijkers et al., 2000) or after exposure to arsenite (Cai and Xia, 2008). One possible mechanism could be nuclear translocation of the transcription factor FOXO 3A in response to a certain stress; once in the nucleus, FOXO 3A may induce expression of Bim isoforms. Other studies suggest that Bim activation is dependent on JNK kinase (Okuno et al., 2004; Perier et al., 2007), possibly by upregulation and/or phosphorylation. Indeed, in neurons deprived of growth factors, Bim is regulated post-translationally by phosphorylation (Putcha et al., 2003), thus enhancing its pro-apoptotic properties. Degradation of Bim by ubiquitination was shown to be a protective mechanism in ischemic preconditioning (Meller et al., 2006).

Bid is a pro-apoptotic protein that acts as a “death ligand”, by which TNF/Fas death receptor activation is coupled to downstream events. These receptors activate caspase 8, resulting in cleavage of Bid to truncated Bid (tBid) (Plesnila et al., 2001). tBid, in turn, inserts into the mitochondrial membrane by itself or helps induce Bax oligomerization and pore formation, further inducing cytochrome c release and activation of apoptotic mechanisms downstream of mitochondria (Korsmeyer et al., 2000). Bid might be an early player in hypoxia–induced apoptotic processes (Yin et al., 2002), since
mice deficient in Bid are more resistant to ischemia induced by middle cerebral artery occlusion. Other BH3-only members may act in parallel with Bim and tBid; Noxa and Bad bind selectively only to certain pro-apoptotic Bcl-2 family proteins (Youle and Strasser, 2008). Reports of Bid or Bim activation in progenitor cells are scarce. Bid was found to be cleaved following oxidative stress by caspase 2 (Tamm et al., 2008) but not following cytosine arabinoside-induced apoptosis (Leonard et al., 2001) in neural precursor cells.

**Pro-apoptotic BH multidomain proteins Bax and Bak**

Bax resides mostly in cytoplasm, either as a monomer (Hsu and Youle, 1998) or sequestered by a Bcl-2 pro-survival protein (Wang et al., 2004). Upon activation, Bax translocates to mitochondria, where it inserts into the outer mitochondrial membrane using its C-terminal domain. Bax then forms dimers or oligomers by itself or with Bak, which has a constitutive mitochondrial localization. These oligomers form large pores in the outer membrane, thus allowing the extrusion of intermembrane mitochondrial proteins into the cytosol. In the central nervous system Bax seems to play the dominant role in cytochrome c release (White et al., 1998). Bak is not expressed in neurons, and its splice variant, N-Bak, does not induce apoptosis (Sun et al., 2001).

**Apoptotic pathways activated by OGD and ischemia. Intrinsic and extrinsic pathways**

OGD induces a continuum of necrosis and apoptosis, depending on the duration of the insult. In cultured neurons, short durations of OGD (15 min) induce apoptotic signs only, while increasing durations (to 75 min) induce necrosis in half of the neurons (Malagelada et al., 2005).
Ischemia activates the intrinsic death pathway, characterized by release of apoptotic factors from mitochondria. Cytochrome c translocates from mitochondria into the cytosol after ischemia in rats (Fujimura et al., 1998) and in brain slices (Perez-Pinzon et al., 1999), further inducing apoptosome formation, as described in the classical intrinsic mitochondrial pathway. The effector caspases 3, 7 and 9 are activated after ischemia (Chen et al., 1998; Sugawara et al., 2002). Caspase 3 activation is probably the result of cleavage of caspase 3 by caspase 9 (Malagelada et al., 2005). Caspases 3, 7 and 9 are responsible for downstream cleavage of substrate proteins, such as caspase–activated DNases (CAD, thus inducing DNA damage), fodrins (leading to cell proteolysis), or poly (ADP-ribose) polymerase (PARP). PARP activation leads to cellular energy depletion and death. Mice lacking PARP are more resistant to ischemia (Eliasson et al., 1997) consistent with the hypothesis that PARP contributes to ischemia-induced cell death.

An “extrinsic” (outside the mitochondria) apoptotic pathway is also activated by ischemia, since Fas and Fas ligand proteins are increased in animal models of ischemia (Rosenbaum et al., 2000; Jin et al., 2001). Recruitment of Fas-associated death domain protein, FADD, and caspase 8 was seen after focal cerebral ischemia (Won et al., 2002), and upregulation of FADD in the vulnerable CA1 region of the hippocampus was seen after ischemia (Jin et al., 2001). See Figure 1.4 for diagram of the apoptotic pathways.

Proteins in the “inhibitors of apoptosis” family (IAPs, such as X-linked IAP, XIAP) suppress apoptosis by preventing enzymatic activation of capsases and by binding other pro-apoptotic factors such as Smac/DIABLO (Liston et al., 2003). In one study,
release of Smac preceded caspase activation after global ischemia (Sugawara et al., 2002), indicating that IAPs may have a role in reducing cell death after ischemia.

**Endoplasmic reticulum stress**

A decrease in oxygen or glucose levels also causes dysfunction of the endoplasmic reticulum (Ogawa et al., 2007). Several stress proteins are expressed in cells following ischemia. These proteins include the glucose–regulated proteins and oxygen-regulated proteins. These proteins participate in maturation of proteins synthesized or modified in the endoplasmic reticulum. Endoplasmic reticulum dysfunction after ischemic stresses results in accumulation of immature, unfolded proteins. These proteins often form aggregates with ribosomal and more mature proteins (Liu et al., 2005). The “unfolded protein response” (Ogawa et al., 2007) further triggers induction of stress proteins, generalized suppression of protein synthesis, and activation of a protein degradation pathway.

**Excitotoxicity, Ca\(^{2+}\) and calpain**

Among the first events that occur after ischemia is calcium overload. The drop in ATP level induces dysfunction of active pumps, such as Na\(^+\)/K\(^+\)-ATPase, inducing depolarization, which further opens Ca\(^{2+}\) and Na\(^+\) channels (Hansen, 1985). Intracellular Ca\(^{2+}\) accumulation ensues. One proposed mechanism of cell death after ischemia is excitotoxicity, including activation of NMDA receptors, via Ca\(^{2+}\)-dependent mechanisms (Takagi et al., 1993). Increased intracellular [Ca\(^{2+}\)] further induces activation of calpains and cytosolic phospholipase A2, which contribute to cell death. Calpains are Ca\(^{2+}\)-dependent cystein proteases which can cleave cytoskeletal proteins such as fodrin. Activation of calpain was reported following hypoxia-ischemia in striatum, cortex, and
CA1 hippocampus of gerbils, and this event preceded neuronal death (Yokota et al., 2003).

**The role of caspases in cell death regulation of NPCs**

There is growing evidence that caspase 3 is abundant in NPCs and in the immature brain. Caspase 3-deficient mouse embryos exhibit hypercellularity in certain telencephalic regions and have marked developmental abnormalities (D'Sa-Eipper and Roth, 2000; Roth and D'Sa, 2001). These authors found that Caspase 3 (but not Bax or Bcl-xl) regulates the number of progenitor cells in developing brain, whereas postmitotic neurons are under the control of caspase 3 as well as Bcl-xl and Bax. Caspase 3 becomes activated after perinatal hypoxia ischemia in SVZ, where neural progenitor cells reside (Romanko et al., 2004). Hu and colleagues showed that following middle cerebral artery obstruction in a rodent model for stroke, several brain regions including the striatum became immunopositive for caspase 3 (Hu et al., 2000). The role of caspases in adult brain damage from ischemia remains to be defined, since levels of caspases are low in the adult brain and the effects of caspase inhibitors in ischemic injury are variable and controversial.

**Apoptotic pathways specifically activated in NPCs**

The major apoptotic pathway activated in NPCs seems to be the intrinsic pathway. Following staurosporin treatment and oxidative stress produced by 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), there was release of cytochrome c from mitochondria of NPCs from a primary culture and from a cell line (Ceccatelli at al. 2004). Fas ligand and procaspase 8 are expressed in NPCs, but activation of Fas ligand with Fas antibody failed to induce apoptosis. Thus, these studies support an intrinsic pathway that
led to NPC death (Ceccatelli et al. 2004). In neural stem cells and cell lines, DMNQ-induced oxidative stress led to upregulation of p53 protein, activation of caspase 2 upstream of mitochondria, and further activation of the intrinsic apoptotic pathway (Tamm et al., 2008a). Caspase 2 is the most apical caspase activated after genotoxic stress in neural precursor cells (Robertson et al., 2002). Exposure to heavy metals (manganese and methyl mercury) was also toxic to NPCs via a caspase, mitochondrial dependent mechanism (Tamm et al., 2008b; Tamm et al., 2006). Radiation, another genotoxic stress, was responsible for inducing p53 protein and activation of cell cycle check-points in hippocampal neural precursors (Limoli et al., 2004). Taken together, data from the literature suggest that NPCs are vulnerable to different stresses by predominant activation of intrinsic apoptotic pathway.

The goal of the work presented in this dissertation was to determine mechanisms by which stresses involving hyperthermia alone, or combined oxygen glucose deprivation (OGD) followed by hyperthermia damage young neurons and NPCs in vitro. Hyperthermia and ischemia followed by hyperthermia were found to induce brain damage in vivo, especially to the developing brain. In vitro studies showed that hyperthermia induces death in cancer cell lines (Dressler et al., 2006; Nijhuis et al., 2006; Yu et al., 2008), but previous studies of hyperthermia, or hyperthermia following OGD, on NPCs are ill-defined. The present study demonstrates that hyperthermia is especially damaging to young neurons and neural precursors, and that OGD sensitizes these cells to heat-induced damage.
Fig. 1.1 Neural stem cells and progenitors in culture

Only stem cells and neural progenitor cells (altogether referred as neural precursor cells, NPCs) survive in growth factor-enriched medium and proliferate, forming clonal colonies, neurospheres. Upon dissociation and transfer, stem cells and precursor cells form secondary spheres. When transferred to differentiative conditions in culture, neurospheres generate neurons, astrocytes and glia. Adapted from Reynolds and Rietze, (2005).
Fig 1.2
The neural proliferative zones during development and adulthood.

Early during development, the embryonic neural stem cells are neuroepithelial cells (in yellow) forming the ventricular zone, VZ; later subventricular zone, SVZ is formed, while VZ disappears. During adulthood, neurogenesis occurs mainly in SVZ, formed of stem cells, transit amplifying cells and neuroblasts, lying on a layer of ependymal cells. Adapted from Conover and Allen, (2002).
Fig. 1.3.
Factors modulating neurogenesis.

EGF and FGF2 increase neuroproliferation in vivo and in vitro. FGF2 stimulates neuroproliferation and migration of newly born neurons. EGF alone blocks differentiation of some precursor cells. Other positive regulators of neuroproliferation are BDNF, HB-EGF, and VEGF. Transcription factors: B lymphoma Mo-MLV insertion region (Bmi-1) and Nuclear receptor tailless (TLX) are important in maintenance of neural stem cells by preventing expression of differentiation markers. Cell cycle regulators modulate neuroproliferation, as cell cycle inhibitor p27kip blocks cell division. Guidance molecules regulate neuroproliferation, e.g. Eph B2 receptor directly activates, while Ephrin B3 ligand inhibits neurogenesis.
Fig. 1.4
Intrinsic and extrinsic apoptotic pathways.

The intrinsic (mitochondrial-dependent) pathway starts with activation of BH3-only proteins, e.g. BIM and PUMA, which further activate Bax and induce formation of mitochondrial outer membrane permeabilization MOMP. Subsequently, cytochrome c is released into the cytoplasm along with other proapoptotic factors (Smac/DIABLO and AIF). Cytochrome c binds APAF-1 in the presence of ATP to form the apoptosome, which further cleaves caspase 9 to activate caspase 3/7. Caspases activate DNAses, e.g. CAD, responsible for nuclear cleavage, and polymerase PARP, leading to energy depletion, and eventually to cell death.

The extrinsic pathway is induced by activation of cell surface receptors, TNFR1 and Fas, followed by recruitment of FADD protein and activation of caspase 8. In turn, caspase 8 cleaves Bid to tBid, which further induces MOMP. Caspase 8 can directly activate caspase 3, thus bypassing the mitochondrial step.
Chapter 2

Hyperthermia induces cellular damage in NPCs and immature neurons

Summary

Previous *in vivo* studies show that embryonic nervous system is vulnerable to hyperthermia. The present experiments investigate possible mechanisms contributing to this sensitivity. Cultures from embryonic day 15 rat cortex containing nestin (+) neural precursors, NPCs, and young neurons (Tuj1 +) were stressed at 43 ºC for 30-45 min and assayed for survival, proliferation and mechanisms of cell damage. At 4 days post-stress the number of total cells was reduced by ~20%, whereas nestin (+) cells were reduced by ~50%. This suggests that NPCs might be selectively depleted following hyperthermia. BrdU incorporation into newly born Tuj1 (+) neurons decreased by 3 days post-stress, suggesting that neuronal differentiation is reduced after heat exposure.

Tunel staining increased from ~ 5% in non-stressed controls to 23-25 % at 5 and 18 h post-stress. Cleaved caspase 3 was detected in ~30 % of cells at 18 h post-stress. Early signs of nuclear damage included: increased phosphorylation of histone H2A, apoptosis-inducing factor (+) nuclear protrusions and nuclear picnotic changes, all occurring within 6 h post-stress. The protein synthesis inhibitor cycloheximide (1-20 µg/ml) protected against death when applied either during or after stress. The stress also produced early and persisting mitochondrial depolarization. Thus progenitor cells and immature neurons in culture are vulnerable to hyperthermia and exhibit early signs of nuclear damage. Caspase 3 activation, nuclear changes and the saving effect of a protein synthesis inhibitor all suggest an apoptotic death process.
Background

Hyperthermia may induce neural tube defects in developing human brain and may exacerbate ischemia-induced damage in the adult brain. During certain ages of embryonic development, hyperthermia can produce severe brain damage in rodents (Cawdell-Smith et al., 1992; Hinoue et al., 2001), suggesting that NPCs and young neurons are especially sensitive to heat.

The adult brain is also vulnerable to heat stress. Memory impairments are often seen after heat stroke in humans. Damage to NPCs and immature neurons may contribute to these memory deficits, since NPCs are needed for some forms of hippocampal dependent memory (Shors et al., 2001). Hyperthermia often occurs after ischemic stroke, worsening the outcome (Kammersgaard et al., 2002). Other conditions when hyperthermia may damage NPCs and immature neurons are during treatment for brain tumors using implant heating systems (Kobayashi and Kida, 1992). Methamphetamine-induced hyperthermia (Bowyer et al., 1994) may aggravate the neurotoxicity caused by this drug.

Elucidating the mechanisms of hyperthermia-induced damage to NPCs may help in designing procedures to minimize brain damage from hyperthermia. This study examined the effects of a brief hyperthermic stress (43 °C for 30-45 min) applied to embryonic cortical cultures containing both NPCs and neurons within 1 day after plating. Our results indicate that NPCs (nestin +) are especially sensitive to hyperthermia, and demonstrate that hyperthermia-induced cell death exhibits multiple characteristics associated with apoptosis.
Materials and methods

Culture model

Cortices dissected from embryonic day 15 rat embryos were gently dissociated and cultured in Neurobasal medium (Invitrogen, Carlsbad, CA) or a basic nutrient medium, N5 (Nonner et al., 2001). The medium was supplemented with L-glutamine (1 mM, Glutamax, Invitrogen) and with a 55 kD serum fraction that enhances neuronal survival (1 mg/ml (Nonner et al., 2001)). Cells were plated on poly-L-lysine coated Terasaki microwells (Nalge Nunc, Rochester, NY) or glass bottom 35 mm dishes (Mat Teck) at a density of ~500 cells/µl. Cultures were maintained in 5.5% CO₂/94.5% air at 37 ºC.

Most experiments were performed on cultures after 1 day in vitro (DIV). These cultures contained 25% nestin (+) cells (considered to be NPCs); 33% were Tuj1 (+) (a marker for young neurons); and 44% were neurofilament (NF 200) (+), representing mature neurons. The percentage of cells staining for the astrocyte marker glial fibrillary acidic protein (GFAP) was less than 1% at this time, consistent with the small percentage of astrocytes in the developing rat brain at embryonic day 15. There was some overlap between cellular markers, as nestin and Tuj1 co-expressed in 5% of the cells that assumed a neuronal trait while retaining the nestin intermediate filament. Thus, almost all cells in these cultures were NPCs (25%), or cells of neuronal lineage (43-78%), while only 1% were astrocytes.

Heat stress paradigm

Cells were exposed for 30-45 min to 43 ºC in a humidified tissue culture incubator. Measurements using a miniature thermistor indicated that the temperature
reached 42.5 °C within 5 min and 43 °C within 10 min. At the end of the heat stress, cultures were transferred back to a 37 °C incubator.

**Immunostaining**

Cells were washed twice with phosphate-buffered saline (PBS), then fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were blocked for 30 min in 10% donkey normal serum (Jackson Immunolabs, West Grove, PA, USA) in 0.1% triton-X 100 in PBS (PBST), then incubated overnight at 4°C in primary antibodies (diluted in 5% donkey serum in PBST). Cells were then washed three times with PBS and incubated with appropriate secondary antibodies (diluted in 5% donkey serum in PBST) for 2 h at room temperature, then washed and imaged. For some experiments, nuclear counterstaining was done with Hoechst 33342 (10 μg/ml, Invitrogen). Primary antibodies used were: mouse anti-nestin (monoclonal antibody, 1:500, Chemicon, Temecula, CA, USA), rabbit anti-Tuj1 (polyclonal anti-class 3 beta tubulin antibody, 1:2000, Covance, Richmond, CA, USA), rabbit anti-cleaved caspase 3 (polyclonal, 1:100, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-AIF (polyclonal anti-apoptosis inducing factor,) rabbit anti-H2AX (polyclonal, anti-phosphohistone H2AX antibody, 1:250, Upstate) and rabbit anti-Ki67 (polyclonal anti-nuclear Ki67, 1:100, Novocastra, Newcastle Upon Tyne, UK). Secondary antibodies were: Alexa 488 donkey anti-mouse (1:1000), Alexa 555 donkey anti-rabbit (1:1000), Alexa 555 goat anti-rabbit (1:500) and Alexa 647 donkey anti-rabbit (1:500-1:1000), all from Invitrogen.

**Live imaging of nuclear changes**

Cells were exposed to 45 min at 43 °C, then returned to the normal 37 °C incubator for 1 h. Cells were then treated with a low concentration of Hoechst 33342 dye
(5 µg/ml) and imaged on the microscope stage. The environment in the imaging chamber mimicked that in the incubator (5% CO₂, 35.5 °C monitored via thermistor, humidified). Phase contrast and blue images (360 nm excitation / 450 nm emission) were taken every 4 min for 4 h.

**BrdU incorporation assay**

Bromodeoxyuridine (BrdU) is a thymidine analogue that can be incorporated into the newly synthesized DNA strands of dividing cells during S phase of the cell cycle. Brdu incorporation into nuclei is an assay for cell proliferation.

Cultures were heat-stressed within 5 h after plating. Just prior to the heat stress, BrdU (6 µM, Invitrogen,) was added to subsets of cultures and remained present for 3 days. Cells were then fixed with paraformaldehyde and rinsed in PBS as described previously. The BrdU staining procedure included a DNA denaturation step: incubation in 2 N HCl for 30 min at room temperature. Then cells were washed twice for 5 min with 0.1 M sodium borate buffer, pH 8.5, and rinsed with PBS. To detect BrdU incorporation, cultures were incubated overnight with an anti-BrdU antibody (mouse monoclonal, Alexa Fluor 647 conjugate, Invitrogen) diluted 1:60 in PBS. Cells were further washed, blocked in 5% donkey serum PBST and processed for Tuj1 staining. BrdU imaging used 650 nm excitation with emissions collected with a far red band pass filter (~680 nm).

**Tunel (Terminal Transferase dUTP Nick End Labeling) assay**

Cell death was assessed using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI, USA), which measures DNA fragmentation. The recombinant Terminal Deoxynucleotidyl Transferase enzyme (rTdT) forms a polymeric tail at the site of DNA breaks, thus incorporating a fluorescein-marked uridine. Fluorescent-labeled
nuclei are considered to be dead or apoptotic. Monolayer cultures were rinsed in PBS, then fixed for 25 min in 4% paraformaldehyde in PBS. Cells were rinsed in PBS, permeabilized (0.2% Triton in PBS), and incubated with equilibration buffer (provided in the kit). The polymerization reaction was initiated by adding incubation buffer (containing for each 50 µl: 1 µl rTdT enzyme, 5 µl nucleotide mix and 44 µl equilibration buffer) and was allowed to proceed at 37 ºC in the dark. Cells were also stained for nestin and Hoechst.

**PI exclusion assay**

Dead or dying cells incorporate propidium iodide (PI). Cultures were incubated for 10-15 min in medium containing PI (15 µM, Sigma, St. Louis, MO, USA) and Hoechst 33342 (16 µM). Fluorescence was imaged using a X microscope objective and a CCD camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu-City, Japan). Using this objective, a field comprised most of a Terasaki microwell (1.3 mm²), so that almost all cells in each well were counted. Computer macros written in Image Pro software (Media Cybernetics, Bethesda, MD, USA) controlled field selection by an OptiScan motorized microscope stage (Prior Scientific Instruments, Rockland, MA, USA). Separate images of each well were taken using 530 nm excitation/ 590 nm emission for PI, 360/ 450 nm for Hoechst, and phase contrast. PI (+) cells were considered dead, whereas Hoechst (+), PI (-) cells were counted as living. Fluorescent objects were identified and counted using a computer macro written in V++ imaging language (Digital Optics, Browns Bay, Auckland, New Zealand). The accuracy of the counting was checked by observing the mask of the counted objects identified by the macro and superimposing it on the original picture.
Measurement of $\Delta \Psi_m$ using TMRM

Tetramethyl rhodamine methyl ester (TMRM, Invitrogen) was used to measure changes in mitochondrial potential ($\Delta \Psi_m$). This dye is membrane-permeable, positively-charged and accumulates in mitochondria to an extent that depends on $\Delta \Psi_m$ (Floryk and Houstek, 1999). Cultures in Terasaki wells were incubated with 1 µM TMRM in normal medium for 30 min (the last 30 min of a 45 min heat stress). After incubation, plates were washed twice with PBS and then lysed in 50% DMSO in distilled water to release the TMRM accumulated within mitochondria. The plates were then read in a fluorescence platereader (535 nm excitation, 590 nm emission, Wallac 1420 Victor, Perkin Elmer, Boston, MA, USA). $\Delta \Psi_m$ depolarization was detected as a decrease in fluorescence.

Statistical analysis

Data are expressed as mean ± s.e.m., unless otherwise specified. Groups of two were compared using Student’s unpaired $t$ test. Multiple groups were analyzed using ANOVA followed by a Newman-Keuls post-test. Statistical analysis was performed using Prism software (GraphPad Software, La Jolla, CA, USA).

Results

Hyperthermia (43° C for 30-45 min) reduces BrdU incorporation into young neurons

The proliferative ability of stressed young cultures was assessed using the BrdU incorporation assay described in Methods. Cells positive for both BrdU and TuJ1 were considered to be derived from NPCs that were in S1 phase during BrdU exposure and then differentiated into neurons (Fig. 2.1A). In both the presence and absence of 5 nM fibroblast growth factor (FGF, which increases the number of nestin (+) NPCs, not shown), heat stress reduced the number of cells that were positive for both BrdU and
TuJ1 (Fig. 2.1B). If all mitotic cells were especially vulnerable to heat, then heat-stressed cultures would be predicted to exhibit fewer BrdU-labeled cells than non-stressed cells. However, in these experiments ~10% of all cells were BrdU (+) in both control and stressed cultures (data not shown). Thus the heat stress preferentially reduced the number of BrdU (+) TuJ1(+) cells. The heat stress may have killed some of the NPCs, or inhibited their differentiation into TuJ1-expressing neurons.

**Hyperthermia reduces the number of nestin (+) cells**

Figure 2.2A shows that heat stress reduced the percentage of cells that were nestin (+). Even in non-stressed cultures, the number of nestin (+) cells declined over time, possibly due to lack of FGF and/or to differentiation of NPCs, but in stressed cultures nestin (+) cells declined more rapidly. Fig 2.2B shows that more than 80% of total cells survived at 96 h post-stress (as assessed by nuclear integrity with Hoechst labeling), whereas stressed cultures contained only ~50% of nestin (+) cells with preserved nuclear integrity compared to controls. This suggests a preferential depletion of nestin (+) cells.

**Hyperthermia increases Tunel staining in both total and nestin (+) cells.**

Tunel is a marker for cell death and nuclear damage. Fig 2.3A shows representative panels of control and heat-stressed cells taken 5 h after stress cessation. Many more cells were positive for Tunel (red) in stressed than in control cultures. At 5 h post-stress, 22% of total cells were Tunel (+), as compared to only 5.8% in controls (Fig 2.3B). Some of the dead/dying cells were NPCs, since 4% of the nestin (+) cells costained for Tunel (arrows in Fig. 2.3A), compared to only 1% in nonstressed cultures (Fig 2.3C).
**Hyperthermia induces caspase 3 activation**

Caspase 3 is an effector caspase, activated upon cleavage by upstream caspases into a 17 kD peptide (Nicholson et al., 1995). Caspase 3 activation was assayed 18 h after stress by staining for cleaved caspase 3, along with Hoechst and nestin. Fig 2.4A shows representative micrographs taken from heat-stressed and control, non-stressed cells. Heat stress induced a significant increase in overall caspase 3 activity (red) compared to controls (Fig. 2.4A, B). Surprisingly, very few nestin (+), caspase (+) cells were detected (0.5% cells in control and 0.3% cells in stressed cultures, from an average of 333 ± 5.8 nuclei counted per field, n=20-28). A possible explanation for the relative scarcity of double-immunostained cells is that cleaved caspase 3 is a late apoptotic marker, labeling mostly cells that have already sustained damage to structural proteins and have lost immunoreactivity for nestin.

**Heat stress induces early nuclear protrusions containing AIF in nestin (+) cells**

Apoptosis inducing factor (AIF) is a mitochondrial protein with oxidoreductase activity (a flavoprotein), normally confined to the mitochondrial intermembrane space. An insult may cause AIF to translocate from mitochondria to the nucleus (Daugas et al., 2000). This process is dependent, at least in part, on Poly ADP ribose polymerase 1 (PARP1). Upon entry into the nucleus, AIF and endonuclease G induce DNA fragmentation and chromatin condensation (Hong et al., 2004).

Figure 2.5A, B show AIF immunoreactivity in control and heat-stressed cultures co-stained for nestin and nuclear DNA (Hoechst). Standard fluorescence microscopy (Fig. 2.5A) and confocal microscopy (Fig. 2.5B) of control cells showed a cytoplasmic AIF pattern (colocalization with intermediary filament nestin, white arrowhead in Fig.
Within 6 h after heat stress, many nestin (+) cells displayed apoptotic features, such as shrunken cytoplasm and fragmented nuclei, with what appeared to be AIF protrusions into the nuclei (white arrows in Fig. 2.5A, B). Colocalization studies with the mitochondrial marker Mitotracker Red revealed a distribution pattern similar to that of AIF in stressed cells (not shown), suggesting that AIF had a mitochondrial and/or cytoplasmic (rather than nuclear) localization. We did not see uniform nuclear AIF staining as expected in the case of nuclear translocation, but the AIF (+) protrusions into the nuclei indicate cellular and nuclear damage.

Heat stress increased both the total number of cells and the number of nestin (+) NPCs that displayed nuclear AIF (Fig 2.5 C). The number of Tuj1 (+) cells with nuclear AIF was not changed after stress. These data suggest that nestin (+) cells were selectively susceptible to heat stress.

**Heat stress induces early apoptotic nuclear changes**

Time lapse imaging of living cells stained with a low concentration of Hoechst was used to study early nuclear changes following heat stress (Fig. 2.6 A). Arrows indicate a cell nucleus that became condensed within 4 h post-stress. Non-stressed cells did not show this nuclear condensation.

**Heat stress induces phosphorylation of histone H2AX**

Immunohistochemical staining for phosphorylated histone H2AX was used to assay for DNA damage following heat stress. Histone H2AX is rapidly phosphorylated following DNA damage induced by ionizing radiation (Rogakou et al., 1998), as well as in the chromatin surrounding double strand breaks caused by external damage or by dysfunctional replication (Fernandez-Capetillo et al., 2004). Fig. 2.6 B shows that nuclear
immunostaining for phosphorylated histone H2AX increased within 6 h following heat stress, an additional sign of early nuclear damage induced by heat.

**Survival after heat stress is improved by blocking protein synthesis**

Cycloheximide, Chx, a protein synthesis inhibitor, can inhibit apoptosis (Deshmukh and Johnson, 1998). In the experiment of Fig 2.7 A, cell survival (assessed by PI exclusion at 20 h post stress) declined from 69.4±3% in non-stress control to 31.3±1.3% after heat stress. Post stress application of Chx (1 and 20μg/ml) increased survival to almost 50%. Figure 2.7B demonstrates that Chx also reduced cellular death when applied before the stress and washed off 40 min post-stress, or when applied immediately after stress for 4 h. These results show that blocking protein synthesis, even after the stress, can reduce hyperthermia-induced death. Chx might have protected by reducing the formation of nascent proteins that are easily denatured by heat. However, when applied after stress termination, Chx was probably inhibiting new expression or upregulation of pro-apoptotic proteins.

**Hyperthermia produces mitochondrial depolarization**

Mitochondrial depolarization often accompanies severe cell damage, and occurs in cell lines following hyperthermia (Dressler et al., 2006). Changes in mitochondrial membrane potential (ΔΨm) were analyzed using a platereader assay of TMRM fluorescence in stressed and non-stressed sister cultures (see Methods). Figure 2.8 demonstrates that TMRM fluorescence decreased immediately after stress, indicating ΔΨm depolarization. This decrease in ΔΨm was still present 20 h post-stress.
Discussion

Results presented here show that NPCs and immature neurons in culture are vulnerable to brief (30-45 min) hyperthermia at 43º C. Cell survival declined from ~65% in non-stressed cultures to 32% in heat-stressed cultures at 20-24 h (Fig 2.7 A, B). Tunel staining increased significantly at 5 h and remained elevated. Nestin (+) cells were among those damaged: ~4% of nestin (+) cells became Tunel (+) after heat stress, compared to ~1% in control cells (p<0.001). Some cells showed a lobulated nuclear morphology with AIF immunoreactivity, while other signs of nuclear damage included nuclear picnosis and staining for a marker of DNA damage, phospho-histone H2AX. These signs of cell death occurred within 6 h after stress. At later times, 30% of stressed cells exhibited caspase 3 activation. All these data suggest activation of apoptotic mechanisms after hyperthermia.

Correlation with hyperthermia studies in vivo

Our results are consistent with studies in vivo describing the vulnerability of developing brain to hyperthermia. For mouse embryos, brief exposure to hyperthermia (12.5 min at 43ºC) caused neuronal apoptotic death and neural tube defects (Hinoue et al., 2001). Hyperthermia in rat embryos at gestational day 9 resulted in extensive apoptosis in the neuroepithelium, which correlated with ultrastructural changes in mitochondria including swelling and shortening of cristae (Padmanabhan et al., 2006). Neural tube defects seen in mouse embryos after exposure to hyperthermia were the result of cessation of proliferation and cell death (Shiota, 1988).
Sensitivity of NPCs and immature neurons versus older neurons

Previous work in our laboratory showed that young striatal cultures (3 DIV) are more sensitive to heat stress than more mature (11 DIV) cultures: 40% of the young neurons survived at 1 day after 1 h at 43º C, and only 20% survived at 2 days, whereas 80% of the more mature neurons survived at 1 and 2 days post-stress (White et al, 2005). Older neurons were even more resistant to heat stress, such that 3-week-old cultured neurons were not affected by 1 h exposure to hyperthermia, but died with a delay after 2 h at 43º C (White et al., 2003). Thus over time neuronal cultures appear to develop protective mechanisms to withstand heat stress.

The present study found that nestin (+) NPCs are more sensitive to hyperthermia than immature Tuj1 (+) neurons. The pattern of cytoplasmic and AIF (+) protrusions into nuclei was observed in nestin (+) cells after heat stress, but not in Tuj1 (+) neurons. Tunel staining showed significant cell death in the whole cultures. Some of the dying cells were nestin (+), although only a small percentage of nestin (+) cells were Tunel positive (~ 4%). The relatively low number of cells showing colocalization for nestin and Tunel may be due to technique limitations: Tunel is a late marker for cellular death, when cytoplasmic proteins, such as nestin, might have been destroyed.

Mechanisms of cell death in immature cultures. Caspase activation

We present evidence that caspase 3 was activated in immature cultures after exposure to hyperthermia. Hyperthermia induces early caspase 3 activation in several different kinds of cells, including a lymphoma cell line (Yu et al, 2008), Jurkat cells (Milleron and Bratton, 2006) and in older cultured striatal neurons (White et al., 2003).
This finding is consistent with the caspase 3 activation we found in NPCs, but the role of caspase 3 in heat stress is not clear. In some studies, inhibitors that blocked caspase 3 activation failed to rescue the cells (Milleron et al., 2006, White et al., 2003) suggesting that other death mechanisms may operate in parallel with caspase 3 activation. One possibility is caspase 2 activation, an early event after heat shock (Bonzon et al., 2006). Caspase 2 may further activate downstream caspases and caspase-activated DNAses, which could cleave chromatin.

**Nuclear damage**

The literature offers many examples of nuclear damage following stresses (Yu et al., 2008). Nuclear damage was evident by 6 h in the heat-stressed immature cultures studied here. It is not known whether this nuclear damage is a primary event caused directly by the heat stress, or a secondary event caused by activation of apoptotic processes and/or increases in reactive oxygen.

AIF translocates to the nucleus after heat stress in glioma cells exposed to 43 and 45 °C (Fukami et al., 2004), and AIF is known to activate nuclear DNAses (Dawson and Dawson, 2004). In our study, AIF (+) protrusions into fragmented nuclei were seen 6 h after stress, in contrast to more uniform pattern of post-stress nuclear AIF staining reported in the literature (Plesnila et al., 2004). AIF (+) nuclear protrusions, seen in ~33% of nestin (+) cells, were interpreted as a sign of nuclear lobulation and fragmentation. This is in agreement with other evidence of nuclear damage.

Heat-stressed embryonic NPCs and immature neurons exhibited signs of nuclear damage and cell death early after stress. This suggests that hyperthermia may directly damage DNA, perhaps at particular stages of cell cycle. However, nuclear damage can
also occur downstream of caspase 3 via caspase-activated DNAase (Enari et al., 1998), through AIF release from mitochondria (Hong et al., 2004), or as a consequence of reactive oxygen production secondary to mitochondrial damage (Giulivi et al., 1995). Thus the primary site(s) of heat stress-induced damage remains unknown.

**Requirement for protein synthesis**

Hyperthermia-induced death in the young cultures studied here was greatly reduced by protein synthesis inhibitor Chx (Fig. 2.7). In contrast, Chx did not save mature neuronal cultures exposed to 120 min at 43 °C (White et al., 2007). This difference suggests that apoptosis requires upregulation of pro-apoptotic proteins in young, but not in more mature neuronal cultures. Future studies using transcription inhibitors (e.g. actinomycin) may confirm such protein synthesis-dependent cell death.

The mechanism(s) underlying this hypothesized upregulation of pro-apoptotic proteins are not yet known, but this mechanism might contribute to the high sensitivity to heat stress in young neural cultures. Upregulation of proapoptotic proteins (e.g. Smac) was a requirement for apoptotic death of young sympathetic neurons in response to NGF withdrawal (Johnson et al., 1998). Also, in NPC cultures exposed to OGD followed by mild hyperthermia, Chx had a saving effect (see Chapter 3) and blocked the upregulation of Bim (data not shown). Thus studying upregulation of Bim, Smac and other pro-apoptotic proteins in immature cultures exposed to heat stress might help determine the critical early damage that makes the immature cells (and presumably NPCs) sensitive to hyperthermia. Other candidates include Bcl-2 family members Bax and Bid, since these proteins were increased in cell lines after hyperthermia (Nijhuis et al., 2006; Peng et al., 2008; Yu et al., 2008),
Mitochondrial depolarization

Heat stress induces mitochondrial depolarization in several different cell types. For example, mitochondria of cancer cells depolarized when challenged with increasing temperatures (Dressler et al., 2006), and mitochondrial depolarization was seen 6-8 h after heat stress in lymphoid cell lines (Yu et al., 2008; Nijhuis et al., 2006). In the present study, TMRM fluorescence decreased immediately after the heat stress, and this decrease persisted for at least 20 h post-stress (Fig. 2.8 B). These results are similar to those obtained with more mature neurons, which also showed rapid and persisting mitochondrial depolarization after heat stress (White et al., 2007).

Some cell lines undergo a mitochondrial-dependent apoptotic pathway when challenged with heat stress. This apoptotic pathway included mitochondrial depolarization (Dressler et al., 2006; Nijhuis et al., 2006; Yu et al., 2008), cytochrome c release (Yoo and Lee, 2008; Yu et al., 2008), activation of Bax (Nijhuis et al., 2006) and caspase 3 activation (Milleron and Bratton, 2006). In other cases Bax was unchanged, but Bcl-2 levels were reduced (Setroikromo et al., 2007).

Our data suggest that NPCs and immature neurons activate an apoptotic pathway following heat stress. Differences from the reported heat–induced death in other cell types and mature neurons include the prominence of nuclear damage early after stress and the survival-promoting effects of Chx. This effect of Chx suggests that apoptotic processes in immature neuronal cultures depend on as-yet-unknown inducible apoptotic protein(s). Elucidating the nature of these proteins may be of clinical relevance in attenuating the deleterious effects of hyperthermia on NPCs and immature neurons.
Fig. 2.1
Heat stress (30 min) reduces bromodeoxyuridine, BrdU, incorporation into immature striatal neurons (Tuj1+) in the presence or absence of fibroblast growth factor, FGF.
A) Tuj1 (green), Hoechst (blue) and BrdU (red) staining of control, non-heated (upper row) and heated (lower row) cells. In the merged images BrdU incorporation was seen in both Tuj1(+) cells (arrows) and also in non-neuronal cells (arrowheads).
B) percentage of young neurons, Tuj1(+), showing BrdU labeling. Numbers above each bar represent wells counted. *: p<0.05, **: p<0.001, compared to non-heated controls.
Fig 2.2
Heat stress (30 min) reduces the number of nestin (+) cells
A) Data are expressed as nestin (+) cells as a percentage of total cells, Hoechst (+) positive cells. p<0.001, 16-30 fields were counted for each group
B) Specific depletion of nestin (+) cells 96 h after heat stress. Number of viable cells (non picnotic, Hoechst-labeled nuclei) in stressed cultures normalized to controls. There were 10.8 +/- 1.4 nestin (+) cells per field in the heated group vs 22.5 +/- 2.1 in control, sister cultures, n=16-19 fields analyzed.
Fig. 2.3
Tunel staining increases in total and nestin (+) cells within 24 h after hyperthermia.
A) Control (top) and heat-stressed (bottom) cultures at 5 h stained for nestin (green), Tunel (red) and Hoechst (blue). Arrows indicate examples of nestin (+), Tunel (+) cells.
B, C): Percentage of Hoechst positive (all cells, B) and nestin (+) cells at 5 h post-stress (C) colabeled with Tunel. p<0.001, t test. Numbers above bars represent fields analyzed (140 +/- 12 cells were counted per field).
Fig. 2.4
Heat stress increases caspase 3 activation.
A) Representative panels of controls and cells exposed to 30 min heat stress and fixed 18 h after stress. Caspase staining (red) is increased in the heated group; however, no nestin (+) cells (green) costain for caspase.
B) Percentage of caspase 3 (+) cells in control and heated cultures; **: p<0.001
Fig. 2.5
Hyperthermia induces nuclear AIF positive protrusions within 6 h after stress
A) Immunostaining for nuclear AIF increases after heat stress. Cultures exposed to 45 min stress were fixed 5 h post-stress. Nestin (+) cells (green) displayed fragmented nuclei (arrow) and AIF protrusions (red) into the nuclei in stressed cultures.
B) Confocal imaging of same control (upper panel) and heated (lower panel) cultures. Each row represents images taken from consequent focal planes, 1 µm apart. One nestin (+) cell (green, arrowhead), in control shows perinuclear AIF localization (red) corresponding to cytoplasm. A stressed nestin (+) cell (white arrow, lower panel) displays nuclear lobulation and immunopositive AIF cytoplasmic protrusions into the nucleus.
C) Heat stress increases both the number of total cells and the number of nestin (+) cells that exhibit nuclear AIF, but did not increase the number of Tuj1 + cells staining for AIF. (n=12-17 fields counted, 39.5 +/- 13.2 cells were counted per field).
Fig. 2.6
Hyperthermia induces nuclear changes early after stress.

A) Cells were exposed to a 45 min stress, then returned to 37 °C for 1 h. Cells were then treated with Hoechst dye (5 μg/ml) and imaged every 4 min on a warmed stage. Illustrated are representative phase (upper row) and Hoechst fluorescence (lower row) images taken from 0h 0 min to 3 h 16 min thereafter. Some nuclei (arrow) become shrunken and picnotic within 4 h after cessation of stress.

B) Hyperthermia increases staining for phospho-histone H2AX, an indicator of DNA damage. Cultures heated for 45 min were fixed 6 h post-stress and stained for phospho-histone H2AX and Hoechst 33342. The number of phospho-histone H2AX (+) cells was normalized to the number of nuclei per field. ** p<0.001, t-test. There were 10.1+/−1.2 histone H2AX (+) nuclei per field in control versus 25.5 +/- 1.9 in heated group; one field comprised ~ 450 cells. Numbers above bars represent wells analyzed.
Fig. 2.7

Cycloheximide, Chx, treatment applied either during or after stress increases survival of stressed cells.

A) Survival, assayed by PI exclusion, of cells that were heat-stressed for 45 min, then treated with 1 or 20 µg/ml Chx 3h post stress. Survival was assayed at 20 h.

B) Survival of cells in which Chx (1 or 20 µg/ml) was applied immediately before heat stress and washed off 40 min after termination of stress (“heat Chx1” and “heat Chx20” groups, or was applied after cessation of stress and washed off 4 h later (“heat Chx 1 after” group). Survival was assayed at 24 h. Each data point represents mean of 5-14 fields analyzed, **: p< 0.001, compared to stressed culture not treated with Chx.
Fig. 2.8
Early and persisting depolarization of mitochondrial membrane potential after 45 min heat stress, assayed by TMRM fluorescence
TMRM fluorescence was reduced immediately after heat stress, compared to initial reading, and remained low 20 h after stress. Data point represent mean values, after background subtraction, of n=12-24 wells analyzed, **: p<0.001
Chapter 3

An in vitro model for ischemia-hyperthermia induces apoptotic changes in neural precursor cells

Summary

Fever that frequently follows stroke correlates with poor recovery. We used oxygen-glucose deprivation (OGD, 2 h) followed by a 41°C heat stress (T, 1.5 h) as an in vitro model of ischemia followed by fever to study mechanisms underlying death of neural precursor cells, NPCs cultured from rat embryos. Survival assays performed on NPCs grown as monolayer cultures and colony-forming neurospheres demonstrated that the hyperthermia alone produced relatively little damage; however, the combined stress (OGDT) produced significantly more damage than OGD alone. Cell death occurred gradually (over 2 days), and was accompanied by caspase activation.

Post-stress application of cycloheximide, Chx, or general caspase inhibitors (especially qVD-OPH) reduced cell death, but specific inhibitors of caspases 2, 3, 8 or 9 were ineffective. OGDT led to upregulation of the proapoptotic protein Bim within 4 h after stress, as well as redistribution of Bax from cytoplasm to mitochondria within 6 hr. There was no significant change in levels of the anti-apoptotic protein Bcl-xL. Persisting mitochondrial depolarization, measured using the fluorescence of tetramethyl rhodamine methylester, TMRM, began within 3 h following the combined OGDT stress, but not following individual OGD or T stresses alone. These findings of caspase activation, increased proapoptotic proteins, and mitochondrial depolarization suggest that the
combined OGDT stress kills neural progenitor cells via apoptotic mechanisms that include activation of mitochondrial death pathways.

**Background**

Following stroke, 30% of patients experience fever (temperature greater than 37.5 °C) within the first 24 hr, and up to two thirds of patients experience fever within 72 h. Fever after stroke correlates with an aggravated outcome, including increased mortality and greater neurological deficits (Reith et al., 1996; Castillo et al., 1998). Similar detrimental effects of hyperthermia are observed in animal models of stroke: elevation of whole body temperature for 3 h following brain ischemia in rats resulted in a 2.6-fold increase in the number of damaged hippocampal neurons (Baena et al., 1997) and increased infarct volumes (Kim et al., 1996).

Neurogenesis may be part of an important repair mechanism after stroke. Ischemia increases neurogenesis in human brains (Jin et al., 2006). In animal models moderate brain ischemia stimulates proliferation of neural stem and progenitor cells in hippocampus and the subventricular zone (SVZ), both in vivo (Liu et al., 1998; Zhang et al., 2001, Yang and Levison, 2006) and in vitro (Jin et al., 2002; Horie et al., 2008). Newly formed neurons, oligodendrocytes and astrocytes migrate to and repopulate damaged brain regions in rodents (Zhang et al., 2001; Arvidsson et al., 2002). These newly-born neurons are functional in rodents (Bendel et al., 2005).

Although moderate ischemia can stimulate proliferation of NPCs, more severe ischemia can damage them. Hypoxia/ischemia induces brain damage resulting in rapid necrosis, within hours after insult, as well as delayed death in the SVZ (Levison et al., 2001; Romanko et al., 2004). Hyperthermia can produce severe brain damage in rodents.
during certain stages of embryonic development (Cawdell-Smith et al., 1992; Hinoue et al., 2001), suggesting that NPCs and young neurons are especially sensitive to heat. If the NPCs of the adult brain are as sensitive to hyperthermia as those in the embryo, hyperthermia following ischemia may increase damage to the adult NPCs.

We used an in vitro model for ischemia followed by fever: OGD followed by hyperthermia, T, and studied the effect of this combined stress, OGDT, on survival of NPCs. This combined stress induced a gradual decline in NPC survival, and reduced their proliferative capacity. Yet the surviving cells retained their ability to differentiate into several different kinds of cells including neurons, astrocytes and oligodendrocytes. The OGDT stress induced activation of caspase 3, and pan-caspase inhibitors were protective, although more selective inhibitors of specific caspases were not protective. Stress-induced mitochondrial depolarization, upregulation of Bim and translocation of Bax from cytosol to mitochondria all suggest mitochondrial involvement in this death pathway.

Material and Methods

Monolayer and neurosphere cultures

Cortical cells from embryonic day 15 rat embryos were mechanically dissociated and cultured at ~5x10⁴ cells/ml at 37 °C in Neurocult medium (Stem Cell Technologies, Vancouver, BC, Canada) or DMEM/F12 medium enriched with 2% B27 (Invitrogen, Carlsbad, CA, USA). The culture medium was supplemented with epidermal growth factor (EGF, 20 ng/ml) and fibroblast growth factor (FGF, 10 ng/ml), (Chemicon, Temecula, CA, USA). The experiments used neurospheres or monolayer cultures.
prepared from dissociated neurospheres. Cells grown as neurospheres were passaged every 2-3 days into fresh medium with growth factors. Experiments were performed on cells at passage 2-3. At least 80% of the cells from the neurosphere cultures stained immunohistochemically for nestin, a marker for NPCs.

**Stress protocol**

For most experiments, stresses were performed on neurospheres. For OGD, passage 2-3 neurospheres were washed twice in medium lacking glucose (125 mM NaCl, 2.8 mM KCl, 1.5 mM MgCl₂, 0.05 mM MgSO₄, 2 mM CaCl₂, 0.83 mM NaH₂PO₄, 24 mM NaHCO₃, 2 mM HEPES) by gently spinning down the neurospheres at 1500 RPM for 1 min and resuspending. Cells were incubated in hypoglycemic medium for 2 h at 37 ºC in an anoxic chamber, perfused with humidified 95% nitrogen, 5% CO₂ (Panickar et al., 2005). Following OGD, the hypoglycemic medium was replaced with prewarmed normal DMEM/F12 medium or Neurocult and the cells were placed in either a 37 ºC incubator (OGD group) or a 41 ºC incubator for 90 min (OGDT group). Nonstressed control cultures and hyperthermia-only group (T) received 2 washes to control for the mechanical stress associated with medium changes, but remained in control culture medium. The hyperthermia-only group (T) experienced only the heat stress.

Following the stress(es), neurospheres were dissociated using a Neurocult dissociation kit (Stem Cell Technologies). Alternatively, enzymatic dissociation was done for 3 minutes at 37 ºC in 0.05% trypsin in EDTA (Invitrogen), and stopped by application of 4% cold bovine serum albumine solution (BSA), (Sigma, St Louis, MO, USA), 0.02M HEPES in 1X EBSS buffer (Invitrogen). Single cell suspensions were obtained by straining through 40 µm cell strainers (VWR, West Chester, PA, USA) to
obtain single cell suspensions in DMEM/F12 (Ricard et al, 2006). Cells were plated into poly-L-lysine-coated Terasaki microplates (Thermo Fisher Scientific, Rochester, NY, USA) at a density of ~500 cells/µl in DMEM/F12 for survival assays. Unless otherwise specified, growth factors were excluded after stress to prevent cell proliferation from obscuring measurements of stress-induced cell death.

In the imaging experiments using TMRM and PhiPhiLux, stresses were applied to NPCs in monolayer cultures prepared from neurospheres dissociated as described above. Cells were plated onto poly-L-lysine-coated glass bottom dishes and incubated in the presence of growth factors for 18 h before stress application. At least 70% of these cells showed nestin immunoreactivity.

**Assays for survival and ability to form neurospheres**

For the propidium iodide (PI) exclusion survival assay, cells stressed as neurospheres were dissociated immediately after stress and plated in Terasaki microwells. PI (10 µg/ml, Sigma,) was added to label nuclei of dead or dying cells and Hoechst 33342 (10 µg/ml, Invitrogen) to label all nuclei. These dyes were added to living cells at 4, 8, 18, 24 and 52 h post-stress and incubated for 10-15 min before imaging. Fluorescence images corresponding to each dye were captured with a 10X objective: (excitation 530 nm, emission 590 nm for PI, 360 nm excitation and 450 nm emission for Hoechst). The fluorescent objects in each image were counted and colocalization of red (PI) and blue (Hoechst) objects was determined using a computer macro written in V++ imaging language (Digital Optics, Browns Bay, Auckland, New Zealand). Survival was expressed as percentage of healthy cells (blue, non-red) normalized to all nuclei per field (blue objects).
Cell survival was also assessed by an enzymatic method that detects fluorescent signals proportional to the relative number of living and dead cells in a population (MultiTox Fluor Multiplex Cytotoxicity Assay, Promega, Madison, WI, USA). Cells were incubated with 6 µl assay reagent/well in Terasaki wells for 45 min. Measurements of viability (excitation 400 nm, emission 505 nm) and cytotoxicity (excitation 485 nm, emission 520 nm) were taken using a plater reader (Wallac 1420 Victor, Perkin Elmer, Boston, MA, USA). The viability index was calculated for each well as a ratio of viability/cytotoxicity values. This ratio was independent of cell density and correlated well with survival data obtained from the PI survival assay described above.

To assess neurosphere-forming ability, neurospheres that had undergone different stresses were dissociated, passed through a strainer and single cell suspensions cultured in 35 mm tissue culture dishes at ~5 cells/µl in Neurocult medium enriched with EGF (10 nM) and FGF (10 nM). The number of seeded cells was counted in Hoechst-stained sister wells. Stem and progenitor cells were allowed to form neurospheres for 7 days, after which colonies were collected, gently spun down and transferred to 96 well plates for counting. Neurospheres were counted from phase images of each well and scored according to their diameter into small (50-90 µm), intermediate (100-150 µm) and large (>150 µm) neurospheres.

The ability of the cells from the stressed neurospheres to differentiate into different cell types was assayed by dissociating the stressed neurospheres to form suspensions of single cells as described above. These cells were then plated in sister Terasaki wells in N5 medium enriched with 5% horse serum without added mitogens.
(EGF, FGF). The cells were allowed to differentiate for 8 days and then fixed and stained for cell-specific markers: Tuj1 (neurons), GFAP (astrocytes), Rip (oligodendrocytes).

**Immunohistochemistry**

Cells grown in Terasaki microplates or glass bottom dishes were washed twice with phosphate buffered saline, PBS, then fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were blocked for 30 min with 10% donkey serum (Jackson Immunolabs, West Grove, PA, USA with 0.1 % Triton-100 in phosphate buffered saline, PBST), then incubated overnight at 4°C in primary antibodies diluted in 5% donkey serum in PBST. After incubation with primary antibodies, cells were washed three times with PBST and incubated with appropriate secondary antibodies (diluted in 5% donkey serum in PBST) for 2 h at room temperature, then washed and imaged. Nuclear counterstaining was done with Hoechst 33342 (10 μg/ml, Invitrogen). Primary antibodies were mouse nestin (1:500), rabbit Sox-2 (1:100), both from Chemicon and porin monoclonal (1:500, Mitosciences, Eugene, OR, USA). Secondary antibodies were Alexa 488 donkey anti-mouse (1:1000) and Alexa 555 donkey anti-rabbit(1:1000), both from Invitrogen.

**Assays for caspase activation**

The pancaspase indicator SR-VAD-FMK FLICA is thought to interact with the enzymatic site of many activated caspases (including 1, 3, 5, 6, 7, 8, 9) via its recognition sequence (VAD). Cells were loaded with FLICA reagent diluted 1:150 in culture medium (LIVE Red Caspase Detection Kit, Invitrogen). After 1 h incubation at 37°C, cells were fixed and immunostained for nestin. Rhodamine fluorescence was detected using a Leica microscope (excitation 530 nm, emission 590 nm).
Caspase 3 activity was measured using the fluorogenic substrate PhiPhiLux-G1D2 (Oncoimmunin, Gaithersburgh, MD, USA), which is cleaved by caspase 3-like proteases at DEVD sites (Telford et al., 2002; Komoriya et al., 2002) to yield a fluorescent product within cells, thus permitting live imaging (excitation 488 nm, emission collected with a 535 nm band pass filter). Cells were loaded for 45 min with 5µM substrate diluted in control medium. PhiPhiLux-positive cells (containing active caspase 3) were measured as a percentage of total cells (identified on the phase image).

Western Blots

Cultures were treated with lysis buffer (50 mM Tris HCl, 1% NP-40, 0.25% Na deoxycholate, 1mM EDTA) containing protease inhibitors. Lysates were stored at -80°C until used. Protein was quantified using the Coomassie /Bradford method (Pierce, Rockford, IL, USA). Equal amounts (25-50 µg) of protein were loaded on each lane using a loading buffer containing β-mercaptoethanol. Samples were run on precast gels containing 10% and 15% Tris Hcl (BioRad, Hercules, CA, USA), transferred to nitrocellulose membranes, blocked with Odyssey blocking buffer (Licor, Lincoln, NB, USA), then incubated overnight with primary antibodies including anti Bim (rabbit polyclonal, dilution 1:1000, Chemicon), anti-Bid (rabbit polyclonal, dilution 1:1000, Chemicon), anti Bcl-xL (mouse monoclonal, 1:1000, RD Systems, Minneapolis, MN, USA), with anti β-tubulin as loading control (rabbit polyclonal, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed in TTBS buffer (50 mM Tris-HCl, pH 8, containing 0.05% Tween 20 and 150 mM NaCl) followed by addition of appropriate secondary antibodies: IRDye rabbit 800 and IRDye mouse 700
(Licor). Fluorescence was read and quantified using Odyssey system and software (Licor).

**Assessment of mitochondrial membrane potential**

TMRM (Invitrogen) is a membrane-permeable, positively-charged dye that accumulates in mitochondria to an extent determined by the potential across the inner mitochondrial membrane (Floryk et al, 1999). NPCs cultured as monolayers were loaded with 40 nM TMRM 30 min before initial imaging, and the 40 nM TMRM remained present during imaging (excitation 568 nm, emissions collected using a 590 nm long pass filter). Initial pictures were collected before stresses, immediately following stress application; cells were maintained in a 37ºC incubator between imaging sessions. Fields selected for analysis were re-located by their x,y coordinates on a computer controlled microscope stage. Regions of interest, ROI, were chosen within cell contours on corresponding phase images and average TMRM fluorescence for each ROI was calculated using a V++ macro. After background subtraction, TMRM fluorescence for each ROI was normalized to the initial pre-stress value.

**Statistical analysis**

Data are expressed as mean ± s.e.m., unless otherwise specified. Multiple groups were analyzed using ANOVA followed by the Newman-Keuls post-test. Groups of two were compared using Student’s unpaired t test. Statistical analysis was performed using Prism software (GraphPad Software, La Jolla, CA, USA).
Results

OGDT reduces both survival and colony-forming ability

NPCs cultured as neurospheres were exposed to the OGD stress, or OGD followed immediately by hyperthermia at 41 °C for 90 min (OGDT), or the hyperthermia alone. After the stress the neurospheres were dissociated to single cells and plated at very low density as described in Methods. Survival was determined using the propidium iodide (PI) exclusion assay. Representative micrographs in Fig. 3.1 A, B show that at 18 h post-stress there were fewer surviving cells (PI-excluding, blue) and more dead/dying cells (PI-labelled, red) in the OGDT group than in non-stressed controls. Survival curves (Fig. 3.1 C) showed a modest decrease in survival of non-stressed cells, from 81% at 4 h to 61% at 52 h. Cultures subjected to hyperthermia alone (T) had slightly lower survival rates than non-stressed cultures between 8 and 24 h post-stress, but were similar to non-stressed controls by 52 h. Cells subjected to OGD alone showed decreased survival at all assayed times (P<0.01 for each time point). Survival of cells subjected to the combined OGDT stress was similar to that of OGD-only cells for the first 18 h post-stress, but then became significantly worse at later times (24-52 h, P<0.001), with only 35.9 % of OGDT cells (SD+/−3.5; n=18) versus 43.3% of OGD cells (+/−5.8; n=16) surviving 52 h post-stress. The data represent the mean of 3 different experiments. These results indicate that OGD was the more severe of the two individual stresses, and that the addition of mild hyperthermia following OGD contributed to additional death beginning 24 h post-stress.

Figure 3.2 shows results of an experiment in which stressed neurospheres containing NPCs were dissociated to single cells that were then plated at low density and allowed to form colonies for 7 days. This neurosphere assay is an established method for
characterizing survival of neural stem and progenitor cells, based on their ability for self-renewal and proliferation (Reynolds and Rietze, 2005). The number of intermediate-sized neurospheres were not affected by individual or combined stresses. Compared to non-stressed controls, both individual and combined stresses reduced both the total number of neurospheres and the number of small neurospheres. Large-diameter neurospheres were significantly reduced after hyperthermia alone and after the combined stress, but not following OGD alone. The proliferative effect expected after OGD alone (Jin et al., 2002) was seen with a subset of very large neurospheres (>250 µm, not shown). But this proliferative effect appeared to be annulled by subsequent hyperthermia since the combined OGDT stress gave a reduction in the number of large neurospheres compared to control or the OGD stress (P<0.01). Interpretation of this data is complicated by potential stress-induced shifts in the size of neurospheres produced by neural stem cells and more restricted progenitors, as well as the death of some of the cells. But it is clear that the total number of NPCs capable of giving rise to neurospheres was reduced by the OGD stress alone and further reduced by the hyperthermia following OGD.

A differentiation assay was used to analyze whether the stressed cells still retained the ability to form neurons, oligodendrocytes and astrocytes (Fig. 3.3). After culture in differentiative medium containing serum, the control cells gave rise to ~ 43% Tuj1 (+) neurons, 30% GPAF (+) astrocytes and 2% Rip (+) oligodendrocytes. The remaining cells were probably mature neurons that lost immunoreactivity for Tuj1 and progenitor cells. The percentage of Tuj1 (+) neurons, GPAF (+) astrocytes and Rip (+) oligodendrocytes were not significantly different among experimental groups. This
suggested that potentiality of stressed stem and progenitor cells was largely preserved after exposure to individual or combined stresses.

**OGDT-induced cell death requires caspase activation and protein synthesis**

Micrographs of monolayer cultures in Fig. 3.4 A show that the combined OGDT stress increased the number of cells (including nestin-positive NPCs, in green) that stained with SR-VAD-FLICA red, indicating activation of caspase-like proteases. Fig. 3.4 B shows that this increased staining was significant 3 h post-stress, and persisted until at least 18 h post-stress.

Activation of caspase 3 was assayed using the specific substrate PhiPhiLux, whose cleavage generates a fluorescent peptide. Fig. 3.5 A demonstrates that some of the stressed NPCs (that were immunopositive for Sox-2 after fixation) stained with PhiPhiLux during live imaging. Fig. 3.5 B shows that the number of cells with activated caspase 3 was significantly increased 6 h post-stress. Live co-imaging experiments using both SR-VAD-FLICA and PhiPhiLux revealed that some, but not all, SR-VAD-FLICA-positive cells also stained with PhiPhiLux, suggesting that SR-VAD-FLICA detects caspase 3 activity, as well as activity of some other proteases/caspases (data not shown). This is consistent with the ability of SR-VAD-FLICA to detect several other caspases in addition to caspase 3 (Lee et al. 2008).

If caspase activation is required for the cell death induced by the combined OGDT stress, then pan-caspase inhibitors should reduce cell death. Table 3.1 shows that pan-caspase inhibitors applied immediately post-stress increased survival assayed by PI exclusion 18 h post-stress. qVD-OPH was effective at the lowest concentrations (10-20 μM); z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) was effective at 100 μM,
but not 50 μM. Similar doses of these two inhibitors were required to prolong survival in older neuronal cultures exposed to a 2 h heat stress at 43 °C (White et al. 2003). Thus at least some of the cell death induced by the combined OGDT stress was caspase-dependent.

Figure 3.6 A shows that 10 μM qVD-OPH applied 1 h after the combined OGDT stress significantly reduced the number of FLICA-positive NPCs measured 10-24 h post-stress. The survival of sister plates evaluated at 24 h revealed that qVD-OPH restored cell viability to levels comparable to that in non-stressed cultures (Fig. 3.6 B).

Figure 3.7 shows the results of a neurosphere experiment designed to test for long-term saving effects of pan-caspase inhibition. qVD-OPH applied after the combined OGDT stress increased the total number of neurospheres measured 7 days post-stress. Most of this effect of qVD-OPH was due to an increases in the number of large (>150 μm) colonies; the number of medium and small colonies was not altered by qVD-OPH. The survival-promoting effect of qVD-OPH in this 7 day neurosphere assay was thus long-lasting, but was quantitatively less than the nearly 100% saving observed with the 24 h survival assay in Fig. 3.6 B. Either the survival-promoting effects of pan-caspase inhibition diminished over time, or more NPCs may have survived, but lost their ability to proliferate.

Table 3.2 shows results of experiments testing whether inhibitors selective for specific caspases would increase post-stress survival, using the MultiTox Fluor cytotoxicity assay (see Methods). We tested inhibitors specific for caspase 3, caspase 2, caspase 8 and caspase 9, as well as pairwise combinations of the caspase 2 inhibitor with caspase 8 or caspase 9 inhibitors. None of these specific inhibitors, alone or in the tested
combinations, increased post-stress survival at 24 h compared to vehicle-treated stressed groups. Thus inhibitors of specific caspases were not effective, but pan-caspase inhibitors enhanced post-stress survival consistently (in more than 10 experiments).

The evidence for caspase activation presented above suggests that the cell death induced by the combined OGDT stress involves apoptotic mechanisms. In some cases apoptotic cell death requires new protein synthesis and is blocked by the protein synthesis inhibitor Chx. Fig. 3.8 shows that post-stress application of Chx (10 μg/ml), partially protected cells, suggesting that at least some OGDT-induced death depends on synthesis of proteins, e.g., inducible proapoptotic proteins.

**OGDT increases formation of Bim S and translocation of Bax to mitochondria**

Since the cell death produced by the OGDT stress has apoptotic characteristics, and since in vivo brain ischemia can increase pro-apoptotic proteins such as Bim (Ness et al., 2006) and truncated Bid, tBid, (Plesnila et al., 2001), we assayed the effects of OGDT on levels of selected pro- and anti-apoptotic members of the Bcl2 family of proteins. Western blots in Fig. 3.9 A suggest increased expression of two forms of Bim, Bim S (16 kD) and BimEL (26 kD) following OGDT. The upregulation of BimS following OGDT was significant at both 4 and 21 h post-stress, and the value at 4 h was significantly greater than that measured for OGD alone (Fig. 3.9 B, C). Hyperthermia alone (T) also induced a significant increase in BimS expression at 21 h post-stress.

There was a tendency for increased post-stress expression of the active form of Bid, truncated Bid (tBid, 15 kD), but this increase failed to reach significance (Fig 3.9 D-F). Stresses produced no change in expression of uncleaved Bid (Fig. 3.9 E, F), or of an anti-apoptotic protein, Bcl-xl (Fig. 3.10).
Bim and tBid induce downstream Bax oligomerization and permeabilization of the outer mitochondrial membrane (reviewed in Zhou and Chang 2008), and during apoptotic death the cytosolic protein Bax often translocates to mitochondria. Fig. 3.11 A illustrates an experiment in which the effect of the OGDT stress on the cellular distribution of Bax was analyzed by immunostaining 6 h post-stress. Co-localization of immunostaining for Bax and a mitochondrial membrane protein, porin, was used to quantify Bax translocation to mitochondria. Non-stressed cells showed punctate porin staining and relatively diffuse Bax staining. OGDT-stressed cells showed a more punctate pattern of Bax staining and increased colocalization of porin and Bax, as quantified in Fig. 3.11 B. Bax localized to mitochondria might reflect formation of Bax-containing pores, a process associated with cytochrome c release.

**OGDT stress induces early, sustained mitochondrial depolarization**

Mitochondrial potential ($\Delta\Psi_m$) was assessed using TMRM fluorescence, as described in Methods. Monolayer cultures of neural progenitor cells were loaded with TMRM and the fluorescence of each cell in the field was measured before stress application and at different times following stress termination (Fig. 3.12 A,B). Stress reduced the TMRM fluorescence, indicating mitochondrial depolarization. This reduction was evident immediately after the stress, and persisted for at least 22 h thereafter (Fig. 3.12 C). TMRM values for the OGDT group were significantly lower than those for either individual stress group (OGD alone or T alone) at all post-stress times. Depolarization immediately after stress may reflect an increased proton leak (decoupling), reduction of electron transport and/or opening of the mitochondrial permeability transition pore. The sustained depolarization following the combined OGDT stress suggests irreversible
damage to mitochondria, perhaps downstream from Bax-mediated pore formation in the outer membrane and release of apoptotic factors including cytochrome c and Smac which activate cascades that cause further mitochondrial damage.

**Discussion**

As detailed in the Introduction, the fever that frequently follows ischemic strokes correlates with greater persisting brain damage and dysfunction. The *in vitro* model studied here shows that even mild hyperthermia can increase damage to NPCs following OGD: 2 h OGD followed by 1.5 h at 41 °C produced significantly greater damage to NPCs than OGD alone, even though the hyperthermic stress by itself produced relatively little damage. Addition of the hyperthermic stress following OGD had both short-term effects, such as increased levels of BimS and mitochondrial depolarization within the first post-stress day, and longer term effects, such as increased cell death measured 2 days post-stress, and reduced colony-forming ability measured 7 days post-stress (Fig. 3.2).

The reduction in the number of neurospheres produced by the combined OGDT stress correlated with increased cell death of nestin-positive cells. However, other effects on remaining NPCs might also contribute to the reduction in neurosphere formation and/or size. Reduced ERK phosphorylation correlated with reduced proliferation of NPCs exposed to 4 hr of an OGD stress that did not produce cell death (Kalluri et al., 2007). Sung and colleagues found that proliferation of neural precursors decreased immediately following 4 h of OGD, but increased by 24 h following reoxygenation (Sung et al., 2007). Thus OGDT stress might temporarily reduce proliferation by surviving cells, addition to killing some NPCs.
**OGDT stress results in increased BimS levels and mitochondrial depolarization**

Levels of BimS were increased within 4 hr after the combined OGDT stress. The mechanism(s) underlying the early increase in BimS is not known, but low ATP levels due to mitochondrial dysfunction might reduce Akt-dependent phosphorylation of FOXO3a; dephosphorylated FOXO3a translocates to the nucleus and contributes to Bim upregulation e.g. (Sunters et al., 2003). Inhibition of protein synthesis with Chx blocked this increase in Bim and reduced cell death. Upregulation of BimS and/or other proapoptotic proteins can increase cell death by promoting Bax oligomerization and translocation to mitochondria (Gillespie et al., 2006), and indeed Bax translocation was increased within 6 h after the combined OGDT stress.

Other pro-apoptotic proteins might also contribute to this activation of Bax. For example, truncated Bid (tBid) is formed by cleavage of Bid by upstream caspases as caspase 8 (Plesnila et al., 2001). Bid cleavage by caspase 2 has been proposed as a mediator of cell death in response to heat stress (Tu et al. 2006). Cleavage of Bid by an upstream caspase would be consistent with the robust saving produced by the pan-caspase inhibitor qVD-OPH, which inhibits many upstream caspases including caspase 8, which can cleave Bid. However, it seems unlikely that formation of tBid was sufficient to cause the cell death produced by the combined OGD-T stress, because although there was a tendency toward elevated tBid levels in stressed cultures, these elevations did not reach significance. Specific inhibitors of caspase 8 and caspase 2, even when combined, did not increase cell survival after the OGDT stress. Also, given the abundance of Bid and little change in its level following the OGDT stress (Fig. 3.9 E), the survival-
promoting effect of Chx would not have been expected if caspase-mediated tBid formation were sufficient to produce death.

**OGDT stress produces early activation of caspase-like proteases in NPCs**

Upon activation/oligomerization by pro-apoptotic proteins, Bax forms channels in the outer mitochondrial membrane that mediate the release of cytochrome c, apoptosis initiating factor (AIF) and Smac/DIABLO (reviewed in Milleron and Bratton 2007). The released cytochrome c and Smac/DIABLO is expected to cause activation of caspase 9 via APAF-1 and thus result in activation of caspase 3. Consistent with this scenario, cleavage of a fluorescent caspase 3 substrate probe, PhiPhiLux, indicated activation of caspase 3 within 6 h post-stress. The pan-caspase probe SR-VAD-FLICA demonstrated increased caspase activation within 4 h post-stress, and this increased activation persisted throughout the first post-stress day.

qVD-OPH is a broad-spectrum caspase inhibitor with a nontoxic profile and better efficacy than the widely used pan-caspase inhibitor zVAD-fmk (Caserta et al., 2003). In a B cell line, qVD-OPH was found to inhibit three major caspase-dependent pathways: caspase 9/3, caspase 12, and caspase 8/10 (Caserta et al., 2003). Likewise, in serum-deprived neurons qVD-OPH blocked activation of caspases 2, 3 and 9 (Chauvier et al., 2005). In the present study post-stress application of qVD-OPH did not inhibit the early (3-6 h) increase in pan-caspase activation, but potently inhibited the later (9-24 h) increase. The SR-VAD-FLICA probe can detect activated forms of caspase-like proteases even in the presence of caspase inhibitors (Kuzelova et al., 2007). Thus the fact that qVD-OPH did not inhibit the early post-stress increase in SR-VAD-FLICA fluorescence suggests that this early signal is due to an upstream caspase/protease whose
activation does not require caspase activity. The effectiveness of qVD-OPH in reducing the later increase in the SR-VAD-FLICA signal is consistent with activation of downstream caspases (e.g. caspases 3, 7) by an upstream caspase blocked by qVD-OPH.

Even though upstream caspases are likely to contribute importantly to the death pathways initiated by the combined OGDT stress, inhibitors of upstream caspases 2, 8 and 9 did not increase post-stress survival. It is especially surprising that inhibitors of caspase 9 and caspase 8 did not increase survival, since one of these caspases is usually required for activation of the downstream caspase 3. Milleron and Bratton encountered a similar scenario in their study of the damaging effects of hyperthermia on mouse embryonic fibroblasts: the pan-caspase inhibitor zVAD-fmk protected cells from the hyperthermia, but neither inhibitors for caspase 2 or 8 nor use of cells lacking caspase 2 gave protection (Milleron and Bratton, 2006). They suggested involvement of an as-yet-unidentified upstream caspase-like protease. Cell death can of course be produced by pro-apoptotic proteins acting independently of caspases, but if this were the primary mechanism of cell death, pan-caspase inhibitors such as qVD-OPH would not afford such robust protection.

qVD-OPH protects neurons in both in vitro and in vivo paradigms. In a murine model of stroke, qVD-OPH reduced the number of dying cells in the ischemic penumbra and completely reduced mortality after experimental middle cerebral artery occlusion (Braun et al., 2007). Post-stress application of qVD-OPH was also protective in a model of rodent neonatal ischemia, decreasing infarct volume and improving neurological function assayed 2 and 21 days post- ischemia (Renolleau et al., 2007). The stress-protective effects of qVD-OPH on NPCs demonstrated in the present study may
contribute to these in vivo protective effects. The low toxicity and long (in vitro) half-life of qVD-OPH make this pan-caspase inhibitor a good candidate for treatment of stroke, trauma and neurodegenerative diseases where the prosurvival effect on neurons as well as on neural progenitor cells could be beneficial.

In summary, work presented here demonstrates that sequential presentation of OGD and mild hyperthermia exerts synergistic damaging effects on neural progenitor cells and their ability to proliferate. The combined stress produced increased expression of BimS, Bax translocation to mitochondria, mitochondrial depolarization, caspase activation and delayed cell death, consistent with apoptotic death mechanisms. Post-stress application of a pan-caspase inhibitor offered robust and long-lasting protection.
Chapter 3 Figures

Fig. 3.1
Combined OGDT stress reduces survival of neural progenitors more than either stress alone.
A, representative pseudocolor images of nonstressed control cells (upper panel) and cells subjected to the OGDT combined stress at 18 h post-stress (lower panel). All nuclei were stained with Hoechst dye (blue). Propidium iodide (PI, red) is taken up by dead/dying cells.
B, Post-stress time course of survival for cells subjected to individual and combined stresses, plotted as the percentage of healthy cells (blue, non-red) normalized to the total number of nuclei per field. ** indicates significant difference from control, nonstressed cells, p<0.001. ♦ indicates significant difference from T, p<0.001, ■ indicates significant difference from OGD group, p<0.001. Note that error bars represent standard deviation. Plotted data represent the mean of 3 independent experiments, in each of which 5-6 culture wells were analyzed per group. Time scale shifts between 24 and 26 h.
Fig. 3.2
Combined OGDT and individual OGD and T stresses reduce the ability of progenitor cells to form new colonies (neurospheres, NS), assayed 7 days post-stress. * and + indicate significant difference from non-stressed control, p<0.01, p<0.001, respectively. ♦ indicates significant difference from OGD alone, p<0.001. Data represent means ± SEM from 3 experiments in which a total of 35-41 microscope fields were assayed per group.
The differentiation ability of surviving neural progenitor cells is maintained after combined or isolated stresses. Following the stress neural progenitor cells were cultured in N5 medium containing 5% horse serum. Formation of neurons, Tuj1 (+) cells, astrocytes, GFAP(+), and oligodendrocytes, Rip(+), was determined 8 days later by immunostaining.

A) representative pictures of control culture co-stained for cellular markers Tuj1 (green, upper), GFAP (red, middle), Rip (green, lower panel) and nuclear marker Hoechst (blue, all panels).

B) quantification of cell lineage distribution within experimental groups. No significant differences were seen in the percent distribution of neurons, astrocytes or oligodendrocytes (n=7-12 wells analyzed per group, 160+/13.3 cells counted per well).
Fig. 3.4
A) Pseudocolor micrographs of cultures prepared from OGDT stressed (top row) and non-stressed NPCs (bottom row), stained with SR-VAD-FLICA (red), 6 h post-stress, then counterstained for nestin (green) and Hoechst (blue). Arrows in merged and individual micrographs indicate cells positive for both nestin and FLICA.
B) Time course of post–stress increase in SR-VAD-FLICA fluorescence, mean ± SEM.
Data are expressed as percent of corresponding controls. ** indicates difference from non-stressed cells, p = 0.001. Data were pooled from 3 separate experiments, with at least 5 culture wells assayed at each indicated time.
Fig. 3.5
Combined OGDT stress activates caspase-3 in neural progenitor cells, assayed using PhiPhiLux fluorescence.

A) Phase and fluorescent micrographs of the same microscope field, first with live imaging for PhiPhiLux fluorescence at 6 h post-stress (left), then after fixation and staining for Sox2 (right). Arrow indicates a cell that exhibited both PhiPhiLux and Sox2 staining.

B) Quantification of PhiPhiLux fluorescence in stressed and non-stressed cultures at 6 h post-stress, mean ±SEM, * p = 0.04. Data were pooled from 2 different experiments, with 5-10 fields analyzed at each time (50.3 ±8.2 cells were counted per field).
Pan-caspase inhibitor qVD-OPH reduces late activation of caspases (A) and increases survival (B) after combined OGDT stress.

A) SR-VAD-FLICA-positive cells (expressed as percent of nestin-positive cells) as a function of post-stress time in the presence or absence of qVD-OPH (10 μM, added 1 h post-stress; remained present until addition of SR-VAD-FLICA reagent). qVD-OPH was also added to non-stressed cultures.

B) Survival assayed using Multitox Fluor at 24 h post-stress in the presence or absence of qVD-OPH (10 μM, added 1 h post-stress), normalized to survival of non-stressed cultures.

Both A and B plot mean ± SEM (in some cases error bar is embedded in the symbol), asterisks in A indicate significant difference from stressed cultures not treated with qVD-OPH, * p<0.01, ** p<0.001. In A, n=6-8 cultures for each group and time analyzed; similar results were obtained in at least 2 other experiments at 10, 18 and 24 h, and in 1 experiment at 3 and 6 h. Data in B represent the average of 5 separate experiments, each performed with n=5 culture wells per group, ** p<0.001.
Fig. 3.7
Pan-caspase inhibitor qVD-OPH increases the number of large (>150µm) and total neurospheres (NS) formed after OGDT stress. qVD-OPH (10µM) was added post-stress and remained present for 7 days. * significant difference between presence and absence of qVD-OPH in stressed cultures, p<0.05; mean ± SEM for 2 experiments, n=7 wells per group.
Fig. 3.8
Cycloheximide partially protects from OGDT-induced cell death. Cycloheximide (Chx, 10 μg/ml) or qVD-OPH (20 μM) were added immediately post-stress, with % survival assayed 20 h later by PI exclusion. + indicates significant difference from stress-only cultures, p<0.001, ♦: p<0.01 from Chx-treated stressed group, n=6 wells per group. Neither Chx nor qVD-OPH affected survival of non-stressed cultures. Similar results were obtained in a separate experiment in which survival was assayed by the MultiTox Fluor assay (not shown).
Fig. 3.9
Effect of individual (OGD or T) and combined (OGDT) stresses on expression of proapoptotic proteins Bim and Bid.

A and B, representative Western blots of lysates from neurospheres obtained 4 (A) or 21 (B) h after the indicated stress, immunostained for Bim S (16 kD) and Bim EL (26 kD). C, Bim S expression normalized to tubulin loading control and plotted as % of expression in non-stressed control cultures, mean ± SEM for 3-4 blots. * p<0.05 compared to non-stressed; ♦ p <0.05 compared to OGD alone. D, E and F, comparable blots and quantification for full-length Bid (22 kD) and truncated Bid (tBid, 15 kD).
Fig. 3.10
Individual and combined stresses produce no significant change in expression of an anti-apoptotic protein, Bcl-xl.

A) Representative Western blot from lysates prepared 4 h post-stress.

B) Bcl-xl expression normalized to tubulin loading control and plotted as % of expression in non-stressed control cultures, mean ± SEM for 5 blots.
Fig. 3.11
Combined OGDT stress increases Bax translocation to mitochondria. 
A) micrographs of non-stressed (upper panels) and OGDT-stressed (lower panels) cells fixed 6 h post-stress and stained for the mitochondrial protein porin (green) and Bax (red). Yellow in the overlay shows increased porin and Bax co-localization in stressed cells. 
B) % of porin-positive granules showing Bax co-localization, quantified using a 
V++ macro. ** p<0.01, n = 22-26 fields. Similar results were obtained in a 
different experiment at 6 h.
Fig. 3.12
Combined OGDT stress increases mitochondrial depolarization, assayed using TMRM fluorescence.
A) and B) phase (left column) and fluorescence (right column) images collected from OGDT-stressed (A) and non-stressed (B) cells, with the same field imaged 4 h before, and 0, 4 and 22 h post-stress. Boxes in phase images show regions of interest drawn within cellular contours; the fluorescence of these cells was tracked over time.
C) post-stress time course of changes in TMRM fluorescence (calculated using V++ macro) in non-stressed cells and cells exposed to individual and combined stresses, normalized to the initial TMRM fluorescence of individual cells before the stress. * indicates difference between OGDT group and all other groups, p<0.05, n=15-22 fields.
Fig. 3.13
Timeline of events after OGDT stress.

NPC survival declines rapidly within 18 h after combined OGDT stress, then more slowly up to 52 h. Caspase activation, evidenced by FLICA fluorescence, reaches a peak around 10h, then declines. Activation of apoptotic mechanisms early after stress is also evidenced by BimS upregulation and possibly tBid elevations at 4 h. BimS may facilitate Bax translocation to mitochondria, seen at 6h.

Mitochondrial depolarization is evidenced by TMRM decline in fluorescence immediately after stress. Persistent mitochondrial depolarization seen up to 20 h after stress may reflect structural damage to mitochondria, possibly secondary to formation of Bax pores.

Y axis on left represents percent survival. Scale bars at the right of each inset represent fold increase compared to initial, before stress values.
Chapter 3 Tables

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control, % survival</th>
<th>OGDT, % survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>68.2 +/- 2.2</td>
<td>48.0 +/- 3.9</td>
</tr>
<tr>
<td>qVD-OPH 10 µM</td>
<td>63.3 +/- 1.7</td>
<td>59.9 +/- 1.5 *</td>
</tr>
<tr>
<td>qVD-OPH 20 µM</td>
<td>70.7 +/- 0.9</td>
<td>67.7 +/- 3.5 *</td>
</tr>
<tr>
<td>zVAD- fmk 50 µM</td>
<td>67.7 +/- 1.1</td>
<td>48.7 +/- 3.2</td>
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<tr>
<td>zVAD- fmk 100 µM</td>
<td>71.2 +/- 1.6</td>
<td>56.6 +/- 6.6 *</td>
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Table 3.1
Pan-caspase inhibitors qVD-OPH and zVAD- fmk protect progenitor cells from OGDT-induced death.

Neural progenitor cells exposed to OGDT stress were dissociated, then exposed to the indicated drugs for 18 h, after which survival was assayed by PI exclusion. Mean survival is given as the percent of the total cells that exclude PI (with +/- SD, with 6 culture wells per group). * indicates significant difference from OGDT no treatment group, p<0.01. Neither inhibitor had any significant effect on survival in non-stressed cultures. These data are representative of 3 separate experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (µM)</th>
<th>Survival, % of OGDT stressed, +/- SD (n)</th>
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<tbody>
<tr>
<td>Z-DEVD-fmk</td>
<td>2</td>
<td>93.3 +/- 0.9 (6)</td>
</tr>
<tr>
<td>Z-DEVD-fmk</td>
<td>20</td>
<td>98.6 +/- 3.4 (11)</td>
</tr>
<tr>
<td>Z-VDVAD-fmk</td>
<td>20</td>
<td>98.0 +/- 6.5 (11)</td>
</tr>
<tr>
<td>Z-IETD-fmk</td>
<td>2</td>
<td>95.0 +/- 2.2 (6)</td>
</tr>
<tr>
<td>Z-IETD-fmk</td>
<td>20</td>
<td>95.3 +/- 3.5 (11)</td>
</tr>
<tr>
<td>Z-LEHD-fmk</td>
<td>2</td>
<td>94.2 +/- 1.2 (6)</td>
</tr>
</tbody>
</table>

Table 3.2
Inhibitors specific for caspases 2, 3, 8, or 9 do not save progenitor cells following combined OGDT stress.

Drugs were added immediately after stress, with survival assayed 24 h later, expressed as % of stresses cells in cultures not treated with any drug (Multitox Fluor assay). Caspase 2 inhibitor was Z-VDVAD-fmk, caspase 3 inhibitor was Z-DEVD-fmk, caspase 8 inhibitor was Z-IETD-fmk and caspase 9 inhibitor was Z-LEHD-fmk. Only the pan-caspase inhibitor Q-VD-OPH provided significant protection.
Chapter 4

General discussion

Summary

My experimental studies provide evidence that neural precursor cells (NPCs) and immature neurons are vulnerable to stresses involving hyperthermia. These cells were severely damaged by heat stress alone (30-45 min at 43 °C) or by milder hyperthermia (90 min at 41 °C) following a mild OGD stress. More mature neurons (more than 3 days in culture) are not detectably damaged by either of these stresses (White et al., 2007), suggesting that NPCs and immature neurons are more vulnerable to hyperthermia than mature neurons. Heat stress alone increases death with approx 20% (fig 2.3), and with 10% when the hyperthermia is milder (fig 3.1), while combination of oxygen glucose deprivation and hyperthermia increased death with approx 25-30 % of cells (fig 3.1). Taken together, these data suggest that hyperthermia induces cellular damage by itself and further sensitizes the cells to damage from subsequent stresses as oxygen glucose deprivation.

The stress paradigms we used induced both early and delayed cell death. Common signs of cell damage following intense hyperthermia and OGD included caspase 3 activation and mitochondrial depolarization. Pharmacologically blocking protein synthesis with Chx partially protected 1 DIV cultures from the 43°C stress, as well as NPCs from the combined OGD stress, suggesting that both paradigms induced a type of death requiring synthesis of new proteins.

Reduced proliferation occurred, evidenced by decreased BrdU incorporation (in immature neurons after 43 °C hyperthermia) and by impaired neurosphere formation.
(after OGDT). Although the true neural stem cells comprise only ~1% of the neurosphere population, there is evidence that these cells were also damaged by the OGDT stress, because this stress reduced the number of large colonies formed after stress and formation of large colonies is usually attributed to neural stem cells in a neurosphere assay. Further work is needed to prove damage to neural stem cells. Although all the mechanisms underlying this sensitivity of immature neurons and NPCs are not yet clear, my studies of the death mechanisms activated by the stresses give some insight about mechanisms that might contribute to the greater sensitivity of these young cells.

**Apoptotic pathways activated by heat stress in NPCs and other cell types**

The literature classifies apoptotic death into those using an intrinsic death pathway involving mitochondria and an extrinsic death pathway that operates independently of the mitochondria (see Fig. 1.4). The mechanism of heat-induced death is likely mitochondrial-dependent, since mitochondrial depolarization was seen immediately after stress and persisted 20 h later. The apoptotic events triggered by hyperthermia are summarized in Fig. 4.1.

Hyperthermia affects mitochondria in other types of cells. Cancer cells undergo a mitochondrial-dependent apoptosis when challenged with heat stress; their apoptosis was preceded by mitochondrial depolarization (Dressler et al., 2006; Yu et al. 2008) and cytochrome c release (Yoo and Lee, 2008; Yu et al., 2008). In spermatocytes, an early event in heat-induced damage is redistribution of Bax (Yamamoto et al, 2000). Relocation of Bax to mitochondria was accompanied by cytochrome c release, suggesting that Bax translocation activated mitochondrial pathways (Hikim et al., 2003). The
extrinsic, Fas-dependent death pathway is probably not required for the hyperthermia-induced death of spermatocytes (Hikim et al., 2003).

The caspase 3 activation we found in immature neurons following heat stress is consistent with findings in more mature neurons, where delayed caspase 3 activation was seen (White et al., 2003). In fibroblasts, caspase 3 is robustly activated after heat stress, probably forming amplifying loops (Milleron and Bratton, 2006). Caspases are activated in hyperthermia-induced germ cell apoptosis, and the pan-caspase inhibitor qVD-OPH injected to experimental animals protected from this cell death (Vera et al., 2005).

Thus a common mechanism of heat-induced death may occur in young neurons, germ cells and cell lines: activation of mitochondrial-dependent pathways, ultimately resulting in caspase 3 activation. One common attribute of young progenitors in culture, different cell lines and germ cells could be active division, and this property may render them vulnerable to hyperthermia.

Could heat shock proteins (HSPs) deficiency contribute to the sensitivity of NPCs to hyperthermia?

HSPs protect cells from hyperthermia by acting as protein chaperones and preventing protein aggregation. Thus lower expression and/or induction of HSPs in NPCs and young neurons might contribute to their sensitivity to hyperthermia. Indeed, robust induction of HSP-70 was seen in adult cerebellum where no signs of heat-induced death were detected (Khan and Brown 2002). We could not find data on HSP expression after heat stress in young neurons or NPCs, but other highly sensitive cells (germinal cells) undergo massive apoptosis, with minimal HSP-70 induction (Khan and Brown, 2002). Another study reported upregulation of HSP-70 in testes by hyperthermia, possibly as a protective mechanism (Rockett et al., 2001). On the other hand, HSP-70 does not confer
protection in thymocytes, which undergo massive apoptosis after heat shock in spite of high basal and stress-induced levels of HSP-70 (Khan and Brown, 2002). Studies of HSPs are needed to resolve their possible role in defending NPCs from hyperthermia.

**Mechanisms of OGD-induced cell damage**

According to in vivo studies, the earliest damaging changes during ischemia involve ATP depletion. Within 5 min of brain ischemia ATP levels drop significantly. In neuronal cultures ATP levels drop to ~10% of the control value after 30 min of OGD (Kusumoto et al., 1996; Iijima et al., 2003). This very low level of ATP has several damaging consequences including excessive loading of cells with Na⁺ and Ca²⁺, and block of protein synthesis with nascent proteins caught on ribosomes. Mitochondria depolarize and begin to consume rather than make ATP. These changes set in motion mechanisms that produce permanent damage including oxidation of proteins and lipids, denaturing of proteins, osmotic damage and damage due to activation of Ca²⁺-dependent proteases such as calpains. The next steps leading to cell death are more controversial but include upregulation of pro-apoptotic proteins, release from mitochondria of apoptotic proteins such as AIF (Dawson and Dawson, 2004) and activation of caspases followed by DNA damage, ultimately resulting in cell death. In severely damaged brain regions neurons die rapidly by osmotic necrosis, but in less severely damaged regions neuronal death can occur over several days.

Our data suggest that OGD alone induces some cell damage, evidenced by a decline in cell survival; however, it did not induce mitochondrial depolarization or increase in BimS. Based on data from literature, it is likely that OGD induced a decline in
ATP, triggering some apoptotic pathways discussed above, but further studies on ATP levels in NPCs after stress are needed to elucidate this problem. The relative resilience of NPCs to OGD may be due to their glycolitic metabolism.

**Cell death pathways activated by combined OGD and hyperthermia**

This dissertation presents several lines of evidence that the combined OGD-T stress induces apoptotic cell death. My data indicate that a caspase dependent apoptotic pathway is required for the NPC death, since a general caspase inhibitor, qVD-OPH, greatly reduces this cell death (Fig.3.6). This is generally considered to be strong evidence for an apoptotic death since all known types of death requiring caspase activity are apoptotic. However, this conclusion is dependent on the assumption that qVD-OPH is not acting by blocking other potentially destructive proteins such as calpains or capthensins. Currently available evidence indicates that qVD-OPH is selective for caspases and blocks caspase 8, caspase 9 and caspase 3 activity (Caserta et al., 2003), although it might block another protease such as calpain. Another caspase inhibitor, fmk-ZVAD, also enhanced survival although not as effectively as qVD-OPH (table 3.1).

Other evidence that the cell death is apoptotic is the delayed nature of the death and the nuclear condensation and fragmentation seen following the stress. Cell death from the standard necrotic type of mechanism is usually much more rapid, occurring within hours rather than days after the stress and is usually accompanied by bursting of the cells rather than shrinkage and nuclear condensation. Thus, the cell death observed here is apoptotic type, although a minor fraction of the death that occurs within hours after the stress is likely to be necrotic.
Fig. 4.2A summarizes the findings on apoptotic pathways activated by OGD. OGD activates the intrinsic pathway by inducing Bax translocation to mitochondria and mitochondrial depolarization. This process is enhanced by Bim upregulation. Bcl-xl was not increased after stress, suggesting that Bcl-xl could not counteract the proapoptotic proteins Bax, Bim and Bid. TBid, produced by Caspase 8 or caspase 2 cleavage, was not significantly increased after stress. The apoptotic cascade further proceeds with activation of caspase 3, likely downstream of caspase 9. Active caspase 3 induces nuclear damage, which further contributes to cell death. The general caspase inhibitor qVD-OPH increases survival by blocking activation of downstream caspases, likely caspase 3 and 7, but also by blocking yet unknown apical, or upstream caspases. Protein synthesis inhibitor Chx increases survival rate, by inhibiting expression of proapoptotic proteins such as Bim.

In the present study, OGD appeared to sensitize neural precursors and young neurons to subsequent hyperthermia. Mechanisms underlying this sensitization are not yet known, but are likely to involve prolonged cellular changes produced by the OGD stress. These might include subtle mitochondrial damage, partial depletion of ATP, and/or activation of transcription factors that could then lead to upregulation of pro-apoptotic proteins by the hyperthermia.

A proposed model for synergistic effects of OGD and hyperthermia is shown in Fig 4.2B. OGD induces ATP depletion (Kusumoto et al., 1996), which further causes dephosphorylation of FOXO 3A, a transcription factor whose dephosphorylated form translocates to nucleus to induce Bim upregulation (Sunters et al., 2003). Hyperthermia may enhance the ATP depletion (White, 2005), further contributing to the downstream
apoptotic effects. Hyperthermia can activate Bax dimerization by itself (Pagliari et al., 2005), eventually leading to cell demise.

**Applicability of results from embryonic NPCs to adult NPCs: would these cells respond differently to the stress?**

We used embryonic NPCs as culture paradigm, but the results may be applicable to both embryonic NPCs and adult NPCs. Studies comparing adult versus embryonic NPCs showed that embryonic NPCs give rise to more neurons, compared to adult NPCs, which predominantly form oligodendrocytes (Eucher et al., 2006). However, when embryonic and adult cell types were stressed with amyloid, their differentiation profile was unchanged, such that following stress, the NPCs gave rise to the same proportion of neurons, astrocytes and oligodendrocytes as before stress. Amyloid stress induced similar decrease in mitochondrial function in both adult and embryonic NPCs. These data suggest that embryonic and adult NPCs may have similar responses to stress. Thus our results indicate that OGDT may damage NPCs in both embryonic and adult brain.

**Are rapidly proliferative cells more sensitive to hyperthermia?**

The present study provides several lines of evidence indicating that immature cortical cultures are especially sensitive to hyperthermia, compared to older cultures. In vivo, germ cells, thymocytes, and neuroepithelial cells undergo apoptosis after exposure to heat shock (Khan and Brown, 2002), suggesting that actively dividing cells are prone to heat-induced death. Rapidly proliferating cells in culture are more sensitive to hyperthermia than slowly dividing cells, and this vulnerability is increased with higher temperatures (Johnson and Pavelec, 1972).
Since dividing cells are more sensitive to heat stress, a common mechanism could be damage to the mitotic apparatus. Both spermatogonia undergoing mitosis and primary spermatocytes during the first stage of meiosis are sensitive to heat (Khan and Brown, 2002). Insect cells are most sensitive to heat shock during mitosis, showing disruption of the mitotic spindle, especially of the centrosome (Debec and Marcaillou, 1997). Thermal damage to centrosomal structure is also seen in Chinese hamster ovary (CHO) cells, but cells preconditioned with mild hyperthermia were able to repair the damage (Vidair et al., 1995). Heat shock led to either G1 or G2/M arrest in a glioma cell line (Kuhl et al., 2000).

Damage to the mitotic apparatus could explain the reduced proliferation after heat stress in the immature neuronal cells used in this study; 5% of young neurons were BrdU (+) in control cultures, but only 2.5% of young neurons were BrdU (+) after heat stress. Another mechanism could be a block in S phase of the cell cycle, preventing further incorporation of BrdU. While 45 min at 43 °C damaged at least some of the proliferating NPCs, a slightly longer stress (60 min at 43 °C) severely damaged many immature neurons that were post-mitotic (White, 2005). In contrast, post-mitotic mature neurons in culture were not killed by 60 min at 43 °C (White et al., 2003). Thus cell division may not be the only factor that makes these cultures sensitive to heat stress.

NPCs and immature neurons versus mature neurons: Do these cell populations have different susceptibility to hypoxia-ischemia?

Immature brains are less susceptible to hypoxia-ischemia than adult brains (Zhu et al., 2006). Cytochrome c content increases during development (Blomgren et al., 2003), probably to keep up with the increased energy demand of the mature brain. Thus
increased energy demand in mature neurons may render them more vulnerable to oxygen deprivation.

In culture, immature neurons are less vulnerable to OGD than mature neurons: progenitor cells can withstand 4 h of OGD without substantial cell death (Kalluri et al., 2007). On the other hand, mature neurons suffer severe damage: 60-90 min exposure to OGD killed ~ 60-70% of neurons (Cao et al., 2003; Kusumoto et al., 1996). This difference may be due to increased glycolytic metabolism and lower energy demands in NPCs. Thus, these cells may keep up with essential energy demands and may be more resistant to hypoxia than mature neurons. In our study, OGD produced more damage on NPC cultures than reported by Kalluri and colleagues (2007): 2 h of OGD reduced survival of NPCs from ~65 % in non-stressed cultures to ~45 % at 2 days after stress. Different cell culture systems and OGD protocols may account for the reported variations in survival after OGD. For example, even small differences in residual glucose and/or oxygen might have large effects on survival. Studies of the glucose transporters in NPCs might help address this problem.

Neural stem cells versus restricted progenitors: are these cells differently affected by hypoxia–ischemia?

In vivo studies showed that neural stem cells and restricted progenitors have different sensitivity to hypoxia-ischemia. Perinatal hypoxia-ischemia induced selective death of PSA-NCAM (+) neuronal progenitors in the lateral SVZ, while sparing nestin (+) cells in the medial zone of postnatal rat brain (Romanko et al., 2004). Hypoxia also killed oligodendrocyte progenitors in the periventricular white matter (Levison et al., 2001). These results suggest selective vulnerability to hypoxia–ischemia of at least some
neuronal progenitors and oligodendrocyte progenitors, while some neural stem cells may be more resistant. One possible explanation for selective resilience of stem cells is the presence of glycogen granules that enable these cells to survive through the stress via glycolysis. However, at late times after reperfusion, stem cells were also depleted, possibly due to recruitment of these cells to an astrocytic fate (Rothstein and Levison, 2002).

Our in vitro study is consistent with resistance of stem cells to OGD. Using a colony-forming assay, we found that 2 h OGD reduced the number of small neurospheres formed (p<0.001), probably representing colonies derived from more restricted progenitors, but did not alter the number of large neurospheres, probably originating from true stem cells. Yet the number of large neurospheres was reduced when mild hyperthermia followed the OGD. Thus our data support the hypothesis that stem cells were not affected by OGD alone, but their number (or their proliferative capacity) was reduced by the combination of OGD and mild hyperthermia. This might be due to greater sensitivity of stem cells to hyperthermia, or might represent a sensitization mechanism: OGD further sensitizes stem cells to hyperthermia.

**Differences in cell death between immature and mature neurons**

Immature brains possess more apoptotic machinery than the adult brain. Caspase 3 is found at high levels in the developing brain (Zhu et al., 2005) but its activation following ischemia declines during development (Hu et al., 2000). Other apoptotic effectors such as Bax (Vekrellis et al., 1997) and AIF (Zhu and Blomgren, 2005) are also upregulated in immature brain, suggesting that the immature brain exhibits readiness to
undergo apoptosis (reviewed in Blomgren et al., 2007). Levels of apoptotic proteins are
downregulated in the adult brain, whereas levels of anti-apoptotic Bcl-xl and Bcl-2 are
maintained (Vekrellis, 1997). Although cytochrome c content is lower in young than in
adult neurons, cytochrome c is more readily released from mitochondria after
hypoxia/ischemia in immature brain (postnatal day 5) than in brain at postnatal days 9, 21
and 60 (Zhu and Blomgren, 2005), suggesting that cytochrome c-caspase 3 dependent
death is more prevalent in immature than in adult brain.

The high expression of apoptotic proteins during development may contribute to
the greater sensitivity of young neurons to mild insults. NMDA- or kainate-induced cell
death occurs more readily in neonatal than mature brain (van Lookeren Campagne et al.,
1995). In our study, brief exposure to hyperthermia induced damage of NPCs and
immature neurons, consistent with the greater expression of apoptotic machinery in
young neurons and immature brains. Alternatively, stress induced damage upstream of
the apoptotic mechanism might be greater in the young cells.

**Cycloheximide protects immature, but not old neurons**

The apoptotic death induced in NPCs by hyperthermia at 43º C or combined OGD
followed by mild hyperthermia was partially blocked by pharmacological inhibition of
protein synthesis. This is interesting, because Chx did not save more mature neurons
from heat stress (White et al., 2007). Chx applied within a therapeutic window after
perinatal hypoxia in vivo reduced infarct volume, although the mechanisms remain
unknown (Park et al., 2006). Thus upregulation of pro-apoptotic protein(s) may
contribute to the high sensitivity to stress of younger neural cells both in culture and in
vivo. The mechanisms triggering this proposed upregulation of apoptotic proteins are
unknown, but Chx blocked the upregulation of Bim in one experiment (data not shown). Thus studying the correlation of Chx-induced protection with levels of pro-apoptotic proteins (Bim, Noxa, Puma, Bax, Bid) may help identify pathways critical for apoptosis in neural precursors.

Chx might also protect via additional mechanisms during the stress. For example, it might protect by reducing the amount of nascent proteins prone to misfolding and aggregation (Liu et al., 2005). Ischemia can result in aggregation of these nascent proteins with ribosomes and even mature proteins (Hartl and Hayer-Hartl, 2002; Liu et al., 2005). For proper folding, proteins need chaperones (HSP70 and Hdj1) as well as ATP (Hartl and Hayer-Hartl, 2002). OGD and hyperthermia may deplete ATP levels, thus compromising protein folding for newly born peptides and thereby favoring formation of protein aggregates. Blocking protein synthesis just before, during or immediately after stress may reduce this protein aggregation, thus saving cells from death due to toxic accumulations of protein aggregates.

Relevance to clinical setting

The results of this dissertation have important implications for clinical situations. Hyperthermia during embryonic development is known to damage the nervous system in both rodents and humans. In the adult brain, transient hyperthermia from fever alone or from fever following a stroke may induce damage to neural progenitor cells as well. Hence finding therapeutic approaches to prevent hyperthermia–induced damage are important. One proposed strategy deriving from these studies is using anti-apoptotic agents, such as general caspase inhibitors, e.g. qVD-OPH. In vivo studies already
showed efficacy of qVD-OPH in preventing brain damage after ischemia (Braun et al., 2007; Renolleau et al., 2007). Since this drug is a broad inhibitor, it may have unwanted adverse reactions, which need to be identified carefully. The present study identified Chx as an effective agent to reduce cell death after stress. Although its effects are dependent on concentration, and it is considered toxic at higher levels, administration of strictly controlled doses within certain time windows after stroke may be a potent therapeutic tool.

The data presented here may also have a broad clinical significance for degenerative disease. The OGDT paradigm used here can mimic some aspects of mitochondrial dysfunction seen in Alzheimer’s, Parkinson’s or Huntington’s disease. Extrapolating results from the present study, it is possible that mild hyperthermia aggravates degenerative processes. Also, strategies used here to rescue damaged young neurons and NPCs may be useful for the treatment of neurodegenerative conditions.

**Future experiments**

More work needs to be done to understand several fundamental questions related to mechanisms of cell death following OGD and hyperthermia.

One question concerns the initial event that leads to activation of the apoptotic cascade. Is this initial event an apical caspase or other protease? We found evidence for caspase activation within 3 h after an OGDT stress, so candidates include caspase 2 and caspase 8. Fluorescent substrate-based methods could be used to assay for substrate-specific cleavage by these caspases. Since the hypothesized apical caspase/protease appears to be inhibited by pan-caspase inhibitors, another way to identify it is by
capturing it with biotinylated qVD-OPH. Samples treated with biotinylated qVD-OPH could then be run on an avidin affinity column to purify and thus help identify any proteins covalently bound by qVD-OPH.

Results from this work suggest that Bim is upregulated after OGDT. Further experiments are needed to elucidate the role of Bim in the apoptotic cascade. What leads to Bim upregulation, and is Bim upregulation sufficient to induce death? One pathway for Bim activation is dependent on JNK kinase, which was found to induce Bim EL (Okuno et al., 2004). This idea could be tested by blocking JNK kinase with a selective JNK inhibitor (SP600125), and assaying for effects on Bim protein levels and NPC survival. Knock–down experiments using siRNA could determine whether reducing Bim protein levels protects cells from stress-induced death. Alternatively, if cells continue to die to the same extent Bim may be dispensable and/or act in parallel with other pro-apoptotic factors such as Noxa.

An initial event contributing to OGD-induced sensitization to further hyperthermia might be ATP depletion, as seen for mature neurons in OGD models. ATP measurements after OGD stress will document the time course of the decrease in ATP and possibly reveal a correlation between the initial ATP level and the extent of NPC death.

Preliminary results revealed an increase in intracellular \([\text{Ca}^{2+}]\) in a subset of cells immediately after the combined OGDT stress, but not after the individual stresses (data not shown). Excessive increases in intracellular \([\text{Ca}^{2+}]\) could be an upstream event that triggers activation of Ca-dependent proteases, such as calpains. Attempts to block calpain activation using one specific inhibitor (calpain inhibitor VI) were not successful, but
other methods to identify calpain cleavage products (fodrins) on western blots could be tried. One could also perform an enzymatic assay for calpain activity.

Since the OGD and hyperthermia stresses used here seem to synergistically activate the intrinsic mitochondrial death pathway, drug interventions might be aimed towards blocking key components of this apoptotic cascade. Candidate drugs include caspase inhibitors, inhibitors of mitochondrial permeability transition (e.g. cyclosporin A) and inhibitors of cytochrome c release. But methods for preventing the initial damage from the stresses might complement treatments that block the death pathways and be even more effective in promoting long-term survival of NPCs.
Fig 4.1
Mechanisms of hyperthermia-induced cell damage in immature neurons and NPCs
Hyperthermia activates the intrinsic pathway resulting in mitochondrial depolarization; this event likely induces caspase 3 activation observed 18 h post-stress, followed by cell death. Hyperthermia induces early nuclear damage, evidenced by AIF nuclear protrusions and phosphorylation of histone H2AX. Chx blocks hyperthermia-induced cell death, likely by inhibiting proapoptotic proteins.
Proposed mechanisms of OGDT- induced damage

A) OGDT stress induced activation of the intrinsic pathway, with upregulation of Bim, followed by translocation of Bax to the mitochondria, formation of mitochondrial outer membrane depolarization (MOMP) and mitochondrial depolarization. tBid increase, a result of Bid cleavage by caspase 8, was not significant. Prosurvival protein Bcl-xl, which antagonizes the action of Bim, Bax and tBid, was not significantly increased after stress, suggesting predominance of apoptotic, rather than prosurvival mechanisms. Caspase 3 was activated by caspase 9 downstream of mitochondria, along with other OPH-inhibitable caspases/proteases. Active caspase 3 induces nuclear damage, which further contributes to cell death. Apical caspases /proteases, inhibitable by general caspase inhibitor OPH, are likely activated early after stress. Protein synthesis inhibitor Chx increased survival rate, by inhibiting expression of proapoptotic proteins such as Bim.

B) A model for contribution of individual stresses to cell damage. OGD likely induces ATP depletion, further accentuated by hyperthermia. ATP depletion leads to dephosphorylation of FOXO 3A, a transcription factor that further translocates to nucleus and induces Bim transcription. Bim upregulation can be induced by hyperthermia alone. Bim upregulation further enhances Bax translocation to mitochondria; however, hyperthermia can induce Bax mitochondrial insertion independent of Bim, eventually resulting in cell death.
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