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The Early Events and Long-Term Effects of Perinatal Cerebral Hypoxia-Ischemia

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The Early Events and Long-Term Effects of Perinatal Cerebral Hypoxia-Ischemia

by

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To my loving Appa, Amma, Aravind, and Ernest.

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"We are like dwarfs on the shoulders of giants, so that we can see more than they, and things at a greater distance, not by virtue of any sharpness on sight on our part, or any physical distinction, but because we are carried high and raised up by their giant size."

-Bernard of Chartres

The Early Events and Long-Term Effects of Perinatal Cerebral Hypoxia-Ischemia

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Neonatal hypoxia-ischemia (HI) affects 60% of low birth weight infants and up to 40% of preterm births. Due to the increasing incidence of preterm and low birth weight infants and the lack of adequate treatment for HI, characterization of brain injury after HI remains an extremely relevant area of interest. Severe HI causes immediate necrosis of neuronal cells, and secondary apoptosis of surrounding cells due to neuronal inflammation. Hence, we sought to characterize the biochemical pathways associated with cell death after HI, as well as the extent of neurological injury that is transferred to the next generation. Bax, a cell death signaling protein, is activated after HI, and translocates to the nucleus, ER, and mitochondria. Translocation patterns of Bax affect the cell death phenotype (necrotic or apoptotic) observed. Once activated, Bax is known to oligomerize. However, little is understood about the factors that control translocation

and oligomerization of Bax. We hypothesized that phosphorylation and interaction with kinases determines Bax intracellular localization and oligomerization. Using well-established *in vivo* and *in vitro* models of neonatal HI, we characterized Bax oligomerization and multi-organelle translocation. We found that HI-dependent phosphorylation of Bax determines its oligomerization status and multi-organelle localization, and ultimately the cell death phenotype observed. Cell death and brain injury after HI have been shown to cause long-lasting behavioral deficits. Neurological injuries have previously been shown to cause epigenetic changes, affecting generations to come. By using a battery of behavioral tests on second generation 3-week-old rodents, we found that neonatal HI is associated with behavioral consequences in progeny. Our results suggest an epigenetic transfer mechanism of the neurological symptoms associated with neonatal HI. Elucidating the transfer of brain injury to the next generation after HI calls attention to the risks associated with HI injury and the need for proper treatment to reverse these effects. Understanding the mechanisms of Bax translocation will aid in the rational design of specified therapeutic strategies which could potentially involve altering Bax subcellular redistribution to decrease the irreversible trauma resulting from a prolonged inflammatory response.

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Chapter 1: Introduction

The human brain begins to form three weeks after conception and continues to develop and rearrange itself until death. Still, at birth, the brain is remarkably immature. Consisting of newly differentiated neurons, neural stem cells, and glial cells, the brain is quite vulnerable to environmental factors during perinatal development. The P7 rat is an excellent model of third-trimester human brain development, and therefore ideal for use in examining the damaging effects of perinatal brain injuries. This dissertation focuses on understanding the immediate, as well as the long-lasting, effects of perinatal hypoxia-ischemia.

PERINATAL HYPOXIA-ISCHEMIA

Epidemiology and Causes

Perinatal hypoxic-ischemic encephalopathy (HI) is a major contributor toward infant mortality and brain damage, affecting thousands of infants across the world. The United States ranks seventh in the world in mortality rates due to perinatal hypoxia, with more than one in every 500 births experiencing some degree of HI every year (Jatana et al., 2006, Shankaran and Laptook, 2007, Fathali et al., 2011). Hypoxia can either be caused by a lack of oxygen supply or the inability of a cell to utilize the oxygen. Coupled with ischemia, where there is a lack of blood flow to provide adequate nutrients, cell death is imminent due to the loss of energy production in the cell.

A number of physical, environmental, biochemical, or epigenetic factors can contribute to the occurrence of HI. Major factors that contribute to HI include premature

birth, low birth weight, umbilical cord strangulation, or complications during delivery that disrupt normal cerebral blood flow (Zanelli et al., 2008). More than 60,000 preterm or low birth weight infants are born every year in the US and up to 60% of these infants experience HI (Hamilton et al., 2011),. In recent years, there has been a strong media focus on the alarmingly increasing rate of premature births and low birth weight. Both of these factors can contribute to poor vascularization and delayed lung development, ultimately leading to systemic HI (Curristin et al., 2002). In addition, asphyxiation caused by physical factors such as umbilical cord strangulation can result in up to 50% mortality rate within the newborn period, with survivors suffering major neurological deficits (Sharp and Bernaudin, 2004, Vannucci and Hagberg, 2004).

Nearly 25% of infants who survive HI accrue brain damage and show symptoms of mental retardation, cerebral palsy, epilepsy, and other cognitive or behavioral deficits (Vannucci and Hagberg, 2004, Fathali et al., 2011). In severe cases of HI, mortality can exceed 50%. More than 80% of infants who survive *severe* HI develop neurological deficits. No immediate developmental deficits have been observed for mild cases of HI. However, as these children mature and require the use of higher order brain processing for learning activities and social interactions, a developmental delay is observed (Smith et al., 2008).

Susceptibility of the Perinatal Brain

Currently, much of what we know about how HI affects the brain comes from studying experimental stroke models in adults. However, mechanistic knowledge of the

adult brain cannot easily be applied to neonates. Though the neonatal brain was originally thought to be resistant to hypoxic-ischemic damage, increasing evidence shows that there are key periods of sensitivity during development where the perinatal brain is especially susceptible to HI (Vannucci and Hagberg, 2004). In the immature brain, high levels of protein turnover, oxidation, nitrosylation, and energy consumption, coupled with low levels of antioxidants make neuronal cells especially vulnerable to HI (Ferriero, 2004).

In the neonatal brain, HI produces acidosis, glutamate excitotoxicity, and oxidative stress (Vannucci et al., 1988, Vannucci and Hagberg, 2004). Low cellular energy levels cause depolarization of neurons and release of excitatory amino acids, such as glutamate. Due to lack of energy, reuptake mechanisms are also affected, resulting in glutamate excitotoxicity. Glutamate binds to NMDA receptors, which are abundant in the neonatal brain. Activation of NMDA receptors is especially toxic to neonates, as this can affect neuronal migration and synaptic plasticity and lead to developmental abnormalities. Oxidative stress is exacerbated in the neonatal brain, as a lack of antioxidants and a high level of free iron contribute to generation of hydroxyl radicals via the Fenton reaction (Vannucci and Hagberg, 2004). In addition, activation of neuronal nitric oxide synthase generates nitric oxide, which in turn leads to mitochondria dysfunction and production of more ROS (Vannucci and Hagberg, 2004).

Cerebral glucose metabolism rates depend on the maturity level of neurons (Ferriero, 2004). As such, glucose metabolism rates are much lower in neonates than in adults. Neonates use 90% less glucose than adults during first few weeks after birth. Though glucose metabolism is the key energy producer, neonates can use other sources

such as lactate, ketone bodies (β-hydroxybutyrate and acetoacetate). However, the immature brain can transport glucose far less easily than lactate and ketone bodies. During HI, the brain relies on anaerobic glycolysis and glucose utilization is essential, causing neonates to be especially susceptible to low glucose levels caused by HI. In neonatal rats, maintaining hyperglycemia *during HI* has been shown to alleviate injury, but there is the danger of too much glucose present when the brain returns to normoxia. Hyperglycemia in the recovery stage following HI has been shown to be detrimental (Vannucci and Hagberg, 2004).

Negative Outcomes and Pathology

The injury phenotype after HI is dependent on a number of factors, including the time of exposure, severity of HI, and cause of HI. Additionally, sensitivity to injury varies for each brain region depending on the developmental stage (Ferriero, 2004). Severity can range from mild and short-term to severe HI. Mild HI leads to apoptosis of a small number of cells, and minimal inflammatory signaling (Gill, 2007, Gill and Perez-Polo, 2008). However, with severe cases of HI, immediate necrosis of affected cells leads to inflammatory signaling. Inflammatory signaling ultimately leads to secondary apoptosis of surrounding cells, and a larger injury overall (Gill, 2007).

The approximate point at which a tissue is termed hypoxic depends on the 'critical tissue oxygen tension' value, which is tissue-specific. This value is dependent on the tissue's demand for oxygen and its aerobic capacity (Sharp and Bernaudin, 2004). The brain is the most sensitive to oxygen deprivation, as the levels of oxygenation in the mammalian brain are as low as 1-5% (Sharp and Bernaudin, 2004). Thus, small changes

in oxygenation can have drastic consequences. When exposure to HI occurs late in gestation or in term infants, the perirolandic cortex is most affected (Ferriero, 2004). Cells expressing nitric oxide synthase in the basal ganglia are less susceptible to the injury, but are responsible for oxidative stress and excitotoxicity that leads to secondary cell death (Ferriero, 2004).

In mild cases, a small injury is formed due to an immediate apoptosis of affected cells. In such a case, the injury may be so subtle that it may go undiagnosed. The behavioral consequences may not be expressed until 7-8 years of age and even then, may go unnoticed. For example some individuals who are diagnosed as adults with having Attention Deficit Hyperactivity Disorder, have in fact experienced symptoms since childhood. Severe cases of HI can cause learning, cognitive, behavioral, and social disabilities (Vannucci and Hagberg, 2004).

Clinical Treatments

Currently, limited clinical therapeutic interventions exist for HI including temperature regulation and hyperoxia, both of which have shown conflicting results in ameliorating the injury. Hypothermia has been attempted to alleviate HI since the early 1950s. However, although a number of groups have shown hypothermia to be neuroprotective to some extent, many have not found hypothermia to have beneficial effects (Yager et al., 1993, Dietrich et al., 1993). Low birth weight and very low birth weight infants often already suffer from hypothermia, which reduces blood circulation and is believed to be detrimental to the developing body. Often, hyperthermia is the remedy for this, though it is known to exacerbate injury after HI. More importantly,

hypothermia is not equally beneficial to severe and mild HI. Experiments with the Cool Cap suggest that hypothermia is most effective for short-term or mild HI injuries, but does little to improve conditions after severe HI (Gluckman et al., 2005, Edwards et al., 2010). Lastly, even in cases where hypothermia appears to be effective, no long-term follow up procedures have been implemented, therefore it is unknown whether the benefits are long-lasting.

Another equally conflicting therapy for HI is that of hyperoxia, which consists of administering supraphysiological levels of oxygen to the HI-affected infant. However, the detrimental effects of hyperoxia treatment have been proven be numerous. Hyperoxia is ineffective at reducing cerebral edema after HI, but more importantly, hyperoxia exacerbates injury after HI (Ferrari et al., 2010, Gill et al., 2008). Consistent with our results that hyperoxia has a negative effect on HI injury, Brutus et al. 2009 found that hyperoxia increased serine phosphorylation and resultant inhibition of antiapoptotic protein Bcl-2 (Brutus et al., 2009). This ultimately led to an increased apoptotic cell death after hyperoxia treatment of HI. Excess oxygen is a potent trigger of apoptotic neuronal cell death in the developing brain (Taglialatela et al., 1998, Felderhoff-Mueser et al., 2004). Hyperoxia generates ROS similar to reoxygenation after HI leading to increased oxidative stress (Hu et al., 2003, Felderhoff-Mueser et al., 2004). Consequently, hyperoxia increases activation of cell death signaling, inflammation, and injury by increasing necrotic-like cell death (Gill et al., 2008). Paradoxically, hyperoxia is still in use as the standard treatment for HI. As current therapies are all but ineffective for severe

cases of HI, the aim of this project was to find a new potential therapeutic target to reduce inflammation and injury after HI.

Investigative Models of HI

In Vivo Models

A number of *in vitro* and *in vivo* models of neonatal HI have been developed to investigate the effects of HI on brain development. As the maturity level of a P7 rat and P10 mouse is histologically comparable to that of a third trimester infant, the rodent is an ideal candidate for a neonatal HI injury model (Dobbing and Sands, 1979, Vannucci and Hagberg, 2004, Rice et al., 1981). Comparable to the third trimester in humans, axons and dendrites undergo rapid differentiation and the cortex doubles in volume during the first 20 days after birth in rats (Curristin et al., 2002).

The clinically relevant Rice-Vannucci (R-V) model of perinatal ischemia and the Middle Cerebral Artery Occlusion (MCAO) model are the two most prevalently used *in vivo* models of HI injury. Both models use P7 rat pups, and result in brain injury due to oxidative stress. We use the well-established R-V model for our studies, as it allows us to compare ipsilateral and contralateral results at early timepoints.

The R-V model requires a unilateral permanent ligation of the left common carotid artery of a P7 rat pup, and systemic exposure to 8% oxygen for two hours, accounting for the ischemic and hypoxic elements of the injury, respectively. In this model, systemic blood pressure decreases by 25%, cerebral blood flow is reduced by 40-60% in the ipsilateral hemisphere, and brain damage is mostly isolated to the ipsilateral side of injury. The cerebral cortex, subcortical and periventricular white matter, thalamus,

and hippocampus are the target injury sites in this model. The R-V model results in a severe HI brain injury, causing necrosis, inflammatory signaling and secondary apoptosis. As such, this model is ideal for investigating the role of cell death signaling in injury progression after HI. (Vannucci and Hagberg, 2004).

In Vitro Models

In vitro oxygen and energy deprivation models of HI take a reductionist approach and focus on specific aspects of the HI injury paradigm. The most commonly utilized models are the Oxygen-Glucose-Deprivation (OGD) model and the rotenone injury model of HI. The OGD model most closely resembles the clinical aspects of HI, as it includes both the hypoxic and ischemic components of injury. Additionally, OGD results in necrotic cell death, with apoptotic phenotypes appearing after returning to normoxic conditions (Fordel et al., 2007, Koubi et al., 2005). Though at first glance OGD may seem to be a simple and efficient model for HI, any minor deviation from the protocol can lead to exceeding variabilities in the injury.

Alternatively, a simple solution becomes apparent when considering the rotenone dose modulation injury model (Imamura et al., 2006, Gill, 2007). Rotenone, a mitochondrial complex I inhibitor, produces morphologically different cell death phenotypes according to dosage. In SY5Y neuroblastoma cells, administration of 25μM rotenone produces an apoptotic phenotype, whereas 100μM rotenone produces necrotic-like cell death. As such, this model is ideal for studying the signaling pathways affected by HI that contribute to cell death phenotype.

CELL DEATH AND INFLAMMATION AFTER HI

Programmed cell death is common during development. In fact, it is essential for removal of redundant cells, formation of digits, defense against pathogens, and tissue turnover. Injurious cell death after HI is dependent on the severity of the injury, but portrays primarily apoptotic or necrotic phenotypes. In severe HI, inflammation, induced by cells displaying necrotic phenotypes, plays a key role in exacerbating injury and leading to secondary cell death via apoptosis.

Chemoreceptors in the medulla of the brainstem as well as those located at the carotid artery bifurcation are responsible for detecting systemic oxygenation. At the cellular level, the detection of hypoxia induces the expression of transcription factor HIF1α (Sharp and Bernaudin, 2004). Response to HI in cells includes a barrage of cell signaling, involving key players such as HIF1α, as well as Bcl-2 family proteins. In addition, HI triggers the IL-1→NFKb→Cox-2/iNOS pathway, which leads to increased oxidative stress. Oxidative stress in turn triggers apoptotic and necrotic cell death (Xin et al., 2007). Oxidative stress shifts Bax, a Bcl-2 family member involved in cell death signaling, from the cytosol to organelles, such as the nucleus, mitochondria, and ER (Gill et al., 2008, Gill, 2007). Bax association with the nucleus produces necrotic-like cell death morphologies, leading to monocyte recruitment and chronic inflammation (Gill, 2007, Gill et al., 2008).

Inflammation

Inflammation plays a major role in injury after severe hypoxia-ischemia in neonates (Ferriero, 2004). Mixtures of necrotic and necroptotic cell death trigger the activation of inflammatory cytokines. Glial cells undergo inflammatory activation and can lead to neuronal death (Bal-Price and Brown, 2001). Inflammatory cells trigger the activation of the death receptor, or extrinsic, pathway of apoptosis of surrounding cells (Thornton et al., 2012). Since such a mixture of cell death phenotypes are observed after HI, our goal was to identify the mechanisms involved in necroptotic cell death signaling so that it may be targeted specifically to potentially reduce the inflammatory injury observed after HI.

Apoptosis

The term apoptosis was coined in 1972 by Kerr, Wyllie, and Currie. At the time, they characterized the very clear morphological differences in apoptotic versus necrotic cells and deemed it was because of cell death signaling pathways associated with apoptosis (Hengartner, 2000). Developing tissues are constantly undergoing apoptosis, and apoptosis occurs throughout an organism's life. The developmental stage of organism influences the signaling pathways for cell death (Morrison et al., 2002). Apoptosis is phenotypically characterized by perinuclear chromatin condensation, cell shrinkage, endonuclease-mediated internucleosomal DNA cleavage. Two different pathways are known to trigger these phenotypes, the intrinsic (mitochondrial) pathway, and the extrinsic (death receptor) pathway (Adams and Cory, 2007). Both pathways converge at

the mitochondria, require the activity of caspases, and both pathways are observed after severe HI (Adams and Cory, 2007, Delivoria-Papadopoulos et al., 2008, Youle and Strasser, 2008, Thornton et al., 2012).

The key difference between apoptotic and necroptotic signaling is that apoptotic signaling requires the activity of caspases (Hitomi et al., 2008, Vanden Berghe et al., 2004, Kawahara et al., 1998). Caspases are highly conserved cysteine proteases that are attributed to causing the morphological changes observed in apoptosis. Caspases have an active-site cysteine and cleave substrates after an aspartic acid residue. Major caspases involved in apoptosis include caspase-8, 9, and 3. Cell membrane proteins (like fodrin), as well as proteins such as PAK2 (p21 activated kinase) are cleaved by caspases, which leads to membrane blebbing during apoptosis (Hengartner, 2000). Caspase-3 cleaves nuclear lamins, which leads to nuclear shrinking and budding. The Bcl-2 family of proteins is the major regulators of cell death signaling, and is responsible for regulation of caspase activity.

TNFR family members, which are typically transmembrane proteins with an extracellular cysteine-rich domain and a cytoplasmic death domain, are involved in the extrinsic pathway of neuronal apoptosis during development and after injury (Morrison et al., 2002). During HI, the extrinsic apoptotic pathway is activated via the activity of inflammatory cells (Thornton et al., 2012, Delivoria-Papadopoulos et al., 2008). Briefly, once the ligand binds to the death receptors, the precursor forms of initiator caspases, specifically procaspase-8, are recruited and undergo self-cleavage to form active caspase-8. Caspase-8 subsequently amplifies the signal by cleaving more procaspase-8, as well as

other cell death signaling proteins and kinases. At this point, the Bcl-2 family member Bax is phosphorylated and translocates to mitochondria, where it induces the release of mitochondrial contents such as cytochrome c and SMAC/Diablo. SMAC/Diablo are responsible for sequestering proteins aptly named "Inhibitors of Apoptosis Proteins" in order to perpetuate the apoptotic signaling pathway. Cytochrome c, meanwhile, interacts with caspase-9 to form the apoptosome, and activate the final "effector" caspase, caspase-3. Caspase-3 is ultimately responsible for the degradation of cellular contents and following through with apoptosis (Hengartner et al 2000). The intrinsic pathway of apoptosis is activated by oxidative stress, which triggers the activation and translocation of Bax to mitochondria, and ultimately results in cell death from the release of mitochondrial celluar contents as described above.

Mild HI leads to quick apoptosis of affected cells, leaving the surrounding cells presumably unperturbed (Gill and Perez-Polo, 2008). During severe HI, apoptosis occurs as a secondary response to inflammatory signaling triggered by cells exhibiting necrotic phenotypes. Inflammatory cells trigger the activation of the death receptor, or extrinsic, pathway of apoptosis (Thornton et al., 2012). It is also important to note that while severe HI causes apoptotic signaling, it also causes mitochondrial dysfunction, which leads to a loss of ATP production. Often, since ATP is required for apoptotic signaling, cells that do not complete the apoptotic signaling pathway end up with a change in cell phenotype to portray a "continuum" of cell death. In other words, they are continually starting and halting cell death signaling, and thus remain in an impaired, weakened state for the duration of their existence (Northington et al., 2007).

Necroptosis

Necrosis is classically defined in terms that indicate the absence of apoptotic characteristics, and is essentially a passive process. Necrotic morphology includes loss of plasma membrane integrity, cell swelling, and organelle swelling. The disruption of calcium homeostasis due to oxidative stress and glutamate excitotoxicity plays a key role in necrotic cell death observed after HI (Semenova et al., 2007, Ermak and Davies, 2002, Park et al., 2010, Vannucci and Hagberg, 2004). Both cause a cellular influx of calcium and an extrusion of calcium from the ER, leading to a highly increased concentration of calcium within the cytoplasm. This leads to mitochondrial and nuclear uptake of calcium. Increasing calcium concentrations in the mitochondria cause them to swell and burst, leading to passive, necrotic cell death (Ermak and Davies, 2002). However, calcium signaling also regulates ER-mitochondrial necrotic *signaling* pathways.

In recent years, a new type of necrotic cell death is being observed after HI, and an increasing body of evidence demonstrates that this type of cell death utilizes a distinct signaling pathway (Degterev et al., 2005, Moubarak et al., 2007, Hitomi et al., 2008, Chavez-Valdez et al., 2012). As early as 1997, there was evidence indicating that activation of classic apoptotic death receptors (FAS/TNFR) could lead to cell death with necrotic morphology (Kawahara et al., 1998, Vercammen et al., 1997) . There is also evidence indicating that their activity is involved in caspase-independent cell death, or necroptosis (Kawahara et al., 1998, Hitomi et al., 2008, Vanden Berghe et al., 2004). Not until 2005 did Degterev et al. coin the term “necroptosis” for this phenomenon. Thus, in this dissertation, programmed necrosis will be referred to as “necroptosis” (Degterev et

al., 2005). Necroptosis can be defined as caspase-independent cell death signaling that results in the formation of necrotic morphology, and most importantly, triggers inflammation.

The evidence for necroptotic signaling cascades being separate activities from apoptotic signaling is only increasing over time. Numerous studies indicate the involvement of specific proteins in necroptotic cell death signaling. As stated above, due to changes in calcium concentrations after HI, the ER-mitochondrial necroptotic pathway is activated (Chen et al., 2010). Nix/BNip3L is a "BH3-like" protein localized on the outer mitochondrial membrane (OMM) and ER. At mitochondria, Nix/BNip3L interacts with Bax/Bak and 'stimulates' OMM permeability (Chen et al., 2010). Nix/BNip3L causes cyclophilin-D dependent loss of mitochondrial membrane potential by opening of mitochondrial permeability transition pore, decreased ATP synthesis, mitochondrial swelling, and release of cytochrome c, and ultimately leading to necrotic cell death. RIP-1 kinase is another protein implicated in necroptotic signaling. RIP-1 kinase is required under 'apoptosis-deficient conditions' in order to activate necroptosis (Hitomi et al., 2008). Apoptosis-deficient conditions may be when caspases are inhibited or when ATP is too low (Hitomi et al., 2008). Under apoptosis-deficient conditions, cells incubated with necrostatins, which are allosteric inhibitors of RIP-1, necroptosis can be fully inhibited (Degterev et al., 2005, Hitomi et al., 2008). Cole and Perez-Polo, 2002 showed that the activity of poly-ADP-ribose protein (PARP) is a key regulating factor of apoptosis versus necroptotic signaling (Cole and Perez-Polo, 2002). Activated by DNA strand breaks, PARP-1 uses up NADPH and ATP pools within the cell, potentially

leading to necroptotic cell death. Both apoptosis and necroptosis are triggered by PARP overactivity, but if cellular ATP levels are maintained after injury, cell death is shifted to apotosis rather than necrosis (Cole and Perez-Polo, 2004, Cole and Perez-Polo, 2002). Moreover, activation of PARP-1, ER Ca^{2+}-activated calpains, and Bax have been shown to be essential for AIF-mediated programmed necrosis (Moubarak et al., 2007). The Bcl-2 family member, Bax has been shown to play a role in necroptotic cell death after HI, in that Bax translocation to the nucleus and ER lead to a more necroptotic cell death (Gill et al., 2008). In a study using zvad.fmk, a universal caspase inhibitor, to produce apoptosis-deficient conditions, Hitomi et al 2008 identified hundreds of genes involved solely in necroptotic signaling (Hitomi et al., 2008). Necroptotic signaling has been found to occur after ischemic brain injury and excitotoxicity (Hitomi et al., 2008, Degterev et al., 2005). A recent publication describes in detail the role of necroptosis in cell death after HI, highlighting the importance of RIP-1, BNip3L, and Bax in necroptotic signaling (Chavez-Valdez et al., 2012).

BCL-2 FAMILY: REGULATORS OF CELL DEATH SIGNALING

Bcl-2 family

Cell death signaling is regulated by a superfamily of highly conserved proteins called the Bcl-2 Protein Family, shown in Figure 1. The first family member to be discovered was the family's namesake, B-cell lymphoma-2 (Bcl-2), an antiapoptotic protein. The 17-plus family members are divided into two groups: pro- and anti-

apoptotic, that interact with each other to balance cell death and survival signaling (Adams and Cory, 2007). The proapototic group is further subdivided into Bax-like proapoptotic proteins that contain at least BH1, 2, and 3 domains and another proapoptotic set of proteins that contain only the BH3 motif and are aptly named BH3-only proteins. All Bcl-2 family members contain at least one conserved Bcl-2 homology domain (BH1-4), from which the BH3 domain is used for interaction between subfamilies. Bcl-2 family members can homodimerize and heterodimerize between the two groups (pro-and anti- heterodimers), and interact with numerous Bcl-2 family proteins at one time. Functional redundancy within the family is quite high, allowing for strict regulation of cell death and survival (Fletcher and Huang, 2008). Overexpression of any of these proteins can tip the scale toward cell death signaling or immortalization and resistance to cell death.

Bcl-2 family members are highly involved in intrinsic and extrinsic apoptotic cell death signaling. The BH1, 2, and 3 domains of Bcl-2 fold into a globular interactive hydrophobic pocket, where hydrophobic regions of other Bcl-2 proteins can bind. In healthy cells, this hydrophobic pocket on Bcl-2 proteins is occupied by binding to Bax and inhibiting its cell-death signaling activity (Fesik, 2005, Hinds and Day, 2005). Stress-induced cell death requires Bax-like proapoptotic proteins and BH3-only proteins for signaling. Two main models of interaction between Bcl-2 family members are accepted: the direct and indirect activation of pro-cell death proteins (George et al., 2010). During direct activation BH3-only proteins directly interact with Bax/Bak to promote conformational changes and translocation.

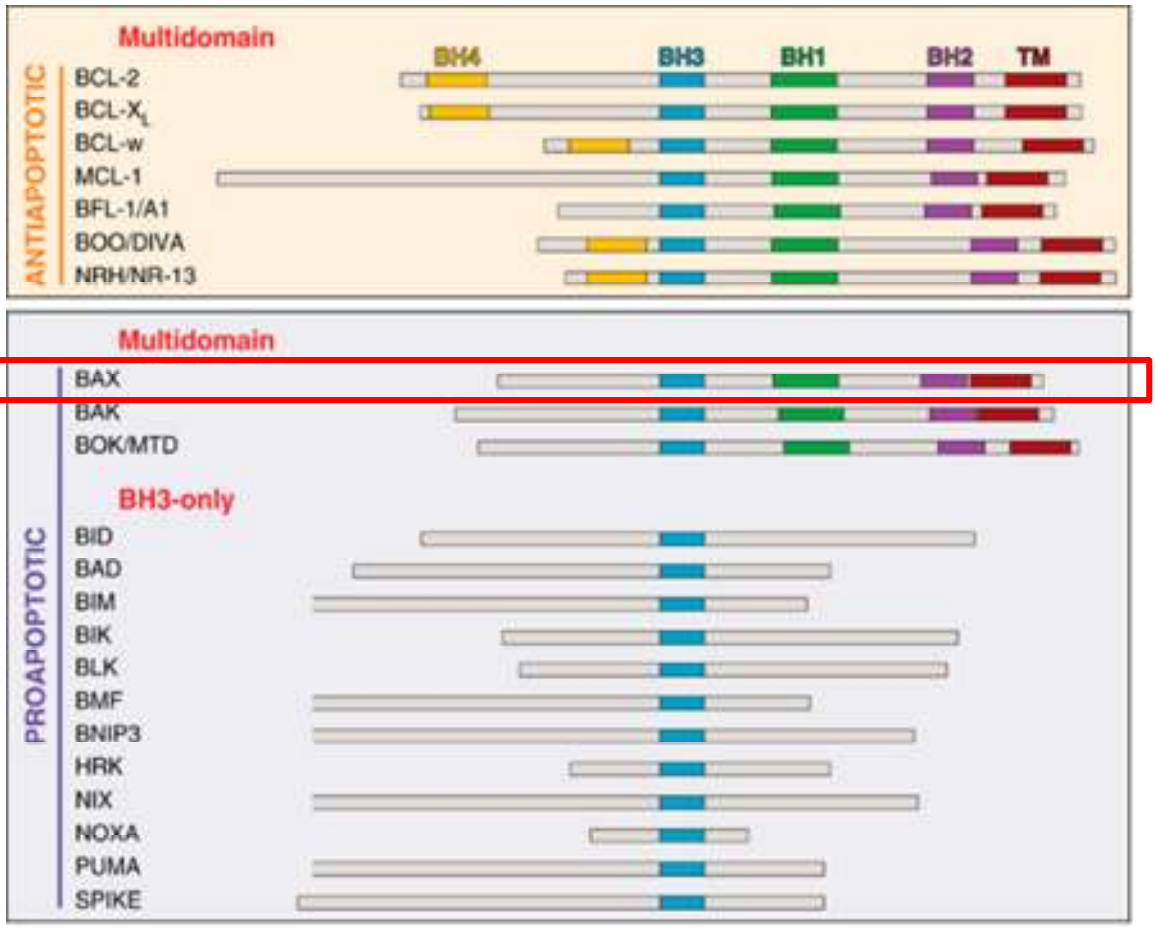


Figure 1. ***Bcl-2 Protein Family.*** Regulators of cell death, the Bcl-2 protein family, are subcategorized as antiapoptotic and proapoptotic . And, depending on the number of Bcl-2 Homology (BH) domains a family member has, it can be further characterized into BH3-Only or Multidomain Proteins. This project's focus is on Bax, a proapoptotic multidomain protein. *Modified with permission from Borner et al (2006).*

In the more commonly recognized indirect activation model, BH3-only proteins detect cell stress and interact with pro-survival Bcl-2 family proteins to inactivate and occupy them, so that Bax-like antiapoptotic proteins can carry on cell death signaling (Fletcher and Huang, 2008). Essentially, BH3-only proteins compete with Bax to bind with Bcl-2.

Bax

The first portion of this dissertation project focuses on the pro-cell death protein Bax and the idea that phosphorylation affects its localization and oligomerization status. In order to understand both the idea and the importance of this idea, it is imperative to form a good understanding of the protein's characteristics. The first pro-cell death protein discovered in the Bcl-2 family, Bax is a 192-amino acid, 21 kilodalton protein that, in its dormant state, is found in the cytoplasm of healthy cells (Oltvai et al., 1993).

Function in cell death signaling

Classically, the role for Bax in cell death has been focused on interaction with the mitochondrial membrane. In healthy viable cells, Bax is found in the cytosol as a free-floating monomer with its BH3 domain hidden in its hydrophobic cleft (Adams and Cory, 2007, Wolter et al., 1997, Oltvai et al., 1993, Youle and Strasser, 2008, Fletcher and Huang, 2008, Fletcher et al., 2008, George et al., 2010). Opposing literature denotes that in healthy cells, Bax bound to Bcl-2 is in the cytosol (Fletcher et al., 2008, Willis et al., 2005, Willis et al., 2007, Willis and Adams, 2005, Xin and Deng, 2005). Bax localization is concentrated near cellular organelles including mitochondria, nucleus, and endoplasmic reticulum. Upon activation of cell death signaling, the interaction between Bcl-2 and Bax is disrupted, presumably by interaction of BH3-only proteins to occupy Bcl-2. The vast majority of literature on Bax activity focuses on the mitochondrial pathway. Typically, once freed from Bcl-2 and activated, Bax translocates to mitochondria, the powerhouse and arsenal of the cell, homo-oligomerizes, and leads to

release of a number of apoptotic proteins such as cytochrome c and Smac/DIABLO (Hengartner, 2000, Adams and Cory, 2007, Newmeyer and Ferguson-Miller, 2003).

Though having been investigated for decades, the exact details of Bax activity are still not fully understood. For example, a highly argued issue is the timing of Bax oligomerization once activated, and whether it occurs before or after mitochondrial permeabiliziation. Another questionable idea is that of *how* Bax permeabilizes the mitochondrial membrane, whether it forms pores on the membrane or interacts with channels to allow the release of cytochrome c and other prominent cell death signaling proteins such as SMAC/Diablo (George et al., 2010, Antignani and Youle, 2006). The predominant view is that of pore formation via Bax oligomerization.

In addition to the mitochondrial role for Bax, mounting evidence suggests that Bax interacts with other organelles, such as the nucleus and ER, to activate different signaling pathways (Adams and Cory, 2007, Zhang, 2008, Godlewski et al., 2001, Hoetelmans et al., 2000, Karlsson et al., 2007, Dohm et al., 2006, Mandal et al., 1998, Raffo et al., 2000). In our HI model, we have found Bax translocates to mitochondria, nucleus, and ER. Whereas translocation to mitochondria has been found to result in apoptotic-like cell death, translocation to the nucleus and ER result in necrotic cell death morphologies (Gill, 2007). Figure 3 illustrates the translocation of Bax to mitochondria during low stress, mild HI, and the translocation of Bax to the nucleus during times of high oxidative stress. Additionally, caspase-independent Bax translocation has been

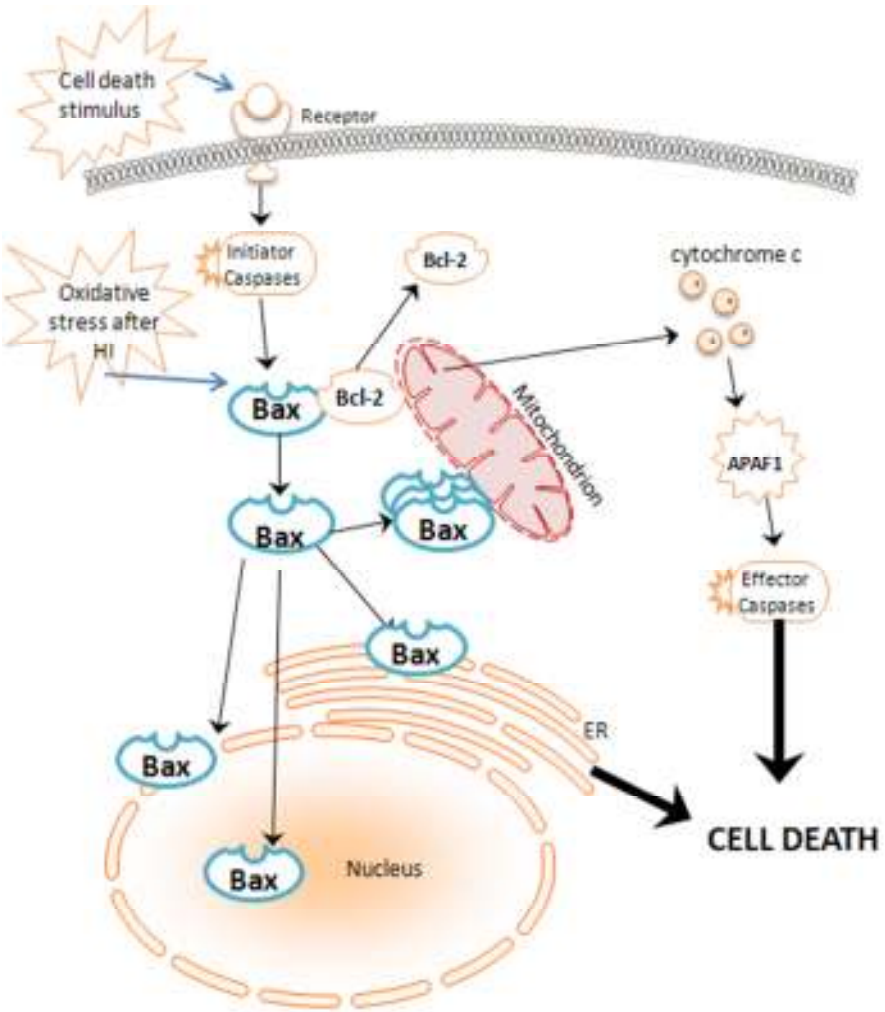


Figure 2. General Role of Bax in Cell Death Signaling. Bax can be activated through the intrinsic or extrinsic system, indicated here by "Oxidative Stress after HI" and "Cell Death Stimulus," respectively. During severe HI, both are activated, thus exacerbating the cell death signaling. Once activated, Bax has been found to translocate to mitochondria, nucleus, and ER, and ultimately lead to cell death and inflammation.

reported by numerous groups, implicating the chance of Bax activity in necroptotic signaling described in the previous section (Putcha et al., 1999, Pastorino et al., 2003). As stated previously, necrotic cell death morphology after HI causes inflammatory signaling, secondary apoptosis, and a larger injury overall. Therefore, investigation of Bax translocation patterns may provide a new target for therapeutic intervention after HI.

Though numerous injury paradigms have observed the translocation of Bax to the nucleus, there is little evidence to explain the cause of selective translocation to the nucleus versus mitochondria. Further explained below, we believe that phosphorylation patterns of Bax may influence the translocation of the protein. Investigation of oligomerization was also of interest to us, due to evidence suggesting that Bax oligomerizes at the nucleus, as seen in Chapter 3. Clearly, oligomerization of the protein speaks to its activity at these organelles.

Oligomerization

Oligomerization of Bax has previously been shown to occur in concurrence with activation. However, exactly which comes first, oligomerization or translocation, remains unknown. Once activated, Bax is known to form SDS-resistant oligomers of various sizes, from dimers to tetramers to 12-mers (Epand et al., 2002, Seye et al., 2003, Bleicken and Zeth, 2009, Er et al., 2007). Bax activity in cell death signaling relies on oligomerization to form pores in the mitochondrial membrane. Equally as important is the finding that the C-terminal region, which contains all three phosphorylation sites, is required for Bax oligomers stability (Er et al., 2007).

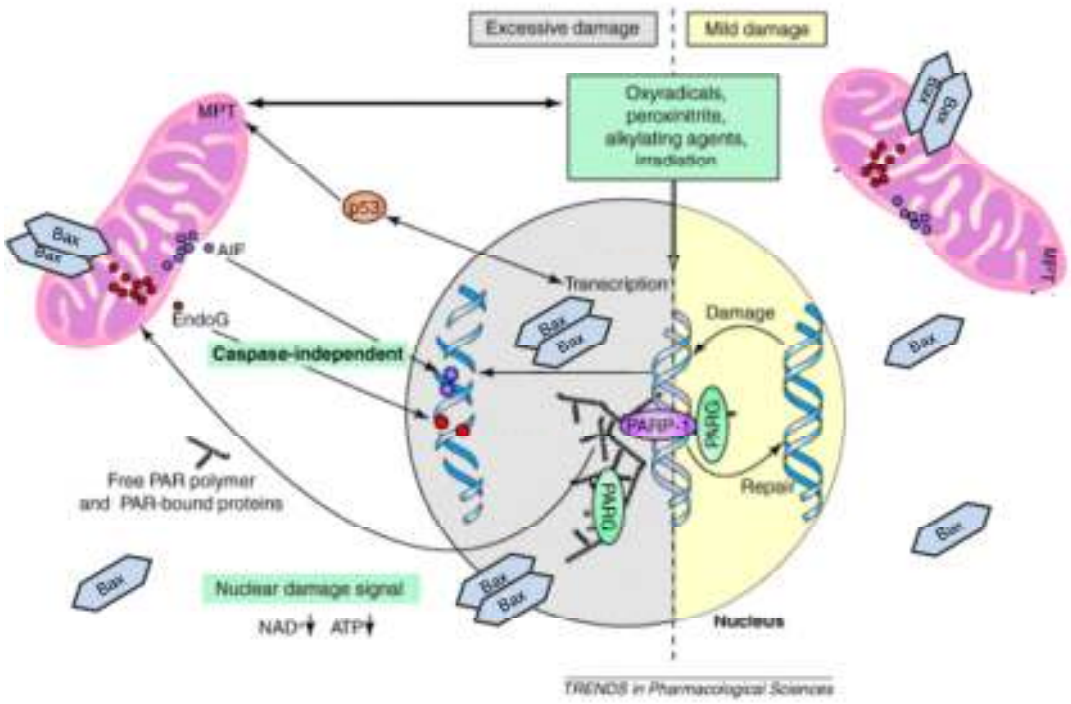


Figure 3 ***Nuclear cell death signaling cascade in caspase-independent cell death.*** When cells suffer only mild damage, Bax is found to translocate to mitochondria. However, as in cases of severe HI, excessive cellular damage causes Bax to translocate into the nucleus. *Modified with permission from Hong et al., 2004.*

Structure

The primary, secondary, and tertiary structures of Bax are visualized in Figure 4. A protein made of nine alpha-helices, α1-9, and numerous hydrophobic regions, the structural conformation changes of Bax play a key role in its activity. The hydrophobic regions of Bax are located at α2, between α4 and α5, and α9, as indicated in Figure 4A. As a Bcl-2 family protein, Bax contains BH1, 2, and 3 motifs, out of which the BH3 motif is used to interact with other Bcl-2 family members. Pertinent to this project, the

three functional phosphorylation sites on Bax are indicated by arrows (Kim, 2005, Kim et al., 2006, Linseman et al., 2004, George et al., 2010, Xin et al., 2007).

Figure 4B hints at the structural rigidity of this protein. On the left is an image composed of 20 superimposed structural diagrams taken via nuclear magnetic resonance. This figure clearly indicates that the most flexible portions of Bax lie toward the N-terminus, the C-terminal alpha-9 region, as well as the BH3 motif region (Figure 4B, circled in black), all proximal to the hydrophobic regions mentioned above. The BH1, 2, and 3 regions form a hydrophobic groove where the α9 helix resides (George et al., 2010, Muchmore et al., 1996, Petros et al., 2004). The α9 helix is proposed to be mitochondrial targeting sequence, but what specifically leads to conformational changes on Bax is unclear. However, evidence indicating that deletion of α9 does not stop Bax from translocating to mitochondria suggests the idea that there are other regions of Bax involved in mitochondrial translocation (George et al., 2010). Some have proposed that α1 might also be a mitochondrial targeting sequence (George et al., 2007, George et al., 2010). The phosphorylation sites flanking the α9 helix are indicated once again with arrows. Oligomerization of Bax occurs via interaction at the α9 helix region, and the homo-oligomerization domain consists of α2, α4, and α5 (George et al., 2010). When Bax is inactive, α1 blocks α9 so that Bax cannot homodimerize (Kim et al., 2009).

Regulation of Activity

Bax activity is regulated in a number of ways, including posttranslational modifications and interaction with proteins such as Bcl-2 and 14-3-3. Posttranslational modifications of Bax include phosphorylation, dephosphorylation, and cleavage.

Although Bax has been found to be cleaved after a number of injury paradigms, Bax cleavage has not been reported to play a major role after HI (Wood and Newcomb, 2000, Godlewski et al., 2001, Karlsson et al., 2007, Yu et al., 2008). Phosphorylation is the central concept being explored in this dissertation; therefore, we will focus on understanding the importance of this posttranslational modification on Bax function.

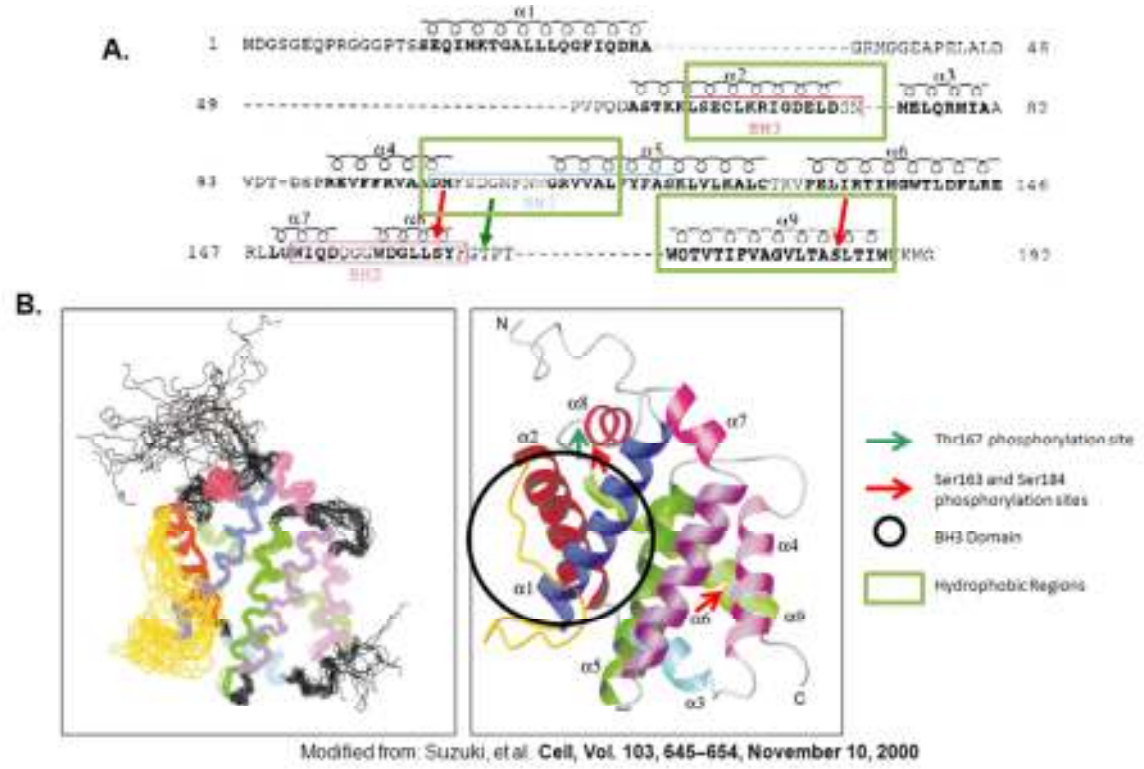


Figure 4. The linear and 3-dimensional structure of Bax. The primary and secondary structures of Bax are shown in A, with hydrophobic regions on the protein indicated with green boxes. B shows the tertiary structure of Bax, with 20 superimposed NMR-derived structures to visualize the structural rigidity of the protein. Phosphorylation sites are indicated in red and green, in flexible regions of the protein. *Modified with permission from Suzuki, et al., 2000*

Three phosphorylation sites on Bax have been shown to affect its function, including Ser163, Thr167, and Ser184. As shown in Figure 5, each site is phosphorylated by a different kinase and leads to different cell death signaling outcomes. Numerous publications have explored the effects of Bax phosphorylation on translocation to mitochondria, but not other organelles (Kim, 2005, Kim et al., 2006, Linseman et al., 2004, George et al., 2010, Xin et al., 2007). Our project focused on phosphorylation at Ser163 and Thr167, as they are known to activate Bax and induce organelle translocation. Phosphorylation at Ser184 by AKT may reduce Bax stability and reduce the half-life of Bax by 3-4 hours by subjecting it to ubiquitination and degradation (Xin and Deng, 2005, Xin et al., 2007, Xin and Deng, 2006, Yamaguchi and Wang, 2001). In various injury models, Bax^{Thr167} has been found to be phosphorylated by c-Jun N-terminal Kinase (JNK), a proline-directed ser/thr kinase activated during stress-induced cell death (Kim et al., 2006, Okuno et al., 2004). Phosphorylation at Thr167 leads to Bax activation and translocation to mitochondria. George et al., 2010 notes that the proximity of Thr167 to α8 makes it particularly interesting, considering that a proline mutation there induces translocation to mitochondria (George et al., 2010). Phosphorylation at Ser167 is induced by GSK3β, however the function of Ser163 phosphorylation has not been shown to be as crucial as Thr167 in cell death signaling (Wang et al., 2010a). Nevertheless, oxidative stress has been shown to cause an overall increase in Bax phosphorylation and translocation to mitochondria (Xin et al., 2007). In a study using the newborn piglet as a model system, Bax was shown to be phosphorylated after hypoxia-ischemia, however exact phosphorylation sites were never specified (Ashraf et al., 2001). Dephosphorylation

also plays a key role in Bax activity, with Protein Phosphatase 2A (PP2A) being the key phosphatase implicated to interact with Bax (Xin and Deng, 2006, Xin et al., 2007, Garibal et al., 2010). In numerous injury models, PP2A has been shown to dephosphorylate Bax at Ser184 and lead to increased Bax activity.

Opening of the α1 helix and exposure of the hydrophobic region requires stabilization, as does keeping Bax in its closed conformation (Arokium et al., 2007). The prime location of the phosphorylation sites near α9 suggests a key role for them in Bax conformation changes, and therefore activity and oligomerization status. For example, phosphorylation at Ser163 or Thr167 may play a role in activation by creating a negative at that region, forcing Bax to open up and expose the α9 helix for interaction with other Bax proteins. Conversely, phosphorylation at Ser184 may impede or obstruct the interaction of α9 with other proteins.

EPIGENETIC MODIFICATIONS AFTER HI

Bax translocation, whether to the nucleus or mitochondria, dictates the type of cell death observed after HI, which in turn affects the severity of injury after HI (Gill, 2007). In our rodent model, the affected brain regions have been shown to result in cognitive and behavioral deficits in 3-week-old rodents (Ferrari et al., 2010). Therefore, we asked the question of how long-lasting these deficits were. As of now, the function of Bax within the nucleus is entirely unknown. It may be possible that Bax is involved, directly or indirectly, in epigenetic regulation of gene expression after HI.

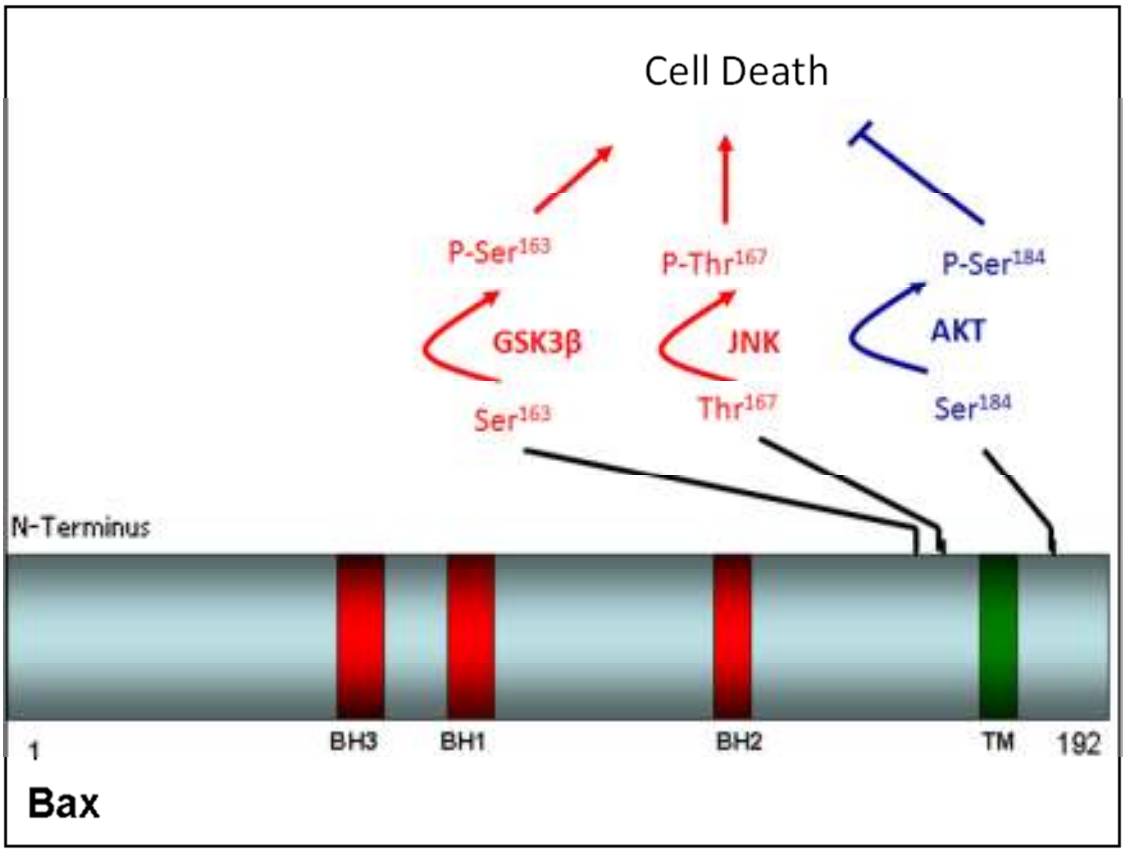


Figure 5. Phosphorylation sites on Bax. Bax contains three known functional phosphorylation sites, which are each phosphorylated by a different kinase. Phosphorylation at Ser163 and Thr167 leads to cell death, whereas phosphorylation at Ser184 renders Bax inactive.

We predicted that the progeny of HI-affected animals may be impaired by the potential epigenetic modifications experienced by their parents, and that these epigenetic modifications are not fully reset during the progenies' development. Undoubtedly, a key component of this hypothesis is the concept of epigenetic inheritance.

Literally, epigenetics means "above/over/in addition to genetics." Scientifically, epigenetics is defined as environmentally-induced modifications in chromosomal

structure that do not directly alter DNA sequence. Figure 6 visualizes the locations that epigenetic modifications could occur. Such modifications include DNA methylation at CpG islands, histone modifications (including methylation, acetylation, phosphorylation, sumoylation, biotinilation, and ubiquitination), nucleosome remodeling, and chromatin interaction with non-coding RNA (Mehler, 2010). Functionally, these modifications can manifest in one or more of the following characteristics: 1. Change cell phenotype; 2. Regulate cell lineage progress; 3. Yield a stably inherited phenotype. Recent evidence suggests that the first and second characteristics may be affected by HI (Failor et al., 2010, Buller et al., 2012, Back et al., 2002, Kuan et al., 2004). However, the exact genetic or epigenetic modifications that lead to change in cell phenotype or regulation of cell lineage remain unknown. Also elusive is the evidence of stably inherited behavioral phenotypes caused by these epigenetic modifications.

Conventional thinking about the reproductive system proposes that germ cells undergo a complete reprogramming of epigenetic modifications so that the next generation starts off with a "clean slate," so to speak. The concept of epigenetic inheritance goes against this idea, suggesting that the epigenome does not undergo *complete* reprogramming in order to produce totipotent cells required during reproduction. Indeed, major epigenetic reprogramming does occur, but some cell-type-specific epigenetic modifications remain unerased and are transferred from generation to generation (Ng and Gurdon, 2008). Plants, such as the toadflax and wild radish have long been known to exhibit this characteristic in order to protect themselves from insects and various environmental factors (Cubas et al., 1999, AA, 2001). Fungi and certain insects

also demonstrate this phenomenon. In mammals, namely laboratory rats, exposure to fungicides have been shown to have an effect on multiple generations, and the hypermethylation status of sperm cells in these animals provide hard evidence toward epigenetic inheritance (Anway et al., 2005, Anway et al., 2006). Epigenetic programming can be affected merely by the social atmosphere during early developmental stages (Szyf and Meaney, 2008). Considering the accumulating evidence suggesting that environmental factors affect generations to come without specifically altering DNA, the concept of epigenetic inheritance is undeniable.

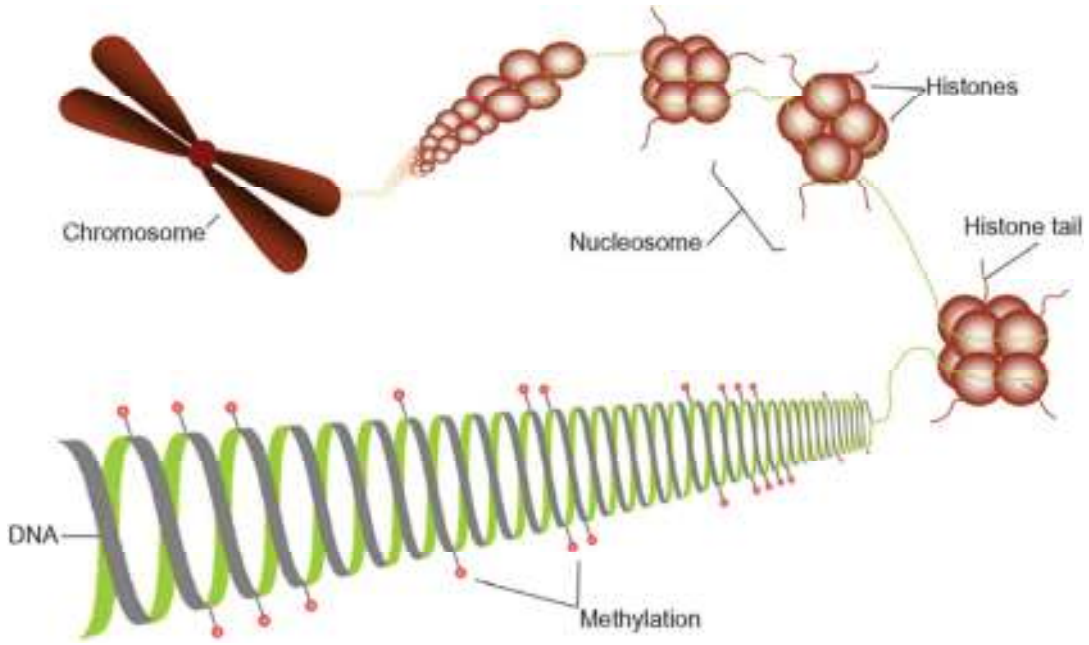


Figure 6. The numerous potential locations epigenetic modifications. Epigenetic modifications can include DNA methylation, histone acetylaiton or methylation, or chromatin interaction with RNAs. *Shown here with permission from Groom A et al., 2011.*

It is widely accepted that environmental factors can affect neuronal development and have a marked effect on behavior (Ma et al., 2010, Juliandi et al., 2010). Epigenetic imprinting via methylation is the key factor to the transfer of epigenetic modifications

from generation to generation. For instance, environmental stress during early postnatal days has been shown to cause epigenetic imprinting in rats, leading to numerous generations of rats exhibiting increased fear response (Zhang et al., 2006, Youngson and Whitelaw, 2008). Neuronal cells are especially susceptible to epigenetic imprinting, as the brain and placenta are the two places where the expression of imprinted genes is the highest (Davies et al., 2007). Therefore, it is reasonable to suggest that epigenetic modifications during early development can lead to inherited behavioral phenotypes.

During early embryonic development, around embryonic day 6 in rodents, the primordial germ line begins to form (Wagner, 2010). In our animal injury model, rat pups are exposed postnatally to the effects of HI. By this time, the next germ line is present, but is not directly exposed to HI. So how could nervous system-targeted postnatal exposure to HI affect the neurological outcome of the next generation? The answer lies in cross-talk between the various components of the endocrine system. Organ system cross-talk is especially critical during early development. Mounting evidence suggests that throughout development, cross-talk exists between the nervous, endocrine, digestive, immune, and reproductive systems (Marchetti et al., 1990). Multiple decades of research indicates that the nervous system and endocrine system are seamlessly integrated and interact, especially in response to environmental stress (Dronca and Markovic, 2011). Undeniably, the reproductive system is certainly influenced by the endocrine system. Prenatal, postnatal, and even adult exposure to environmental toxins have been shown to affect endocrine-reproductive system cross-talk loops and ultimately affect the germ line (Davis et al 2001). Neuroendocrine cross-talk with other endocrine axes is extremely

important to regulate the body's activity and maintain homeostasis. Therefore it is obvious that influences on the nervous system can end up affecting the reproductive system. The direct effects of our HI model on the pituitary gland have not yet been characterized. However, since our injury model directly affects the thalamus and hypothalamus, one can suggest that, beyond cross-talk, at least the inflammatory backlash from HI injury could affect the pituitary gland located just ventral to the hypothalamus and ultimately affect the reproductive system.

The majority of work focusing on the relationship between HI and epigenetics concentrates on how epigenetic regulations affect brain injury after HI. Very few studies have investigated the reverse concept of HI creating epigenetic modifications. Still, evidence exists that hypoxic and ischemic paradigms can cause epigenetic modifications. Oxidative stress from fetal hypoxia has been shown to affect the methylation status and expression of PKCε in cardiac myocytes in a gender-dependent manner (Patterson et al., 2010). Ischemic insults have been shown to affect epigenetic programming of histones in hippocampal neurons (Formisano et al., 2007). Preterm hypoxic insult has also been suggested to play a role in genetic and epigenetic consequences for corticogenesis (Curristin et al., 2002). Two studies even link the epigenetic histone acetylation and downregulation of the amyloid-degrading enzyme neprilysin after hypoxia with susceptibility to Alzheimer's disease in adulthood (Wang et al., 2011, Fisk et al., 2007). Additionally, plenty of indirect evidence points toward epigenetic modifications after neonatal HI. For example, it is well known that over 300 genes are upregulated or downregulated after HI, but the exact mechanisms are not fully understood (Hedtjarn et

al., 2004). Furthermore, HI-influenced epigenetic inheritance and functional phenotypes have not yet been investigated. Based on the evidence presented in this section, we wanted to assess the behavioral phenotype of the progeny of HI-affected animals.

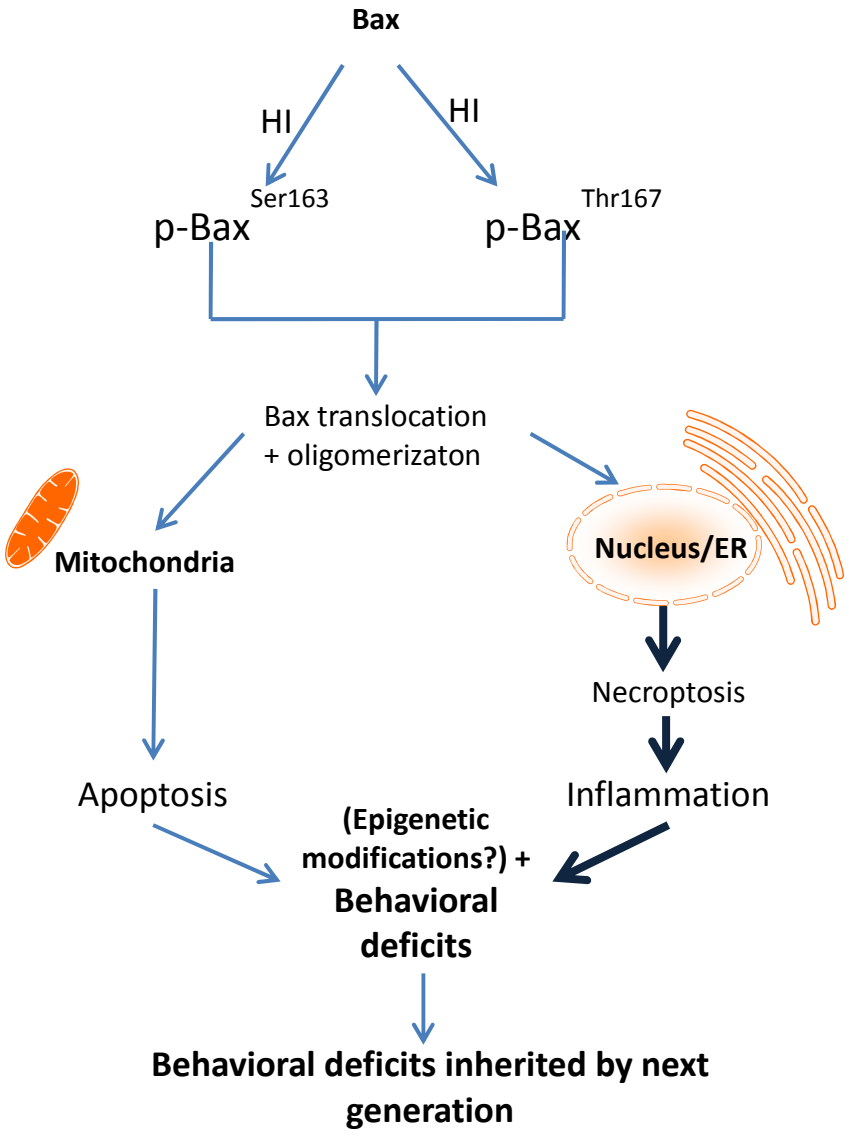


Figure 7. Central Hypothesis. Bax is phosphorylated after HI, which affects translocation to the nucleus or mitochondria. Translocation to mitochondria is associated with apoptotic cell death phenotype, whereas translocation to the nucleus/ER is associated with a necrotic cell death phenotype, inflammation, and secondary apoptosis and ultimately leads to behavioral deficits. These deficits are consequently also expressed in the next generation.

HYPOTHESIS

The overarching goal of this project was to characterize brain injury after neonatal HI. We approached this goal with two different questions in mind: what happens on a biochemical level before cell death occurs, and for how long are the consequences of HI sustained? To this end, our hypotheses were that phosphorylation of the proapoptotic protein Bax is associated with translocation to subcellular organelles, and that the injury sustained after HI carries on to the next generation (Figure 7).

Chapter 3 focuses on a specific protein involved in cell death signaling after HI. Specifically, we wanted to address whether Bax phosphorylation after HI is associated with mitochondrial or nuclear translocation. We also examined how specific kinases affect the oligomerization of Bax. Although Bax translocation to the nucleus has been associated with necrotic-like cell death phenotype, the factors involved in differential organelle translocation are not understood. Our results are the first to show that specific phosphorylation after HI is associated with the subcellular localization of the protein and that Bax interaction with kinases induces oligomerization of the protein. Our results here indicate that Bax may be a candidate for development of a therapeutic target for HI.

Chapter 4 examines whether the behavioral outcomes of injury from HI transfer to the next generation. We report here that the behavioral deficits are indeed transferred to a second generation, and that the susceptibility is gender-biased in second-generation animals. Results from our study provide strong preliminary grounds for future global DNA methylation and epigenetic analysis of HI injury.

With the continuing rise in preterm, low birth weight, and very low birth weight infants, HI remains an extremely prevalent injury with few therapeutic interventions to alleviate its effects on the developing brain. For this project, we aimed to elucidate the novel idea that the effects of HI carry on to the next generation and to identify a potential therapeutic intervention target that could effectively reduce necrosis and inflammation after severe HI.

We are the first to show that Bax phosphorylation is associated with subcellular localization after HI and that the detrimental effects of HI carry over to the next generation.

Chapter Two: Materials and Methods

MATERIALS

All chemicals were purchased from Fisher Scientific and/or Sigma Aldrich. The Pierce BCA assay kit for protein determination was purchased from Thermo Fisher Scientific. Western blot molecular weight markers, western blot membranes, chemiluminescence substrate, and western blot apparati were purchased from BioRad. Bax antibodies were purchased from Cell Signaling and Santa Cruz Biotech. Secondary antibodies were purchased from Southern Biotech. Mitotracker Red CMX-ROS was purchased from Life Sciences. Phospho-Bax antibodies raised against p-Bax^{Thr167} and p-Bax^{Ser163} were custom-ordered from GenScript (Piscataway, NJ). Surgical instruments were purchased from Roboz Surgical Instrument Co. (Gaithersburg, MD), 6-0 surgical silk sutures were purchased form Ethicon (Somerville, NJ). Needles, gauze, filter paper, and absorbent bench pads were acquired from UTMB Materials Management. For atomic force microscopy experiments, detailed in section G, purified Bax protein was acquired from Protein X Labs (San Diego, CA), purified active kinases JNK and GSK3β were acquired from Life Technologies (Grand Island, NY), and pure Amresco Adenosine-5' '-Triphosphate (ATP) Disodium Salt was purchased from Fisher Scientific and stored as directed.

ANIMALS: RICE-VANNUCCI HI MODEL AND BREEDING PROTOCOL

Animal care

All animal procedures were performed according to the UTMB Animal Care and Use Committee (IACUC)-approved protocol # 9102020.We used a modified version of the Rice-Vannucci model of perinatal ischemia (Rice et al., 1981, Vannucci et al., 1988). Pregnant Wistar dams were purchased from Charles River at gestation days between E17-20. Dams gave birth when ready, generally on E21 or E22. On postnatal day 2 (P2), the litter was culled to 10 pups to ensure that all pups received an equal chance to nurse. On surgery day, P7 pups were separated from dams and placed on a heating pad set at 37°C, to mimic the mother's body temperature. Each pup was weighed, sexed, numbered, and categorized into the following groups: Naïve, Sham, or HI. Naïve animals remained with dams and did not undergo surgery or anesthesia, but were weighed, sexed, and numbered on P7 and before tissue harvest. Post surgery analgesics included daily administration of Buprenorphine (Bupernex) and 2 drops of topical anesthetic on suturing to minimize any localized pain for a maximum of three days post-surgery.

Surgical Ischemia Procedure

Littermates were separated into two condition groups: HI and Sham. Pups were placed in 2.5% isofluorane anesthesia chamber for a minimum of 5 minutes. For surgical procedure, pups were moved onto a 37°C heating pad throughout the surgery and anesthetized using 1.5-2.5% isofluorane by nose cone in an air/O_2 mix. For HI pups, aseptic technique was used to make an anterior off-midline incision in the neck. The left

carotid artery was then isolated from surrounding fat and tissue, dually cauterized, and transected between the cauterizations for a permanent ligation. Topical administration of marcaine was provided directly onto the incision, to address any pain or discomfort. The incision was then closed with 6-0 silk suture and cleaned. Topical bitter-flavored skin sealant was applied to the suture to ensure closure of the incision and to deter the dam from removing sutures.

Sham animals were also anesthetized using isofluorane as stated above and placed on a heating pad for surgery. An anterior off-midline incision was made, immediately closed with a 6-0 silk suture, and cleaned. Though an incision was made on sham animals, in order to prevent any minor injuries that could result in ischemia/reperfusion of the left hemisphere, the carotid artery was not isolated.

Anesthesia timings for each pup were monitored, recorded, and never exceeded 45 minutes. After surgery, pups were placed back onto heating pads and monitored for any signs of bleeding until they recovered from anesthesia. If a pup does not nurse or shows no signs of mobility post surgery, the animal was euthanized. Awake pups were then returned to dams, where they were mobile and nursing within 15min, which was our indication that they recovered from the surgical procedure.

Hypoxia Procedure

After 90 minutes of post-surgery recovery, all pups were separated from their mother. HI pups were placed in a hypoxia (8% O_2) chamber for 90 minutes, and Shams were placed on a heating pad in normal air levels of oxygen (20% O_2) for 90 minutes. In the hypoxia chamber, animals were monitored for signs of excessive discomfort or

immobility. Temperature was also monitored and rigorously maintained at 37°C to insure reproducibility. After 90 minutes, all pups were returned to their mothers where they remained until they were sacrificed at specific time points up to 72h after injury. Litters used for breeding of second generation pups for behavior analyses were returned to the animal resource center facility.

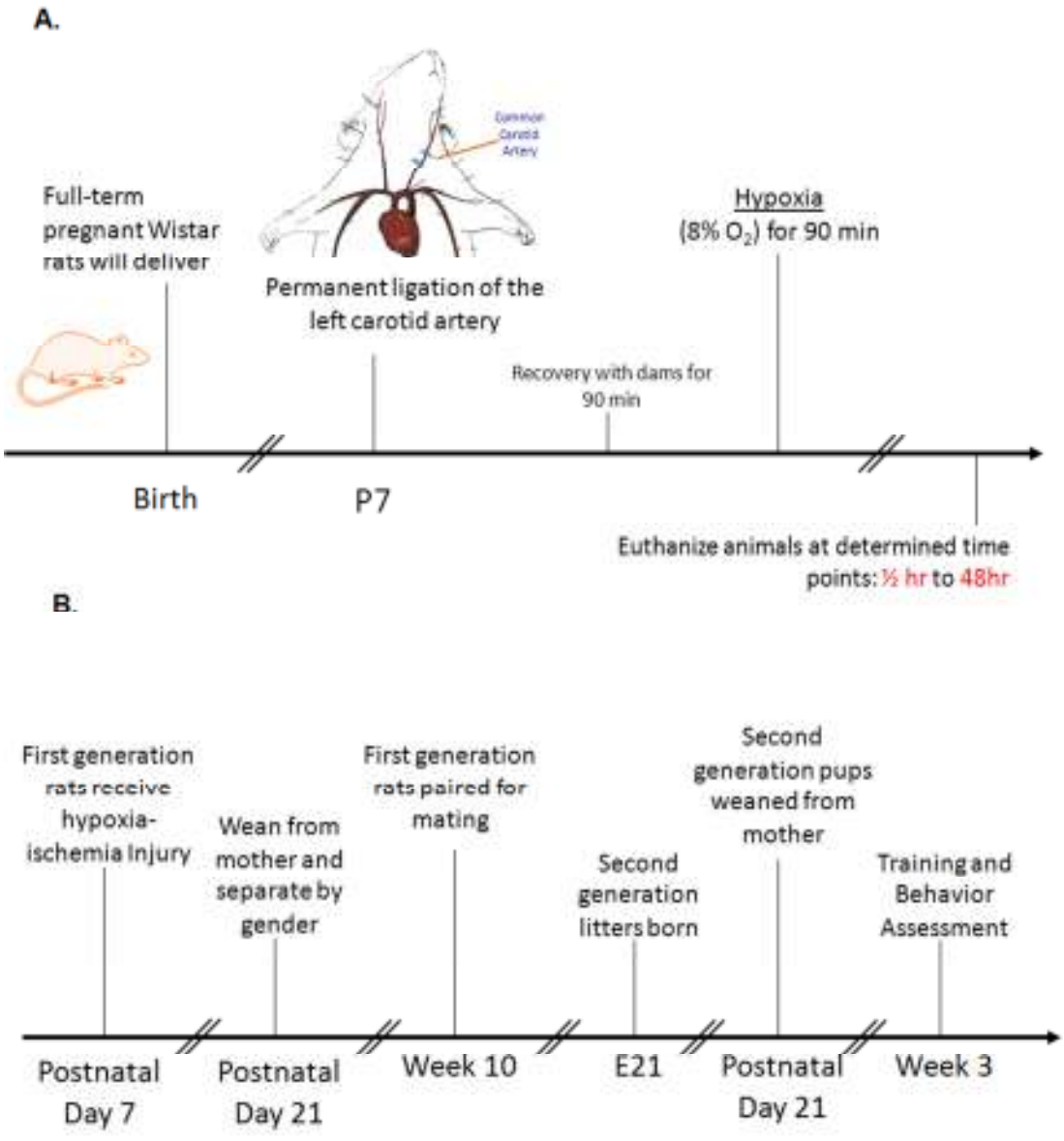


Figure 8 ***Hypoxia-ischemia injury and breeding timeline.***

Tissue Harvest

At pre-assigned time points for sacrifice, pups were euthanized by pentobarbital overdose. For western blots and molecular analysis of tissues, the whole brain was removed and the hemispheres were separated. The parietal cortex (from the anterior tuber cinereum to anterior occipital cortex), hippocampus, and thalamus were isolated in each hemisphere and placed into individual pre-labeled conical tubes. The tubes were immediately flash frozen in liquid nitrogen and stored at -80°C until further analysis.

For immunohistochemistry, rat brains required fixation with 4% paraformaldehyde (PFA). Immediately after euthanization, 3-week-old pups were set up for gravity perfusion. Pups were perfused with a minimum of 150mL 0.9% saline solution at room temperature, to flush out blood. Next, they were perfused with ice-cold 4% PFA for a minimum of 45 minutes. Whole brains were isolated, placed in a 50mL conical filled with ice-cold PFA for 48 hours to ensure proper fixation of tissue. 48 hours after harvest, brains were incubated in 30% sucrose for a minimum of 72 hours. Afterward, brains were blocked anterior and posterior to the thalamus to acquire sections including the parietal cortex, hippocampus, and thalamus.

Animal Breeding

For our behavior analyses in Chapter 5, we required second generation pups. Three pregnant dams were ordered from Charles River, and at P7, each first generation litter was assigned a condition (HI, Sham, or Naïve), and handled as stated above under surgical procedure and hypoxia. After surgery, pups were returned to their mothers and

left under the care of UTMB's Animal Resource Center. On P21, first generation pups were weaned from their mother and separated by gender. On Week 6, pups required further separation as they grew, with a maximum of three females or two males per cage. Wistar rats are mature and ready to mate at 10 weeks of age. On Week 10, rats were paired into harems containing one male and two females. Mates were paired in order to assess all possible crosses between Naïve, HI, and Sham first generation animals. Harems were maintained for up to two weeks, or once a female appeared to be pregnant. Pregnant first-generation females were immediately separated into their own cages to prepare for birth. Generally, second generation pups were born within a month after harems were set up. As before, on P2, litters were culled to ten pups. Second generation pups were allowed to nurse with the mother until P21, when pups were weaned and randomly separated into groups of five per cage. At this stage, pups were not separated by gender. Starting on P21, each pup was handled daily to reduce anxiety from fear and acclimate them to being held. Animals were trained for three days beginning on Day 25, with the final behavior testing day being Day 28.

BEHAVIOR ANALYSIS

Three different behavior assays were conducted to assess sensory-motor coordination impairment in second generation pups. The setup for the Mesh Ascending Test and Bar Holding Test was identical to that of Ferrari et al. 2009. All behavior analyses were carried out between 8am and 4pm, using the same room and setup for each animal. All animals were in room at least 30min before testing, to ensure acclimation to

novel smells and sights. In between each behavior assay, the pups were placed in their home cage to rest.

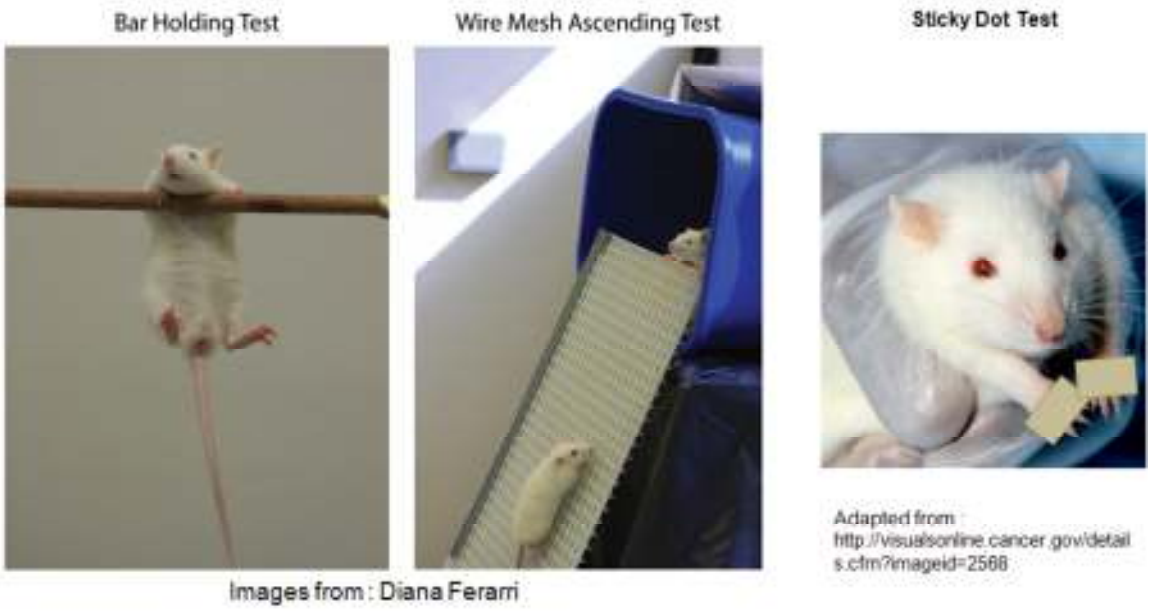


Figure 9. ***Representative images of the behavior assay setups.*** The bar holding test places 3-week old rat pups on a raised bar and assesses their ability to hold on for a maximum of 120 seconds. The mesh ascending test involves a mesh placed at the edge of a table at a 70° angle, ending at the rim of a box containing littermates of the pup to be assessed. The test consists of placing the pup at the bottom of the mesh and determining ascension time. The sticky dot test involves placing stickers on the forepaws of the pups, placing them back in their cage and measuring the time it takes for them to remove both stickers.

Mesh ascending test

To assess sensory-motor coordination, we used the Mesh Ascending Test. A10mm plastic mesh, 45cm high and 15cm wide, was secured at the edge of a table and tilted at an angle of 70° to come in contact with a sturdy plastic box at the top. A cardboard box filled with padding was placed below the table to protect from injury should a pup fall from the mesh. As a stimulus to ascend, four littermates and food pellets were placed in the plastic box at the top. Animals were placed and acclimated in the top box for 5 minutes before training and testing. Each pup was individually were placed at

the bottom of the mesh and the time necessary to ascend was measured for up to 120 seconds. Before testing day, each pup was trained 5 times per day for three consecutive days. On testing day, we performed five separate trials per animal with a resting time of 5 minutes between trials.

Bar holding test

The Bar Holding Test was utilized as another assay to measure sensory-motor coordination. The setup consists of a wooden bar 1cm in diameter and 30cm long suspended 50cm high above a padded soft surface. Each pup was placed atop the wooden bar and time spent on the bar, as well as grasping with forelimbs, was measured up to 120 seconds. If the pup was to balance itself on the corners of the bar, it was immediately moved back to the center. Before testing day, each pup was trained 5 times per day for three consecutive days. On testing day, we performed five separate trials per animal with a resting time of 5 minutes between trials.

Sticky Dot test

To determine the presence of a somatosensory asymmetry, we used the sticky dot test. For this assay, a 1.5cm long piece of one-sided tape was placed on each forepaw of the pup, and the pup was then placed in a clean cage for observation. Three different measurements were recorded for this assay, including paw preference, contact latency, and total time. The first paw contacted by the pup's teeth is considered the 'preferred

paw' and was recorded. Contact latency indicates the time until the pup attempts to remove a sticker. Time elapsed until both stickers were removed was also recorded as "total time." Five separate trials with a resting time of 5 minutes between trials were conducted for each pup, but pups received no training prior to testing day for this assay.

CELL CULTURE AND MAINTENANCE

SH-SY5Y human neuroblastoma cells were grown and maintained in F12 media + 15% donor horse serum. For stock cells, media was changed every other day. When fully confluent, stock cells were dissociated by incubating with Puck's solution (NaCl, KCl, Na_2HPO_4, HEPES, Dextrose, EDTA) for 3-5 minutes and transferred into a 15ml conical. The cells were then centrifuged down at 800 x g and Puck's solution was replaced with fresh media. Cells were then split into two new flasks and grown. To plate cells for experiments, cells were dissociated with Puck's solution for five minutes, spun down, and counted using a haemocytometer. For GFP transfections experiments, cells were plated at a density of $4x10^4$ cells/cm^2.

For cell death experiments, SY5Y were plated at $4x10^4$ cells/cm^2 on 2-well chamber slides and differentiated for 5 days using 10μM retinoic acid. For western blot analysis cells were plated at 1.2 x 10^5 cells/cm^2 in 6-well plates and differentiated for 5 days using 10μM retinoic acid. Rotenone injury was induced by incubating cells with 25 or 100μM Rotenone in fresh media for 0-72 hours.

WESTERN BLOT ANALYSIS

Western blot analysis was used to measure p-Bax protein levels after HI in each organelle. Both SY5Y and cortical tissue were processed for this analysis.

Cell sample processing

An entire 6-well plate was used per sample of SY5Y for subcellular fractionation. Cells were first washed 3x with ice-cold PBS and scraped into eppendorf tubes using an individually-wrapped cell-scraper for each assay condition sample. Cells were spun down for 5 minutes at 800 x g, and PBS was replaced with fractionation buffer. Fractionation was performed in the manner described below, except that the volumes of buffers used were reduced by 50%.

Subcellular Fractionation

In order to separate nuclear, cytosolic, and mitochondrial fractions, a subcellular fractionation protocol was followed. Ipsilateral cortices were weighed and homogenized in 150µl of Fractionation Buffer (FB, 10mM HEPES, 0.5mM EGTA, 2mM EDTA, 10mM DTT, 250mM sucrose, 1 Roche protease inhibitor cocktail pill per 10ml, and 10µL/mL of phosphatase inhibitor). The sample was then centrifuged for 20 minutes at 1000 x g. 50% of the supernatant was then removed and added to tube labeled S1. The original sample was centrifuged again for 20 minutes at 1000 x g. This time, 80% of the supernatant was transferred to S1, and the remaining supernatant was discarded. The pellet, which contained the nuclear fraction, was resuspended in Pellet Buffer (PB, 20mM

HEPES, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF, 1 Roche protease inhibitor cocktail pill per 10ml, and 10µL/mL of phosphatase inhibitor) and incubated on a shaker in the cold room for 30 minutes. This sample was then centrifuged at 14000 x g for 10 minutes, and the supernatant was collected as the nuclear fraction. Meanwhile, S1 was centrifuged at 3300 x g for 10 minutes to discard any remaining nuclear contaminant in pellet form. The supernatant was then centrifuged at 10,000 x g for 20 minutes. Again, 50% of the supernatant was removed and added to a new tube labeled "Cytosolic fraction." The supernatant was centrifuged again for 20 minutes at 10,000 x g to pellet mitochondria. This time, 80% of the supernatant was transferred to the Cytosolic Fraction tube and the remaining supernatant was discarded. The mitochondrial pellet was resuspended in 30µl PB and pulse-sonicated.

BCA Protein determination assay, SDS-PAGE, and blot

The Pierce bicinchonic acid (BCA) protein assay was used to determine the protein concentration of fractionated samples in a 96-well plate according to manufacturer's instructions. 25ug of sample was then aliquoted, mixed with 6x SDS sample buffer (350mM Tris–HCl, pH 6.8, 12.5% 8M urea, 1% 2-mercaptoethanol, 9.3% DTT, 13% SDS, 0.06% Bromophenol Blue, 30% glycerol), and boiled for 10 minutes. Boiled samples were then loaded on a 10% SDS-polyacrylamide gel. The proteins were separated on large gels at 100V for 20 minutes and 120V for 5 hours; mini-gels were run at 90V for 10 minutes, and 100V for 2 hours. The separated proteins in the gel were then transferred overnight onto PVDF membrane (Millipore, Billerca, MA) at 25V in cold room. The next day, to confirm transfer of proteins, membranes were incubated with

Ponceau S for 5 minutes. Membranes were then washed and incubated in blocking buffer (4% BSA in PBS with 0.2%Tween-20) for one hour. Blocking buffer was then discarded and primary antibody diluted in 1% BSA + PBST was added to the membranes. Upon incubation at room temperature for 1 hour, membranes were washed 6 x 10 minutes with PBST. Secondary antibody diluted in 0.5% BSA in PBST was added to the membranes and incubated for 1 hour. Membranes were washed again 6 x 10 minutes with PBST. Enhanced chemiluminescence lighting substrate (ECL) from GE was added to the membranes to visualize the proteins.

MITOTRACKER RED CMX-ROS ASSAY

Mitotracker Red CMXRos was stored and used according to manufacturer's instructions. SY5Y cells were plated in 2-well chamber slides and differentiated as explained in Section D. After rotenone injury, cells were harvested at determined timepoints by washing 3x with 500µl ice-cold PBS. Cells were then fixed by incubating for 10 minutes in 100% methanol at -20°C. Next, the standard protocol for Mitotracker Red CMXRos provided by Life Technologies was followed. Finally, slides were imaged on an epifluorescence microscope.

ATOMIC FORCE MICROSCOPY (AFM)

The purified Bax protein is truncated in the N-terminus, and spans only the 33-171 amino acid region. Its pI is 5.06, with p-Bax pI being approximately 4.93. p-JNK from Life Sciences has a pI of 6.27, thus it can be imaged on a negatively charged mica

substrate using a buffer of pH 7.4. 50µl of stock Purified Bax protein was incubated for 1 hour with 4.6µl stock JNK and 1µl stock ATP in 44.4µl JNK Assay Buffer (500µl of 0.5M Tris, 50µl of 1M $MgCl_2$, 100µl of 0.05M EGTA, 100µl of 100mM DTT).

Similarly, for Bax and GSK3β were incubated for 1 hour. GSK3β was first diluted 1:100 in GSK Dilution Buffer (500µl stock Tris, 250µl stock DTT, 500µl stock BSA), and stock 100µM ATP was diluted 1:10 in GSK Assay Buffer (500µl of 0.5M Tris, 50µl of 1M $MgCl_2$, 100µl of 100mM DTT, 500µl stock Na_3VO_4). Next, 50µl of stock Bax, 5µl of 1:10 diluted ATP, and 17µl of diluted GSK3β were incubated in 28µl of GSK Assay Buffer. As GSK3β has a pI of 8.98, it is positively charged at pH 7.4, and was therefore observed to aggregate onto the negatively charged mica substrate. Therefore, after 1 hour incubation, Bax+GSK3β samples were dialyzed into a sodium tetraborate. HCl buffer with a pH of 9.2, allowing GSK3β to be neutral in charge. As there were no phosphatases in the mixture, stability of p-Bax was not an issue.

Tapping mode imaging was performed by Dr. Andres Oberhauser on his custom-made atomic force microscope. Protocol for AFM sample preparation: absorb 3 µl undiluted sample onto mica substrate for 30 minutes, dry off sample with nitrogen, and image.

STATISTICAL ANALYSES

GraphPad Prism 4 software (GraphPad Software, San Diego, CA) was used for construction of graphs and all statistical analyses. Analysis of significance between two

groups was performed by using the Students t-test. One-way analyses of variance (ANOVA) and a Tukey-Kramer post-hoc test were used to assess significance when comparing multiple groups. For all tests, a p-value less than 0.05 was considered significant.

Chapter 3: Bax Phosphorylation is Associated With Subcellular Localization and Oligomerization after Neonatal Hypoxia-Ischemia

INTRODUCTION

Neonatal HI affects 2-4 out of 1000 births every year. Currently, very limited therapies exist for HI. The extent of the benefits of current therapies is poorly understood and often controversial. Severe cases of HI cause necrosis and inflammation, which ultimately lead to secondary apoptosis of surrounding cells. Mortality rates after severe HI can be as high as 50%, with survivors exhibiting severe behavioral and cognitive deficits. Mild HI leads to apoptosis of a small amount of affected cells, and no inflammation, therefore the injury site is controlled. However, even mild cases of HI create behavioral deficits that go unnoticed until children reach school-age. Therefore, proper care and therapy for HI-affected neonates is imperative. Having a better understanding of the cell death signaling patterns leading to apoptotic versus necroptotic morphologies after HI can help identify a more specific therapeutic target for HI.

Differences in cell death signaling cascades have been implicated in the necrotic and apoptotic phenotypes observed after HI. The key player in both injuries is the Bcl-2 family pro-cell death protein, Bax. Bax has been shown to be involved in both apoptotic and necroptotic cell death signaling, depending on its intracellular translocation patterns (Gill et al., 2008, Gill, 2007). When Bax translocated to mitochondria, cell death after HI displayed apoptotic morphologies. However, when Bax translocated to the ER and

nucleus, necrotic cell death phenotype was observed (Gill, 2007). Until now, the factors influencing the translocation differences were not understood.

Bax has three phosphorylation sites flanking its C-terminal hydrophobic region that have an effect on its functionality. Phosphorylation at Ser163 and Thr167 activates Bax, whereas phosphorylation of Ser184 renders Bax inactive (Xin and Deng, 2005, Linseman et al., 2004, Okuno et al., 2004, Wang et al., 2010a, Yao et al., 2010). Additionally, though Bax does not have a nuclear localization signal, phosphorylation may play a role in conformational changes that target it toward the nucleus. For this reason, we focused on how Ser163 and Thr167 phosphorylation affects translocation of Bax after HI.

Each phosphorylation site of Bax is known to be phosphorylated by a different kinase. In human hepatoma cells, c-jun N-terminal kinase (JNK) was first shown to phosphorylate Bax at Thr167 and have a positive effect on its activation and translocation (George et al., 2010, Xin et al., 2007, Kim et al., 2006). Phosphorylation of Bax at Ser167 is induced exclusively by GSK3β (Xin et al., 2007, Wang et al., 2010b, Wang et al., 2010a, Kim et al., 2006, Arokium et al., 2007). Oxidative stress has been shown to cause an overall increase in Bax phosphorylation and translocation to mitochondria (Xin et al., 2007). In a study using the newborn piglet as a model system, Bax was shown to be phosphorylated after hypoxia-ischemia, however exact phosphorylation sites were never specified (Ashraf et al., 2001).

Additionally, once activated, Bax is known to form SDS-resistant oligomers of various sizes, from dimers to tetramers to 12-mers (Epand et al., 2002, Seye et al., 2003,

Bleicken and Zeth, 2009, Er et al., 2007). Bax activity in cell death signaling relies on oligomerization to form pores in the mitochondrial membrane. Equally as important is the finding that the C-terminal region, which contains all three phosphorylation sites, is required for Bax oligomers stability (Er et al., 2007). Since our main focus was on the p-Bax^{Ser163} and p-Bax^{Thr167} sites, we sought to characterize how JNK and GSK3β affect the oligomerization of Bax using atomic force microscopy.

The central question this chapter addresses is how phosphorylation affects the translocation and oligomerization status of Bax after HI. We observed that Bax phosphorylation is altered after HI, and is associated with organelle localization. Whereas Bax phosphorylated at Thr167 was found increasingly in the nucleus within the first few hours after HI, Ser163 was dephosphorylated at later timepoints, namely at the mitochondria. Additionally, incubation of Bax with kinases known to interact with the protein led to the formation of oligomers. Curiously, the size of oligomeric aggregates differed depending on the kinase. These results pave the way to the possibility of using Bax phosphorylation as a potential therapeutic target.

RESULTS

I. Phosphorylation of Bax at Thr167 is Associated with Oligomerization and Translocation to the Nucleus After HI

Before we assessed the phosphorylation of Bax in HI brain tissue from neonatal pups, we first observed whether Bax phosphorylation is altered in our *in vitro* model of oxidative stress. This model involves the induction of oxidative stress by treating retinoic acid-differentiated human neuroblastoma cells, SY5Y, with varying concentrations of

rotenone, a mitochondrial Complex I inhibitor. Rotenone is expected to induce an apoptotic-like cell death at 25μM, and a necrotic-like cell death phenotype at 100μM. To verify that different doses of Rotenone do indeed cause necrotic and apoptotic cell death phenotypes, we first performed a time-dependent dose response assay. As Rotenone is a mitochondrial Complex I inhibitor, adding it to cells should show a compromise in mitochondrial integrity. Thus, using epifluorescent microscopy, mitochondrial integrity was assessed via intensity of MitoTracker Red CMXRos, a cell-permeant red fluorescent dye that accumulates in healthy mitochondria. When mitochondrial integrity is breached, the dye leaks out form mitochondria and is washed off during processing. As observed in Figure 10, 25μM rotenone causes cells to detach from the surface and shrink as early as 1 hour after treatment. However, mitochondrial integrity is only mildly affected. The cells appear to have apoptotic morphologies described in the introduction, with cells rounding up and beginning to shrink.

On the other hand, 100μM rotenone clearly affects mitochondrial integrity as early as 1h after treatment, as observed in Figure 11A. There is an increasing understanding that when injured, some cells die, others become damaged but ultimately recover, and still others stay damaged permanently but do not die. By 6 hours, the majority of severely injured cells were dead and the remaining cells can be seen reattaching to the plate surface. In addition to mitochondria being affected more severely, Figure 11B and C clearly indicate the morphologically necrotic characteristics, such as membrane swelling and cell rupturing, of SY5Y incubated with 100μM Rotneone. These characteristics were observed only at early timepoints, up to three hours after injury.

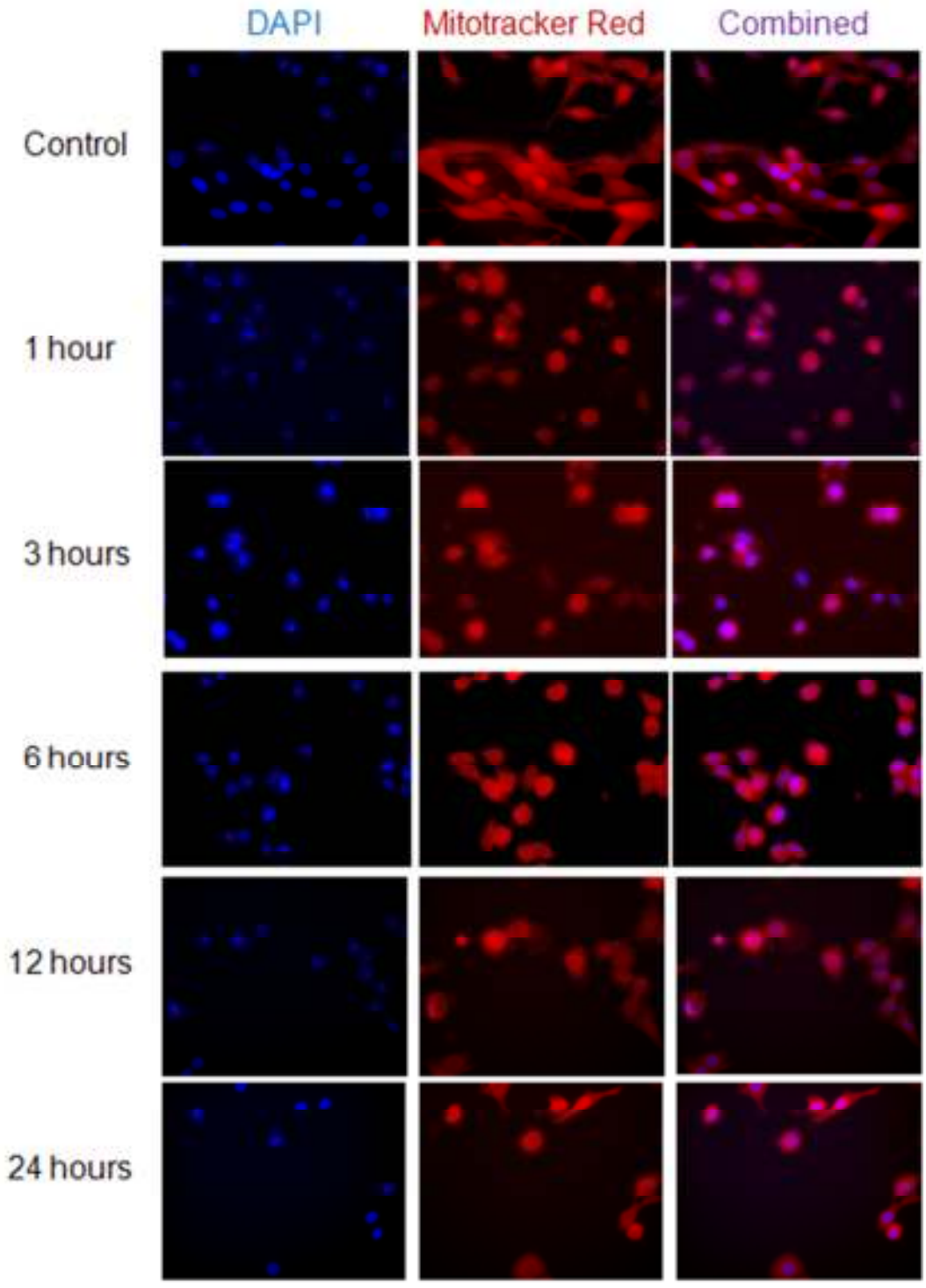
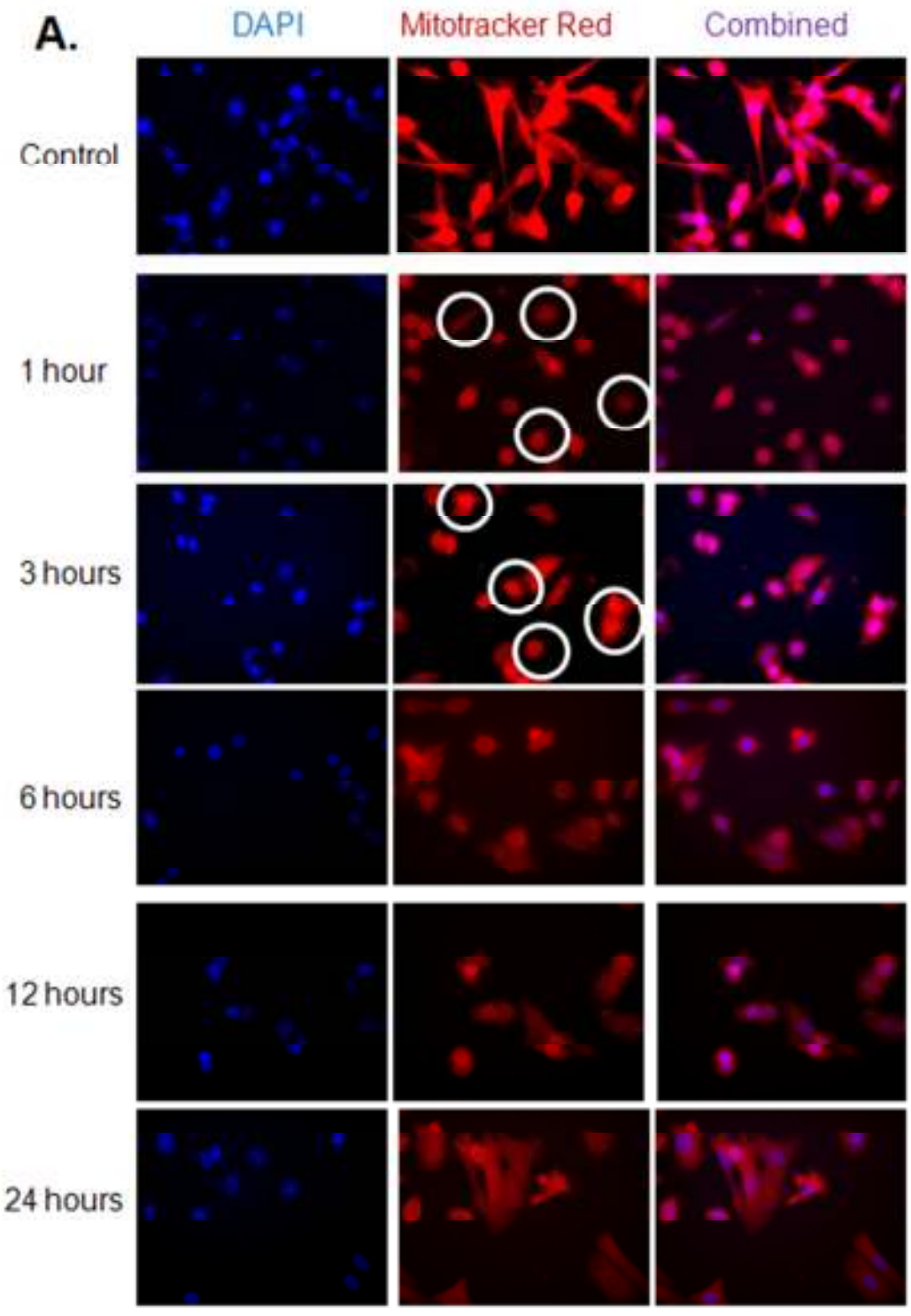


Figure 10. ***Mitochondrial permeabilization and cell death in SY5Y after incubation with 25μM Rotenone.*** SY5Y cells were treated with different concentrations of rotenone and stained with Mitotracker Red CMXROS to show the breach of mitochondrial integrity, which is clearly visible even 1h after Rotenone treatment.


A.
DAPI
Mitotracker Red
Combined
Control
1 hour
3 hours
6 hours
12 hours
24 hours

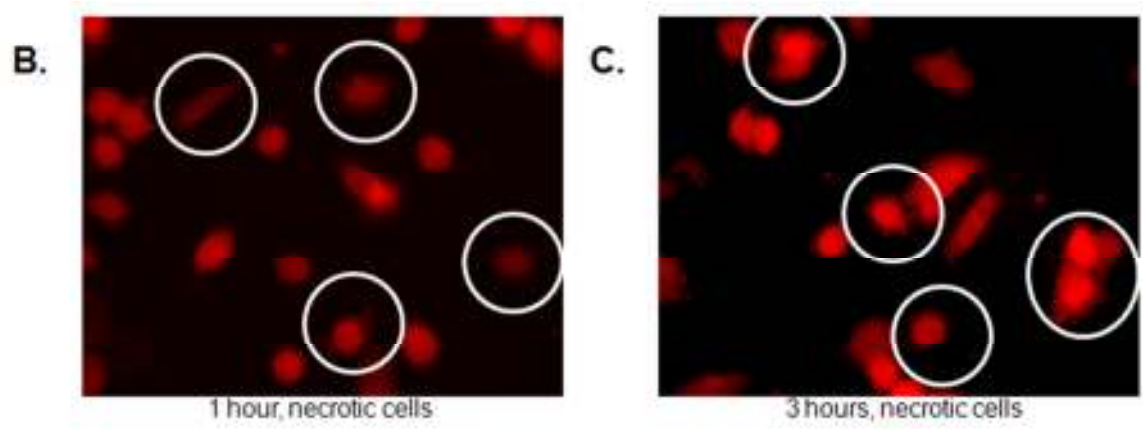


Figure 11. ***Mitochondrial integrity is more compromised in SY5Y after incubation with 100μM Rotenone, than after incubation with 25μM rotenone.*** *A.* SY5Y treated with rotenone at 25μM and 100μM portray two phenotypically distinct types of cell death, apoptotic and necrotic respectively. B and C show a zoom-in of the necrotic cell death morphologies observed after just one hour of injury, that is also apparent at 3h after injury. 40x magnification.

We examined the organelle localization of phosphorylated Bax by performing subcellular fractionations in order to separate the nucleus, mitochondria, and cytosol. Figure 12A depicts the verification of our fractionations using organelle-specific markers. Lamin B1 is an inner nuclear membrane protein and was used to verify the nuclear fraction as pure. For the mitochondrial fraction, CoxIV, a protein involved in the mitochondrial electron transport chain, was used to verify its purity. Bax is also known to be shuttled to the endoplasmic reticulum, however this project specifically focused on the effects of phosphorylation on nuclear translocation. Therefore, during our Subcellular fractionation procedure, we removed any ER contaminants from the nuclear, mitochondrial, and cytosolic fractions. Calreticulin, a folding protein found in the ER, was used to verify that neither the nuclear, mitochondrial, nor cytosolic fractions contain ER.

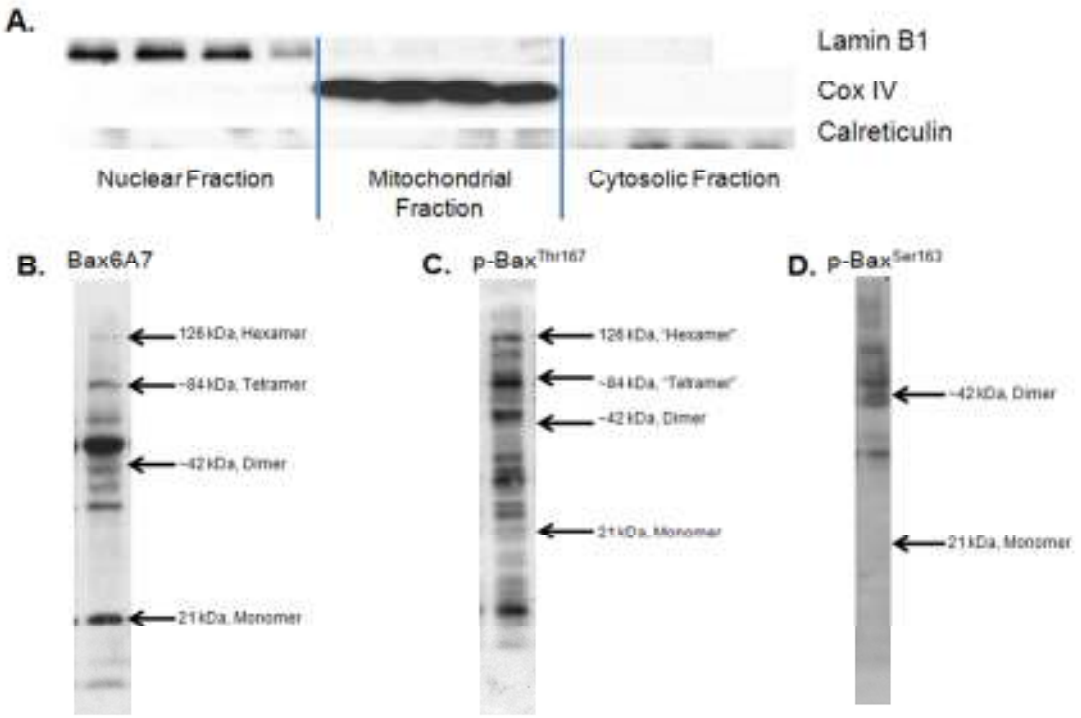


Figure 12. ***Verification of subcellular fractionation shows minimal contamination, and p-Bax bands were identified to assess localization.*** A. Subcellular fractionations were verified using Lamin B1 as the marker for the nucleus, Cox IV as the mitochondrial marker, and Calreticulin as the ER marker. ; B. This representative blot is of active Bax, indicating the bands identified as the monomeric, dimeric, tetrameric, and hexameric forms of Bax.; C. Representative blot using p-Bax^{Thr167}, indicating the bands used to quantify Bax localization.; D. Representative blot using antibody, p-Bax^{Ser163} indicating the bands used to quantify Bax localization.

Figure 12B, C, and D together indicate the method used to identify oligomeric bands of Bax that were quantified for our analyses. Figure 12B depicts a representative western blot of an HI tissue sample probed with anti-Bax(6A7), which only binds to the active conformation of Bax. Bands representing the approximate size of dimeric, tetrameric, and hexameric forms of Bax were selected based on their presence on this blot. Figure 12C and 12D show a representative blot of an HI sample blotted with our custom-made anti-p-Bax^{Thr167} and anti-p-Bax^{Ser163} antibodies, with the same monomeric, dimeric, tetrameric, and hexameric size bands identified.

Oxidative stress is the major constituent of cell injury and cell death signaling after HI. As a pilot experiment, we observed whether severe oxidative stress caused by rotenone affects Bax phosphorylation by subjecting differentiated SY5Y cells to 100μM rotenone. As seen in Figure 13, we observed a significant increase in phosphorylation of Bax at Thr167 in the cytosol, nucleus and mitochondria. The most significant increase in p-Bax^{Thr167} was observed at 0.5 hour in mitochondria, but by 1 hour after HI, there is a sharp decrease of p-Bax^{Thr167} in mitochondria. Consistent with previous results Bax translocation to the nucleus between 0.5 hour and 2 hours after HI, we find here that there is a pattern of increased Bax phosphorylation in the nuclear fraction. This led us to hypothesize that phosphorylation at Thr167 may be involved with translocation of Bax to the nucleus.

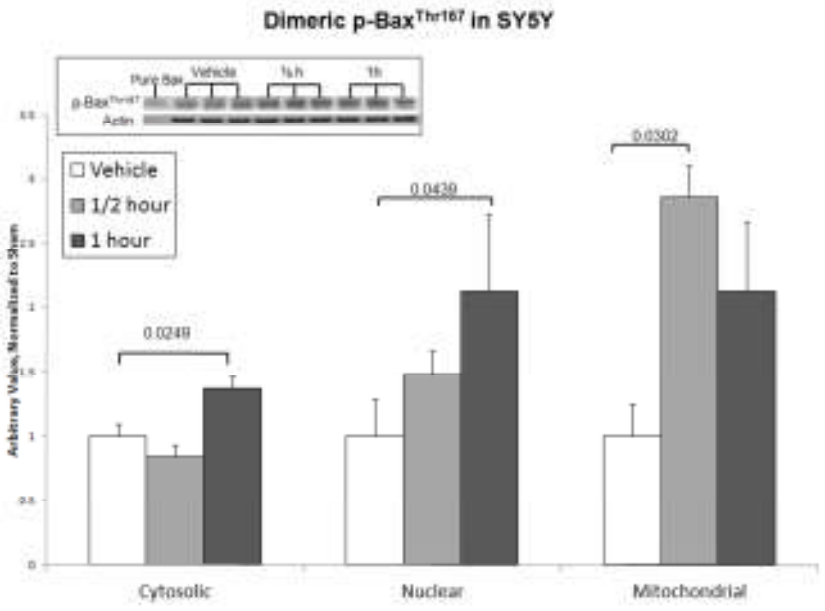


Figure 13. ***p-Bax^{Thr167} translocates to the nucleus in SY5Y after 100μM rotenone.*** Retinoic acid-differentiated human neuroblastoma SY5Y cells were incubated with100μM Rotenone, a mitochondrial complex I inhibitor. Samples were subsequently fractionated and phosphorylation was analyzed using our custom-designed phospho-antibody against p-Bax^{Thr167}. In SH-SY5Y cells incubated with 100μM Rotenone, a significant increase of p-Bax is observed in mitochondria half an hour after injury, and there was a pattern of increase both in the nucleus and cytosol from 0-1 hour after injury. (n=3)

We next observed whether there is an association between p-Bax^{Thr167} and subcellular localization in ipsilateral parietal cortex tissue from our clinically relevant Rice-Vannucci rodent model of HI. Dimeric p-Bax^{Thr167} localizes in the nucleus at early timepoints, consistent with previous reports of Bax localizing in the nucleus between ½ hour and 2hours after HI. We observed a significant increase of p-Bax^{Thr167} in the cytosol at ½ hour after HI. Interestingly, 2h after HI, when Bax has previously been reported to translocate to mitochondria, a significant decrease of p-Bax^{Thr167} is observed in the nuclear fraction. No significant increase of p-Bax^{Thr167} was observed in mitochondrial fractions at any timepoint. At later timepoints, Bax phosphorylation shows no significant changes, indicating that phosphorylation and activity of Bax are early events after HI.

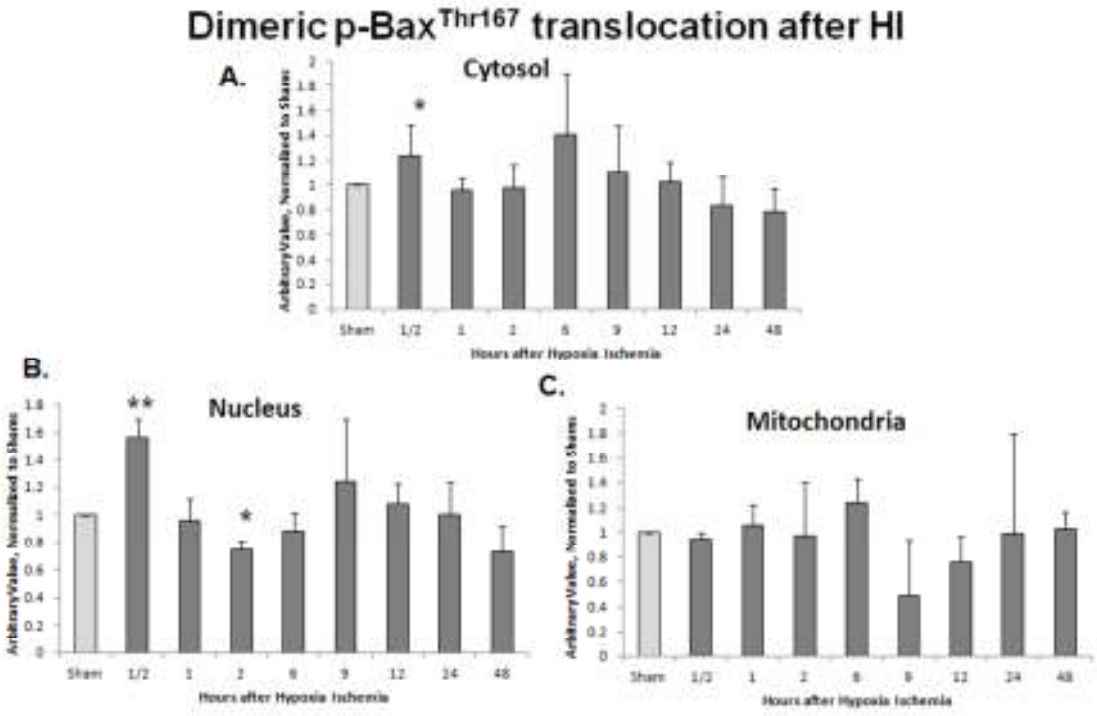


Figure 14. ***p-Bax^{Thr167} translocates to nucleus after HI.*** Densitometric analysis of western blot bands indicate that the dimeric form of p-Bax^{Thr167} increases significantly in the cytosol and nucleus at ½ hour after HI, and decreases in the nucleus at 2 hours after HI. Sham n=3, HI n=4; *p<0.01, **p<0.001 *vs* Sham

Additional analysis of the tetrameric and putatively pore-forming hexameric forms of Bax have shown a similar increase in the nucleus half an hour after HI, and a correlating decrease in mitochondria, as seen in Figure 15. Interestingly, no tetramers were detected in the cytosolic fractions after 6 hours, and after 9 hours in the nucleus and mitochondrial fractions. Furthermore, we found a significant increase of hexamers in mitochondria at 2h after HI.

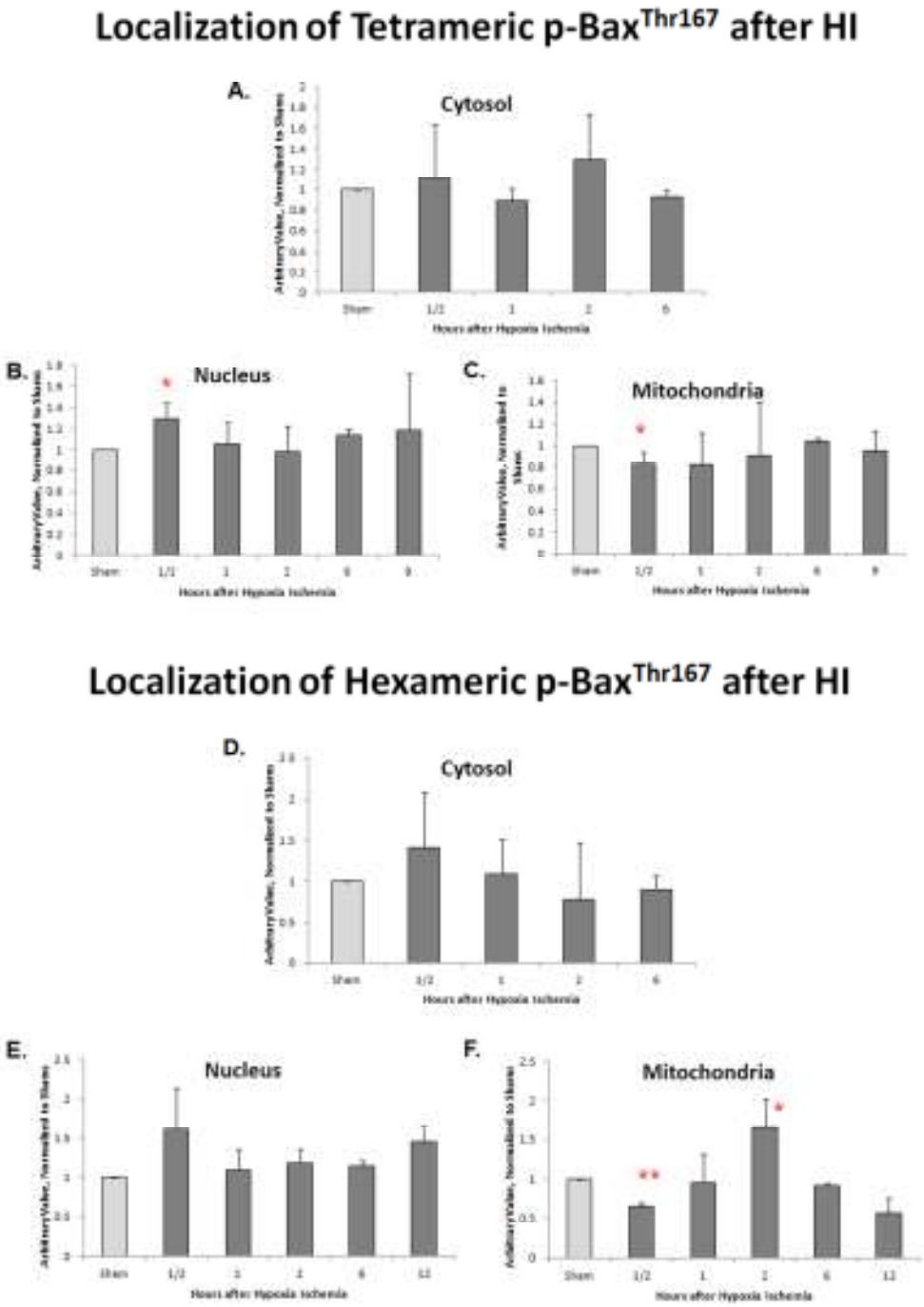


Figure 15. ***Tetramers and hexamers of p-Bax^{Thr167} were increased in nucleus after HI.*** Densitometric analysis of western blot bands correlating to tetrameric and hexameric forms of Bax (84kDa and 126kDa respectively) indicate a significant increase of tetramers in the nucleus at ½ hour after HI, and a decrease in mitochondria at the same time. Hexameric forms of Bax were found to decrease in mitochondria at ½ hour after HI, but show a significant increase in mitochondria by 2 hours after HI. *p<0.01, **p<0.001 compared to Sham

II. Bax^{Ser163} is Dephosphorylated after HI

In addition to p-Bax^{Thr167}, phosphorylation of Bax at Ser163 has been observed to increase Bax activity. However, as indicated in Figure 16, we found no significant increases in the dimeric form of p-Bax^{Ser163}. Quite to the contrary, we observed a significant decrease in p-Bax^{Ser163} levels in mitochondria 6 hours after HI, as well as a pattern of decrease in the cytosol from 1 to 6 hours after HI. This may indicate that dephosphorylation of Bax at later time points may play a role in regulation of Bax signaling activity. No significant changes were observed in time points past 6 hours (not shown).

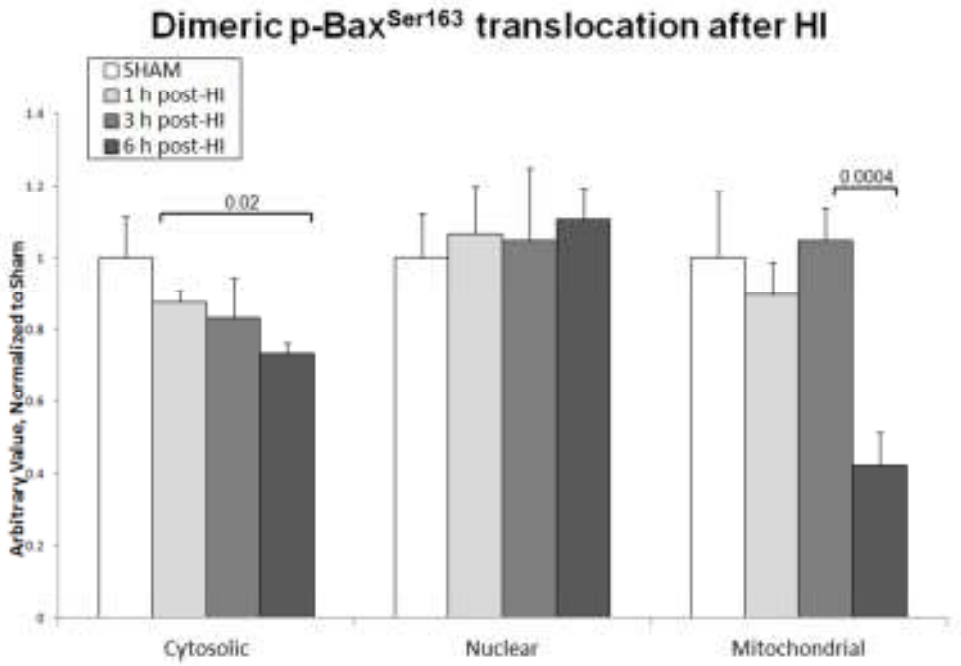


Figure 16 ***p-Bax^{Ser167} decreases after HI*** Densitometric analysis of western blot bands indicate that there is a general decrease of p-Bax^{Ser163} in the cytosol, and a significant decrease in mitochondria at 6 hours after HI. No significant changes were observed at later timepoints after 6h (not shown). n=3

III. Bax Forms Oligomers When Incubated with Kinases

The C-terminal region of Bax has been found to enforce the stability of Bax oligomers (Er et al., 2007). Considering the C-terminal hydrophobic region-flanking location of the phosphorylation sites on Bax, the likelyhood of phosphorylation affecting conformational changes of Bax and contributing to oligomerization is high. We assessed the effects of JNK and GSK3β on Bax oligomerization, as they are known to phosphorylate Bax at Thr167 and Ser163, respectively (Kim et al., 2006, Xin and Deng, 2005, Linseman et al., 2004). In order to assess the oligomerization status of Bax, we used atomic force microscopy (AFM) to visualize the purified and oligomerized proteins.

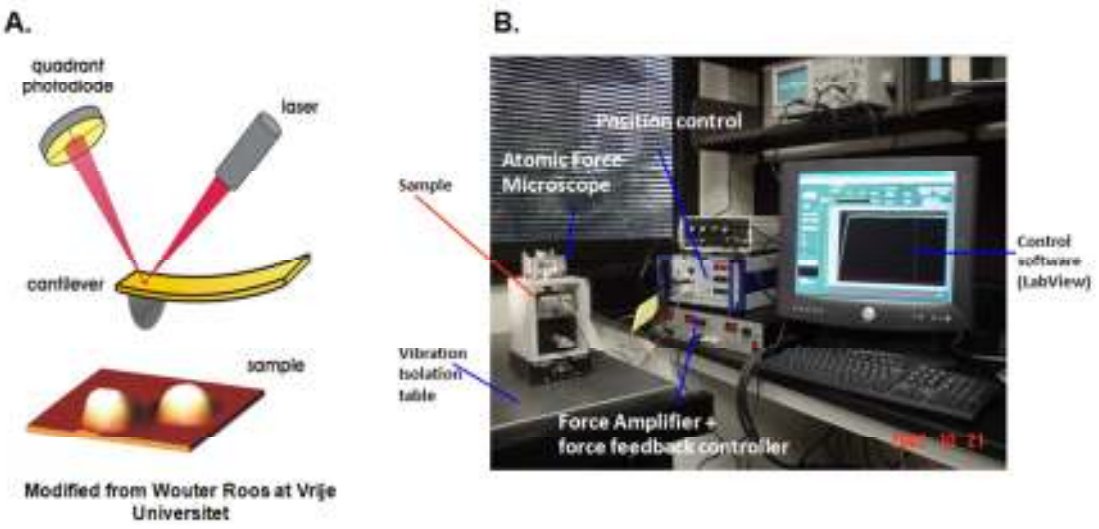


Figure 17. ***Setup for Atomic Force Microscopy.*** A. For Tapping Mode AFM, the setup requires a sample embedded onto a mica substrate, which is tapped on by a cantilever. The quadrant photodiode and laser serve to measure and record the cantilever's movements. B. The microscope is placed on a vibration isolation table and is connected to the force amplifier, and feedback and position controllers. The overall setup is connected to a computer with the appropriate software for analysis, LabView. *Modified from Oberhauser Lecture notes UTMB 2011.*

Figure 17A shows the general concept of AFM. A sample is loaded and adhered onto a mica substrate and placed on the platform within the microscope. Placed at a fixed

distance away from the platform, the cantilever taps continually across the mica substrate. Measurements of protein size are taken by recording the difference in the distance from the cantilever to the surface of the platform. Figure 17B shows the entire setup of the microscope used to obtain the results below. Cantilever measurements are recorded and a topographical map is created, visualizing the protein's size against the mica substrate. The topographical map collected from imaging a sample was interpreted by the software, LabView.

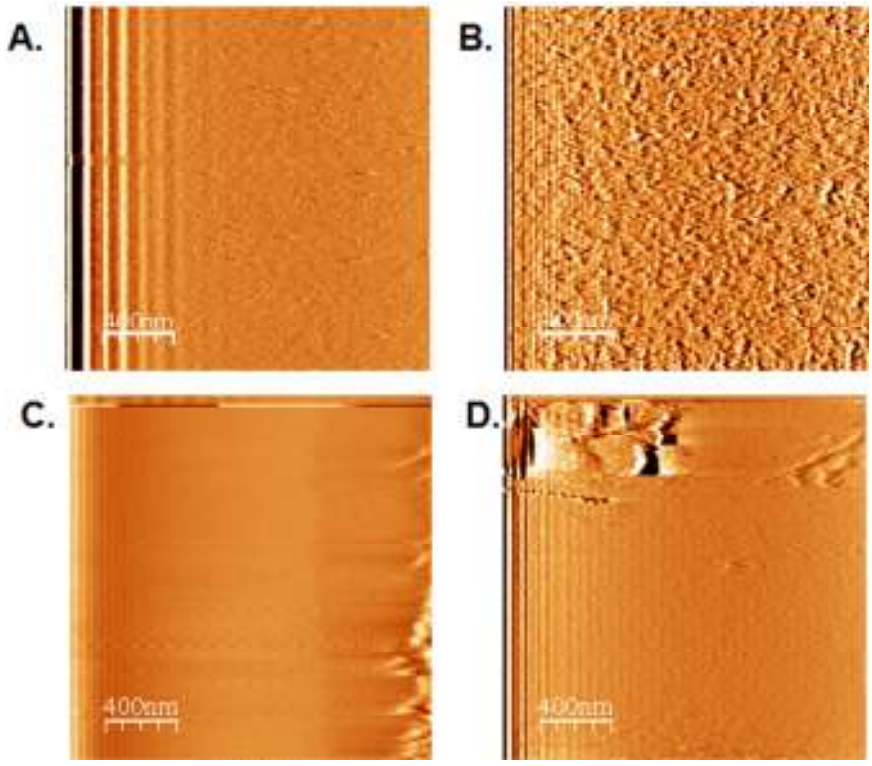


Figure 18. Controls for Atomic Force Microscopy. A. Negative control of purified Bax monomers shows the topography of the mica substrate, but no aggregates. B. Positive control of purified Bax aggregates that form when incubated with the nonionic detergent, DDM. C. Negative control of JNK-only samples show no aggregates. D. Negative control of GSK3-β only samples.

As a negative control, we imaged purified Bax protein alone on the AFM. As expected, results indicate no aggregate formation. There is evidence to support that

nonionic detergents activate Bax by inducing conformational changes and causing oligomerization (George et al., 2010). Therefore, for our positive control, we incubated Bax with the nonionic detergent DDM (n-dodecyl β D-maltoside) to induce oligomerization. As seen in Figure 18B, incubation of Bax with DDM does indeed show aggregate formation. Lastly, as a negative control for kinase expression, we imaged purified active JNK alone (Figure 18C) and GSK3β alone (Figure 18D) and observed no aggregates detectable by AFM.

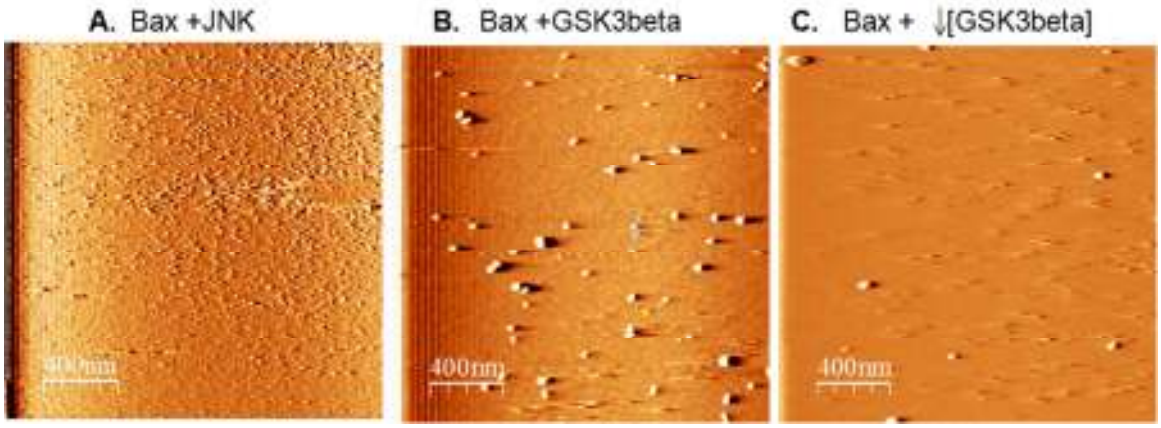


Figure 19. ***Bax incubated for 1 hour with kinases.*** **A**. One hour incubation with JNK produces small aggregates, closer to dimeric and tetrameric forms of Bax; **B**. One hour incubation with GSK3β shows higher order oligomeric species. **C**. Lower concentrations of GSK3β, with respect to the K_m, shows the specificity of the kinase, and indicates a less-than-optimal reaction.

Once our controls were properly established, we assessed the effects of kinase activity on Bax oligomerization. We incubated Bax with JNK or GSK3β as described in Chapter 2, Section G and assessed oligomerization via AFM. As seen in Figure 19A, incubation with JNK induced the formation of small Bax oligomers, whereas incubation with GSK3β induced higher order oligomeric structures of Bax (Figure 19B), implicating that phosphorylation at Thr167 affects conformation and stability of the protein differently than phosphorylation at Ser163. Figure 19C shows the result of incubating

Bax with lower concentrations of GSK3β with respect to K_m. Interestingly, when Bax was incubated with concentrations of GSK3β that were comparable to the Bax + JNK sample (Figure 19C), very few oligomers were apparent, and the ones present were comparable to the GSK3β image in Figure 19B. Clearly, the K_m of the kinase is important in its activity and specificity for Bax.

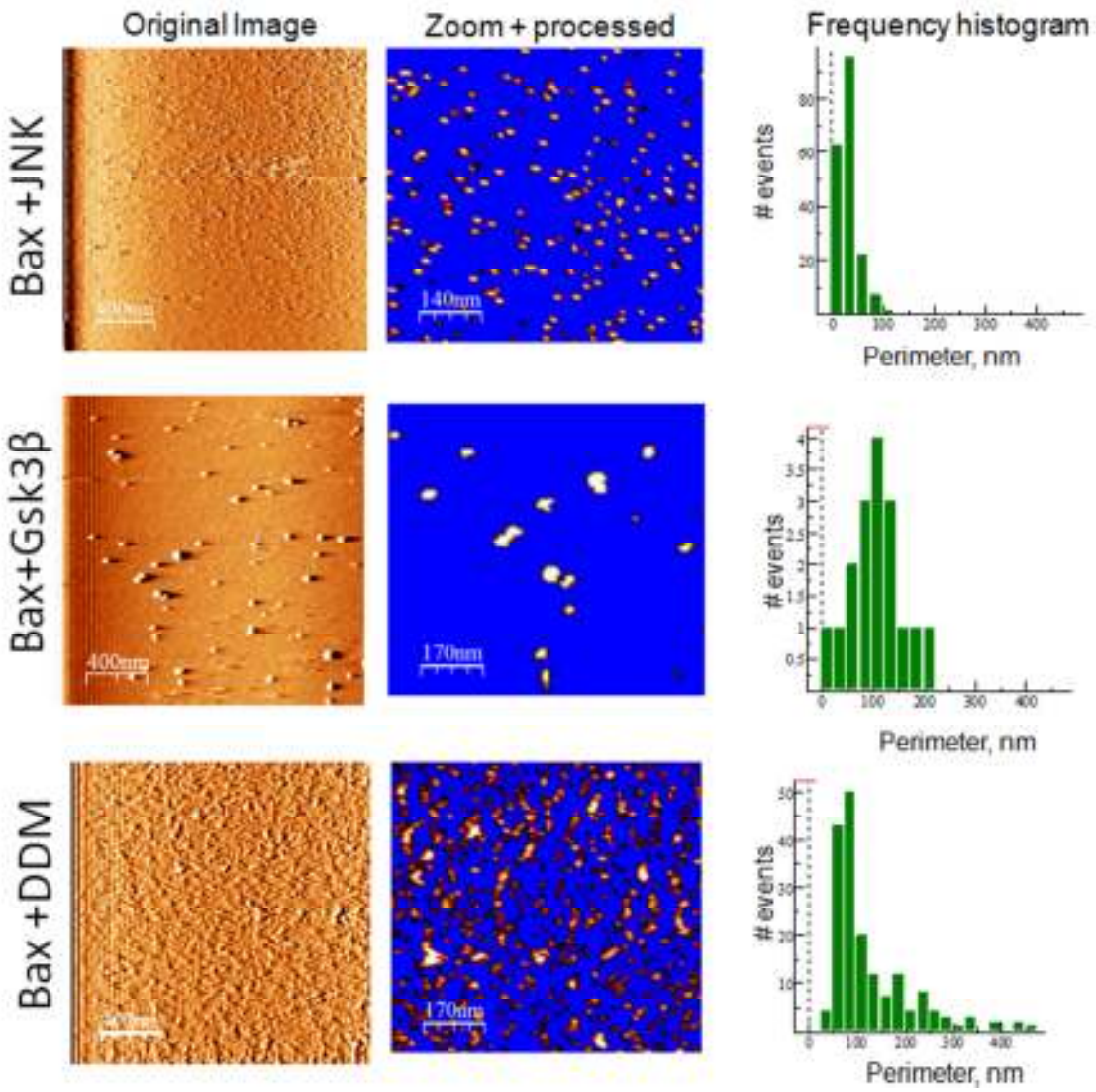


Figure 20. ***Quantification of oligomeric Bax aggregates*** A. Size analyisis shows that these are dimers. B. Topographical analysis of Bax incubated with JNK reveals that most aggregates are within the dimer and tetrameric range.

We further quantified our images using LabView software to determine the size and distribution of different oligomeric aggregates in the sample. We used software to identify the number of occurrences of each oligomeric species, and plotted this onto a frequency histogram. We found that Bax incubated with JNK produces mainly dimeric and tetrameric forms of Bax oligomers. Whereas, Bax incubated with GSK3β produces hexameric and higher order oligomers. Not surprisingly, our control of Bax + DDM produced a range of oligomeric species. Overall, our AFM results indicate a correlation between Bax oligomerization status and interaction with Bax-specific kinases.

DISCUSSION

Our results have shown that Bax is phosphorylated after neonatal HI, and that phosphorylation of Thr167 is associated with nuclear translocation and the formation of higher order oligomers. Dephosphorylation of Ser163 appears to be associated with mitochondrial translocation. Our rotenone injury model allowed us to easily analyze the dose-dependent responses of neuronal cells to oxidative stress, clearly indicated in Figure 11.

Bax phosphorylation was analyzed using custom-made antibodies to p-Thr167 and p-Ser163. In order to identify the correct oligomeric bands of Bax, we performed western blot analysis on ipsilateral cortical tissues and probed the samples with a Bax 6A7 antibody. This antibody is specific to detecting only activated forms of Bax, as the epitope lies in the hydrophobic cleft of Bax, and presentation of the epitope requires a conformational change. Probing with this antibody produced numerous bands, and

oligomeric bands were selected according to the estimated size of Bax oligomers, considering Bax is a 21kDa protein. Thus the band at 42kDa was determined to be the dimer of Bax, the 84kDa band as the tetramer, and the 124kDa band as the 12-amer. No bands were detected correlating with the hexameric form of Bax, which has previously been shown to form pores on artificial lipid bilayers (Antignani and Youle, 2006, Epand et al., 2002, Schlesinger and Saito, 2006). These exact size of bands were also detected when the samples were probed using our custom-made phospho-Bax antibodies, and thus were quantified as the oligomeric forms.

In Figure 13 we observe a significant increase of phosphorylation in the mitochondria half an hour after injury, as well as an increase of cytosolic and nuclear p-bax 1 hour after injury, indicating that oxidative stress and energy depletion by rotenone does induce phosphorylaton of Thr167. This may imply that phosphorylation at Bax^{Thr167} in the mitochondria half an hour after injury may lead to translocation to the nucleus 1 hour after rotenone treatment. However, further analyses must be performed in order to verify such an implication.

We also observed the increase of phosphorylation at Bax^{Thr167} in the nucleus at early timepoints in our HI ipsilateral cortical samples. This data is very consistent with our previous results indicating Bax localization to the nucleus occurs between ½ and 3 hours after HI, and mitochondrial translocation between 3-9 hours after HI. Phosphorylation changes in the nucleus correlate with the timing of Bax localization in the nucleus, thus phosphorylation at Thr167 may be associated with nuclear

translocation. This may imply that Bax translocation to the nucleus is stabilized or initiated by p-Bax^{Thr167}.

Bax oligomers that are larger than dimers are not observed past 9 hours after HI, indicating that Bax translocation and oligomerization are very early activities. This could also be due to the half-life of Bax being 9-12 hours (Xin and Deng, 2005, Wang et al., 2010b, Wang et al., 2010a). Phosphorylation at Ser184 has been shown to significantly decrease the half-life of Bax, therefore it would be interesting to assess the phosphorylation patterns at Ser184 at this timepoint to understand whether this plays a role in Bax activity at later timepoints after HI.

The dimeric form of Bax has been shown to play a significant role in cell death signaling, but since the exact mechanism of organelle membrane permeability by Bax is still being debated, there is also the theory of pore-formation via higher-order oligomers. According to current literature, hexameric Bax can form pores on membranes, therefore the most functionally relevant data in terms of cell death signaling is of the hexamers (lipid bilayer AFM experiments paper source, Swiss group), which show a significant decrease in mitochondria at ½ hour after HI at the same time as a pattern of increase at the nucleus appears. At 2 hours after HI, a significant increase of these oligomers is found at the mitochondria, consistent with previous data indicating that mitochondrial permeability during cell death signaling occurs around 2 hours after injury (Gill, 2007, Gill et al., 2008).

The first sign of any changes in p-Bax^{Ser163} was observed 6h after HI, where we see a significant decrease of p-Bax^{Ser163} in mitochondria, as shown here. As this data

correlates with previous data indicating a decrease in Bax mitochondrial localization by 6 hours after HI, dephosphorylation at Bax^{Ser163} may be involved in destabilizing Bax oligomerization, and shifting Bax out of mitochondria. Again, no significant changes in p-Bax^{Ser163} were observed at later timepoints past 6 hours. Protein Phosphatase 2A has been associated with dephosphorylation of Bax at Ser184, but its 1. Its role in dephosphorylation of the other two sites on Bax has not been examined, nor has the possibility of dephosphorylation by other phosphatases. An interesting avenue of study would be examining the activity of phosphatases after HI.

It is also of importance to note that previous literature has indicated signs of a p18 cleaved form of Bax, especially within the nucleus (Wood and Newcomb, 2000, Karlsson et al., 2007). However, we did not find any p18 band in our nuclear fractions, when probed with total Bax or p-Bax antibodies. This cleavage may be a cell-type or injury-specific phenomenon.

Bax oligomerization plays a key role in its signaling activity; there may be a correlation between oligomerization and localization. Many proteins are known to translocate to organelle membranes after oligomerization. In the case of Bax, the obvious example is pore formation on the mitochondrial membrane. Activated Bax translocates to the mitochondria, oligomerizes, and forms pores which lead to release of mitochondrial contents. Additionally, a classic experiment depicts Bax spontaneously forming pores on lipid bilayers. Therefore, judging that phosphorylated bax lends itself to conformational changes, we asked the question "how does phosphorylation affect oligomerization of Bax?" Consistent with our findings, recent reports suggest that Bax phosphorylation is

directly linked to oligomerization , and that the c-terminal region is required for oligomerization and stabilization of oligomers (D'Alessio et al., 2005, Yu et al., 2008, Er et al., 2007).

As stated in the introduction, Bax's α1 helix blocks α9 and keeps it occupied so that Bax cannot homodimerize (Kim et al., 2009). However, according to our data, absence of α1 helix is not enough to *cause* dimerization, indicated by the Bax-only control in Figure 18B using the N-terminal truncated purified Bax protein. Phosphorylation may play a role in stability of this open form of Bax, lending itself more readily to dimerizing. In addition, previous literature has reported observing oligomers containing up to 22 Bax monomers.

Though our studies have alluded to the fact that HI alters Bax phosphorylation levels at Thr167 and Ser163, which are associated with oligomerization and localization of the protein, there are of course certain limitations to our studies. Further studies are needed to state without doubt that phosphorylation is *required* for subsequent translocation to nuclear or mitochondrial organelles. This could be done by assessing the localization of GFP-tagged mutant constructs of Bax, where the phosphorylation sites are mimicked by a glutamate mutation, or inhibited by an alanine mutation. However, we have certainly shown that phosphorylation and association with active kinases affects the oligomerization status of Bax. Further understanding of this phenomenon could include finding ways to inhibit the phosphorylation of Bax^{Thr167}, oligomerization, and translocation of Bax to the nucleus, thereby averting or reducing the deleterious necroptotic cell death phenotype observed after HI.

Chapter 4: Neonatal HI Causes Behavioral Deficits in Progeny

INTRODUCTION

HI causes numerous detrimental effects to the brain including edema formation, inflammation, and cell death (Ferrari et al., 2010, Gill, 2007). The ability of Bax to translocate *into* the nucleus before necroptotic cell death was observed, and the fact that Bax function in the nucleus is completely unknown originally brought us to the idea that perhaps Bax may act on gene regulation and epigenetic programming. We ultimately asked the question of how long-lasting the impact of HI is on sensory-motor coordination deficits. Based on recent literature, we predicted that the progeny of HI-affected animals may not simply reset their neurological development, but are instead impaired by the epigenetic modifications experienced by their parents.

As stated in the introduction, epigenetic modifications can be manifested by: 1. Changes in cell phenotype; 2. Regulation of cell lineage progress; 3. Yield a stably inherited phenotype. This section is focused on the third manifestation of epigenetics, that of stably inherited phenotypes. In this regard, we assessed the behavioral characteristics of 2nd generation pups of rats that experienced neonatal HI. Results from these data were compared with that of naive and sham 2nd generation pups, as well as behavioral data from 1st generation parents who were directly exposed to HI. Injury from HI has been shown to have detrimental behavioral consequences in rats (Ferrari et al., 2010). In similar behavioral assays as the ones used in this study, sensory-motor coordination has been shown to be significantly impaired in HI animals when compared to shams.

Clinical motivations for testing second generation behavioral deficits associated with neonatal HI include the fact that sensory-motor impairments are well described after neonatal HI, but treatments are still limited. Assessing the devastating extent of the injury's reach serves as a cautionary tale to the risks associated with neonatal HI and provides an incentive to create improved therapeutic measures to treat HI.

For this project, we used a battery of three behavioral assessments of sensory-motor coordination. This study is the first to show that the behavioral deficits observed after HI are transferred to the next generation of animals. More importantly, we have shown that male progeny are more susceptible to inheriting HI-induced neurological deficits than female progeny.

Table 1. ***Fist-generation parent crosses for behavior analysis of second-generation pups.***

Mother	Father	# of total pups
Naïve	Naïve	20
Naïve	Sham	10
Naïve	HI	30
HI	Sham	10
Sham	HI	20
HI	HI	10

RESULTS

I. Progeny of HI animals exhibit major sensory-motor coordination impairment in bar holding assessment

Nulliparous first-generation rats were crossed at their peak mating age, 10 weeks after birth. Table 1 illustrates the crosses that were made, and the number of pups per

cross that were assessed. Rats were handled as described in Chapter 2, Section B.5 and C. A minimum of 10 pups were assessed at 3 weeks of age for each cross.

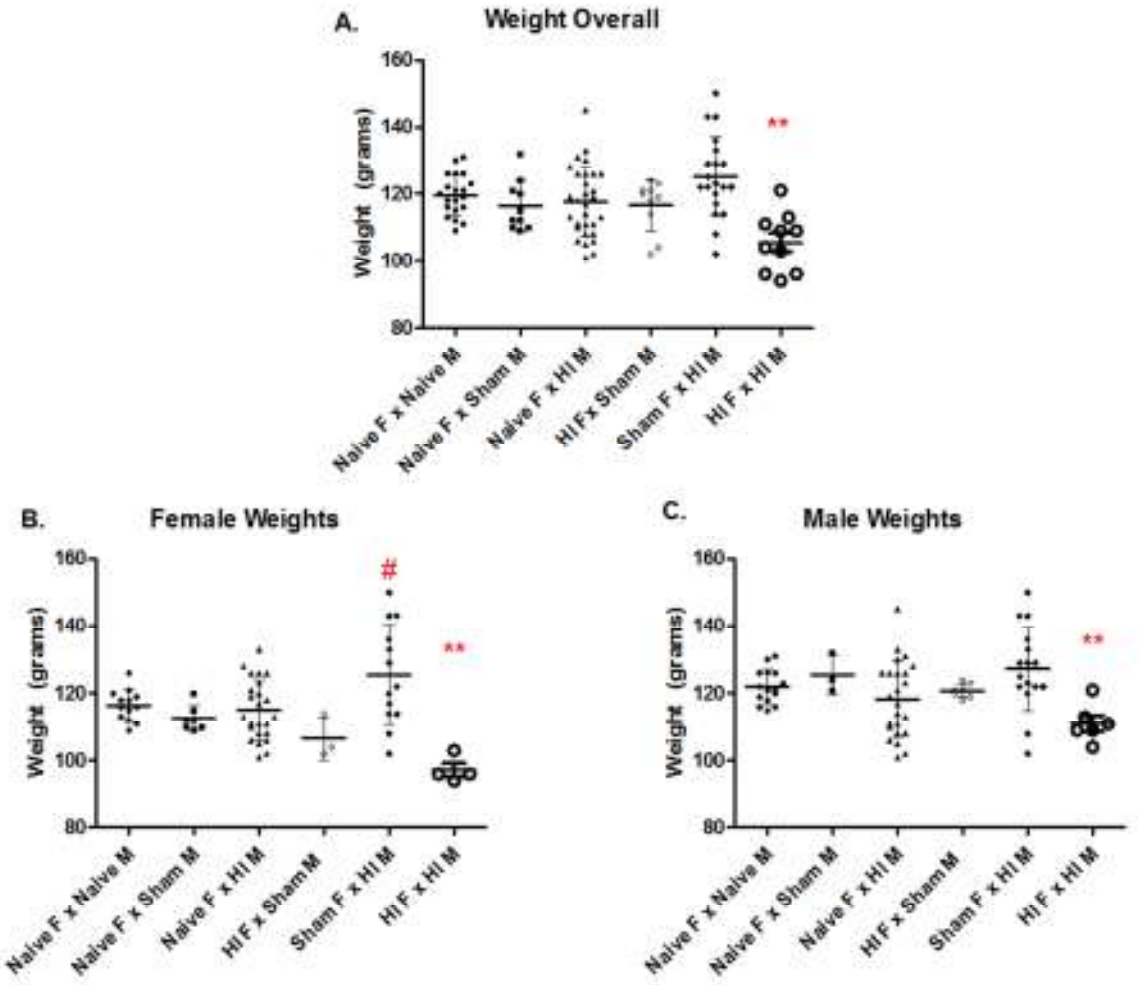


Figure 21. . ***Weight, and therefore developmental progression, of litters is affected by HI.*** A. Overall, weights of second-generation pups appear to be similar for all crosses. B. Female weights vary far more within litters whose parents experienced HI. C. Males show even more variation in weights within litters of parents who experienced HI. ** = $p<0.05$ when compared to Naïve x Naïve; # = $p<0.05$ when compared to Naïve x Sham

Weight gain at this age is associated with general developmental progress. Therefore, we measured weight gain In Figure 21A, there is no significant difference in weight distribution observed, except in HI x HI cross progeny. Remarkably, entire HI x

HI litters are significantly lower in weight. Additionally, when we separate into female and male groups as in Figure 21B and C respectively, it is clear that females are significantly lower in weight than males when compared to all other groups. Moreover, we see that the pups that came from HI parents have a wider distribution of weights in the litters than those from naïve or sham parents.

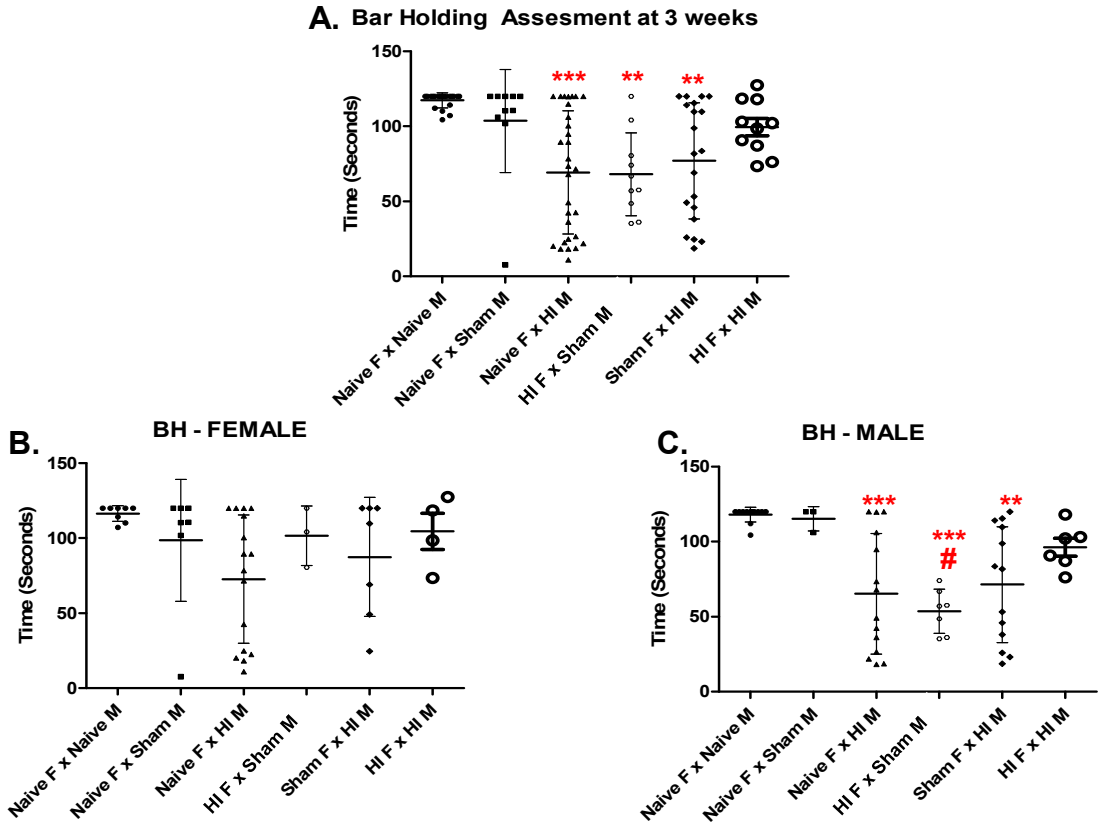


Figure 22. ***Bar holding assessment showed significant impairment in male progeny of HI parents.*** A. Overall, bar holding assessment indicates an impairment in sensory-motor coordination in pups whose parents experienced HI. When separating data into female (B.) and male (C.) groups, it becomes clear that the impairment is mainly seen in males. *** = $p<0.0001$ when compared to Naïve x Naïve; ** = $p<0.05$ when compared to Naïve x Naïve; # = $p<0.05$ when compared to Naïve x Sham

Our first behavioral assessment was the bar holding test, as described in Chapter 2, Section C. Results indicate that all progeny of HI crosses displayed significant behavioral impairment when compared to naive and sham progeny, as seen in Figure 22. HI x HI progeny did not exhibit as much impairment in this test as progeny with only one HI parent. However, they were still slightly more impaired than progeny from crosses using Naïve or Sham animals. When separating the data into female and male groups, it is apparent that the males suffer from behavior deficits more significantly than the females. This is also apparent in HI x HI progeny, though they still performed better than progeny from only one HI parent.

II. Male progeny of HI animals exhibit impairment in mesh ascending test

The mesh ascending test is another indicator of sensory-motor impairment. For this, the animals were placed at the bottom of a sturdy plastic mesh, which was placed at a 70° angle against a safe, dark box at the top. During the first day of training, fear of falling stimulates these pups to climb up to the box. Based on previous work on first generation HI pups, the pups require more incentive to climb the mesh and into the box as they become conditioned during training (Ferrari et al., 2010). Therefore, we placed the pup's littermates in the box so that the pup being tested would have a desire to climb up the mesh and enter the safety of the box.

In Figure 23A, we observed significant impairment only with progeny from HI x HI. However, when separated by gender, male pups from the Naïve Female x HI Male cross show significant impairment when compared to naïve progeny (Figure 23B and C).

Additionally, the impairment in HI x HI progeny is more significant in males than in females.

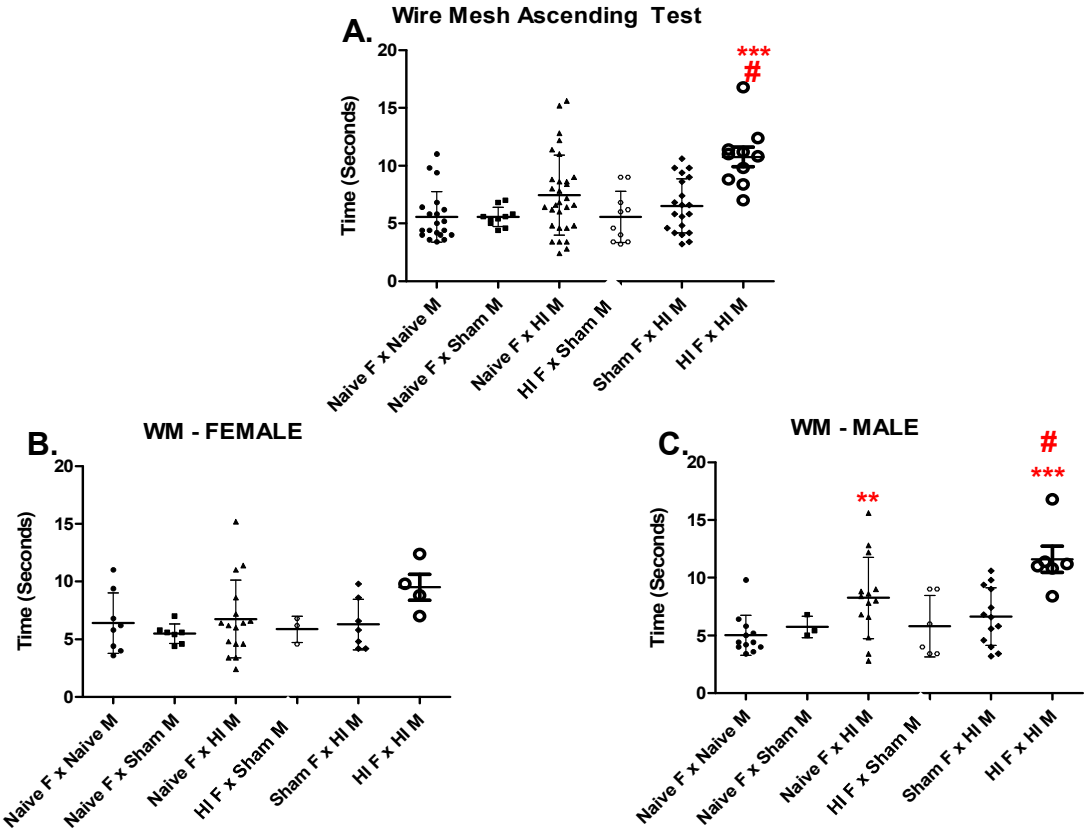


Figure 23. ***Mesh ascending assessment showed significant impairment in male progeny of HI animals when compared to naïve/sham progeny as well as when compared to females.*** A. Overall, the mesh ascending assessment does not indicate any significant differences in behavior of each group. However, when separating data into female (B.) and male (C.) groups, it becomes clear that the sensory-motor, and potentially social. impairment is still apparent in males. *** = $p<0.0001$ when compared to Naïve x Naïve; ** = $p<0.05$ when compared to Naïve x Naïve; # = $p<0.05$ when compared to Naïve x Sham

III. HI x HI pups exhibit more significant delay in motor coordination during sticky dot assessment

Our last test for behavioral deficits consisted of placing stickers onto the forepaws of pups and recording the time it takes for them to acknowledge the stickers by trying to lick them off, which we called contact latency. We also recorded the total time elapsed

before the pups removed both stickers from their paws. No training sessions were possible for this assessment, as the animals quickly began to acclimate to the exercise. However, fear was not an issue since the pups were simply placed back into their empty original cage while they addressed the stickers.

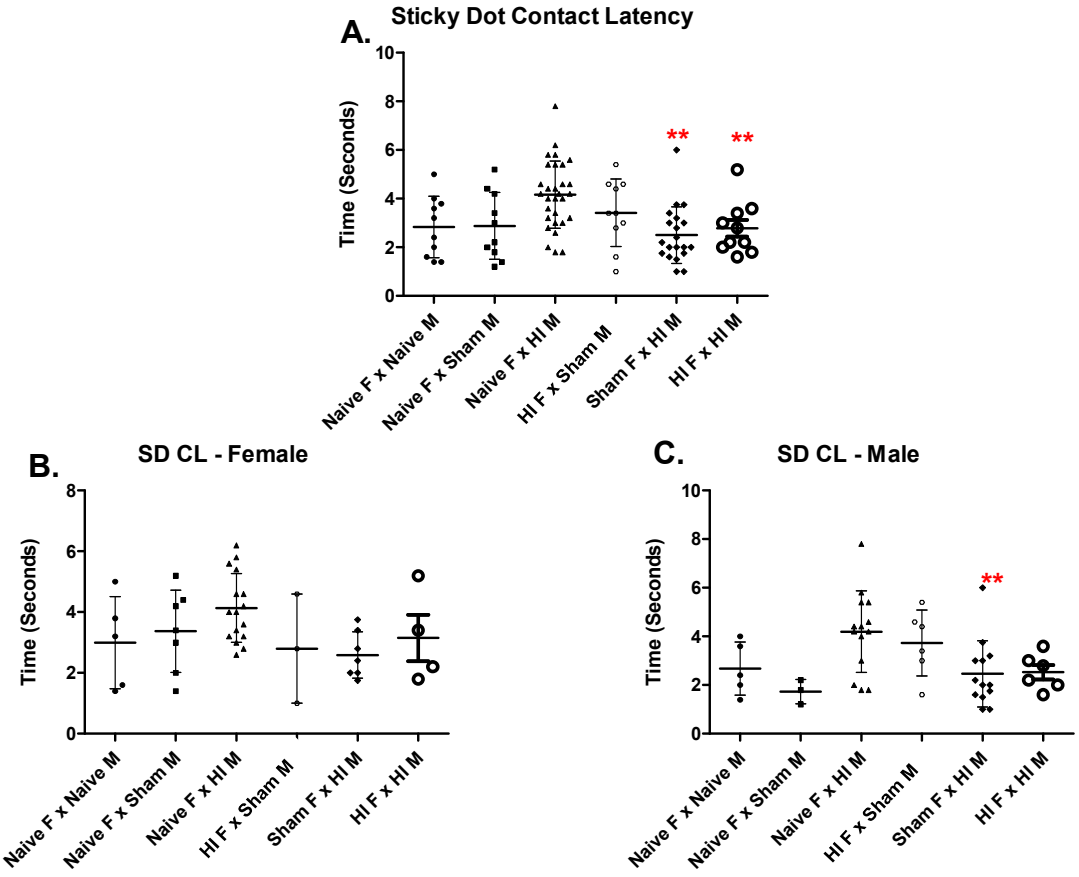


Figure 24. ***Sticky Dot assessment, contact latency, showed more impairment in male progeny of HI animals.*** A. Overall, the sticky dot assessment indicates significant impairment in the Sham F x HI M cross. However, when separating data into female (B.) and male (C.) groups, it becomes clear that the sensory-motor impairment is only apparent in males.
** = $p<0.05$ when compared to Naïve F x HI M

As seen in Figure 24A, no significant differences were observed when comparing progeny with at least one HI parent, and progeny with only Naïve or Sham parents, even

when the data was separated by gender (Figure 24 B and C). Of note is the fact that contact latency was significantly shorter in males from Sham Female x HI Male crosses when compared to males from Naïve Female x HI Male crosses.

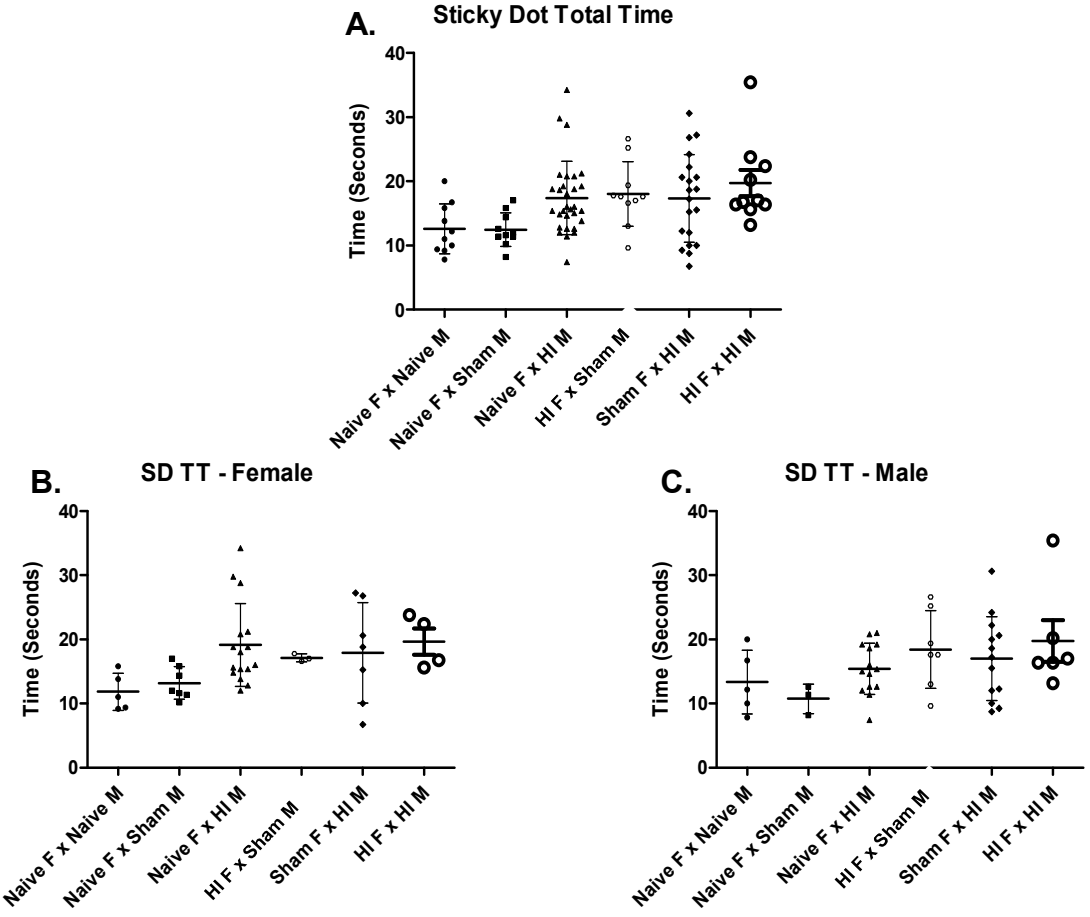


Figure 25. ***Sticky Dot assessment, total time, showed a larger spread of values with progeny of HI parents than from Naïve/Sham progeny.*** A. Overall, the sticky dot assessment's total time does not indicate any significant differences betwewen crosses, even when separating data into female (B.) and male (C.) groups.

Figure 25A indicates that no significant differences were observed between naïve/sham pups and pups from only one HI parent with regards to total time elapsed before both stickers were removed. Interestingly, as with the wire mesh assay, the HI x

HI performed significantly slower than Naïve and Sham progeny. In addition, as with our previous behavior assays, males performed significantly worse than females when compared to sham and naïve crosses, as seen in Figure 25B and C. An additional point to note is that the distribution of values is much wider in animals with HI parents than pups from Naïve and Sham crosses.

DISCUSSION

HI overall may lead to epigenetic changes that manifest in the next generation as behavioral deficits. We embarked on this study to first identify whether there are any observable impairments in behavior. In all animals, fear is always a strong motivating factor in survival situations, and the resulting response can often temporarily cloud any sensory-motor coordination impairments. For this reason, all animals were trained for five times a day for three days before the actual behavioral test. Assessing the progress and behavior of animals during the training period can also be a good indicator of memory impairment.

The overall theme of our results indicates that male progeny are more sensitive to the epigenetic transfer of behavioral deficits than females. This may be attributed to the fragility of the Y chromosome and its potential for methylation (Hill and Fitch, 2012). Sex-specific neuroprotection has been found in numerous injury paradigms, including neonatal HI, in both rodent models and humans (Hill and Fitch, 2012, Giordano et al., 2011). Therefore, it is no surprise that we are observing such significant deficits in second-generation males. The primary factor for cell death after HI is oxidative stress. It was recently reported that paraoxonase 2, a potent antioxidant, is expressed at 2x higher

levels in females than males. This may also explain the gender differences in the manifestation of behavioral deficits. In addition, it is important to note that imprinting occurs at different stages based on gender, so perhaps at the stage of our injury model, males are more susceptible to brain injury than females (Wagner, 2010).

In our data based on the Bar Holding Assessment, unimpaired animals (Naïve and Shams) often learn by the 4^{th} day to balance on top of the bar. This may imply that animals with HI parents have learning and memory deficits, which is associated with hippocampal damage. Another important result to note is that HIxHI progeny fared better than pups with only one HI parent. Further analysis is required to before these results can be interepreted. Though they fared better at the Bar Holding Assessment, HIxHI progeny showed siginificant deficits in the Mesh Ascending Assessment. Their uninterest and unwillingness to mingle enter the box and mingle with their siblings may indicate autistic and antisocial behavior resulting from the oxidative stress damage suffered by both parents.

The limitations of our Sticky Dot Test include the fact that animals cannot be trained before being assessed. Therefore, fear may still be a variable in these tests. However, fear would have led the animals to pay less attention to the stickers and more attention to finding an exit from their cage, but this was certainly not the case. In addition, there were significant differences in time elapsed when comparing progeny from Naïve and Sham pairings and progeny with an HI parent, which indicates that a sensory-motor coordination deficit is in fact present.

As shams of our rodent HI model are exposed to anesthesia, we were curious about whether anesthesia affects sensory-motor coordination in the second generation as well. Therefore, we assessed naïve pups and compared them to shams. As expected, there were minimal differences between progeny from naives and/or shams. Some crosses only had one litter assessed, so the results may be litter-specific. However, the pattern of behavioral deficits observed in pups from HI parents cannot be coincidental.

The fact that our injury model primarily affects the brain of neonatal rats and doesn't directly affect the germ line is both an advantage and a limitation. Based on our behavioral results, it is clear that the germ cells are in fact affected after HI. Our study does not have any direct implication of HI producing epigenetic modifications that are inherited, rather it implies that the behavioral deficits we observe in the next generation may primarily be due to epigenetic modifications arising from cross-talk between the nervous, endocrine, and reproductive systems. Mounting evidence suggests that throughout development, cross-talk exists between the nervous, endocrine, digestive, immune, and reproductive systems (Marchetti et al., 1990). Two to three decades of research indicates that the nervous system and endocrine system are seamlessly integrated and interact, especially in response to environmental stress (Dronca and Markovic, 2011).

It would be interesting to study the effects of global neonatal hypoxia-ischemia on the second and third generation of animals. In the case of global HI, the second generation's germ cells would be directly exposed to HI, therefore analysis of a third generation is necessary to prove epigenetic inheritance as opposed to direct epigenetic

effects of global HI. Here, the advantage of our study shines through in that we can observe the effects of HI in the first generation and relate it to indirect transfer of behavioral phenotype to the next generation.

The next logical step after this study is to assess global methylation patterns in first and second-generation animals of this model. Additional epigenetic tests could include the assessment of histone modifications and protein acetylation occurring after HI. These tests could confirm the presence of epigenetic influence on the inheritance of behavioral deficits observed in the second generation. Undoubtedly, assessing the epigenetic effects of prenatal in utero exposure also holds merit. Lastly, a clinically significant direction to investigate would be to assess how hyperoxia therapy or hypothermia therapy contribute to the reversal of the epigenetic inheritance of behavioral deficits observed after HI.

Chapter 5: Conclusions and Future Directions

Neonatal HI contributes to a vast amount of infant morbidity and mortality. Understanding the mechanisms that result in the detrimental effects of this injury are crucial. From our studies, we conclude that 1. Bax intraorganelle translocation and oligomerization is associated with changes in phosphorylation and 2. Neonatal HI results in significant behavioral deficits that are passed on to the next generation.

BAX

We found that Bax phosphorylation at Thr167 is associated with translocation to the nucleus, in both our *in vivo* and *in vitro* models of HI injury. We also found that Ser163 is dephosphorylated at late timepoints after HI. Until now, dephosphorylation of Bax has only been studied at the Ser184 phosphorylation site. Investigating the dephosphorylation of Ser163 may help elucidate its function in cell death signaling after HI.

Our results strongly indicate a relationship between phosphorylation patterns and localization. However, confirming that phosphorylation is *required* for translocation will ultimately require further analysis. This may include studying the effects of inhibiting kinase activity to observe the effects on Bax localization. However, kinases are quite promiscuous and are required for cell death signaling at many other steps. For example, two main kinases that have opposite effects on Bax activity, AKT and GSK3β, in fact interact with each other. AKT is known to phosphorylate and inactivate GSK3β in multiple cell signaling pathways (Song et al., 2009). JNK is known to phosphorylate

numerous proteins that interact with Bax, such as 14-3-3. Therefore, inhibition of kinases may not be the most specific method of studying Bax activity.

Instead, an excellent method for investigating Bax translocation patterns is that of transfection with GFP-tagged mutants, where the phosphorylation sites are replaced with alanine or glutamate to inhibit or mimic Bax phosphorylation, respectively. Future directions for this project must include the assessment of Bax localization within cells when phosphorylation is tightly controlled. If, in fact, the GFP-Bax mutants were to confirm the necessity for phosphorylation in translocation to different organelles after HI, a new experiment presents itself. At this point, an Oxygen-Glucose Deprivation model or the rotenone injury model could be used to assess whether cell death can be controlled by Bax mutants.

Bcl-2 homologous antagonist/killer, or Bak, is another cell death signaling protein. Bak is known to have an extremely close homology to Bax (Antignani and Youle, 2006, Fletcher and Huang, 2008, Karbowski et al., 2006, Mikhailov et al., 2003). In fact, in many injury paradigms, the presence of both proteins is said to form redundancy in cell death signaling. However, far less is known about Bak activity than Bax other than the fact that Bax and Bak do interact on the mitochondrial membrane and Bak contributes to mitochondrial outer membrane permeability (Karbowski et al., 2006, Kim et al., 2009). Therefore, it would be interesting to study the interactions between Bax and Bak after HI, and whether inhibition of one or the other is enough to halt cell death signaling after HI.

We found that Bax forms higher order oligomers after HI, therefore we assessed the influence of phosphorylation on these oligomers via AFM. AFM suggested that association with the size of Bax oligomers depends on whether Bax is interacting with JNK or GSK3β, indicating that each kinase affects Bax conformation differently. In addition, Bax with c-terminal deletion has been found to still produced apoptotic signaling, but as a monomer (Yu et al., 2008, Er et al., 2007). Therefore, the c-terminal phosphorylation may be involved in stabilization of Bax oligomers and exacerbation of cell death signaling.

Another area of interest is the function of Bax within the nucleus. Despite years of evidence, the presence of Bax at the nuclear membrane as well as within the nucleus is only recently becoming more accepted. However, the function of Bax *within* the nucleus is completely unknown. Bax-P53 complexes have been found in a number of injury paradigms, however, the function of Bax is still unclear.

LONG-TERM EFFECTS OF HI

In the second portion of our studies, we examined the long-term effects of HI and found significant impairment in pups whose parents were affected by HI. Moreover, males appeared to be more susceptible to the generational transfer of behavioral deficits when compared to females of similar background.

Certainly, the next logical step in our behavior assays is to determine the extent of epigenetic changes after HI. To start, global methylation and hypomethylation pattern modifications could be evaluated in first and second generation animals. A potential future experiment could include using Bax -/- animals to observe how lack of Bax affects

behavior after HI both in the first and second generations. The results could elucidate a further understanding of the link between Bax and epigenetic modifications occurring after HI.

As hyperoxia therapy has been found to exacerbate the injuries observed after HI, future directions could also include investigation of the effects of hyperoxia therapy on the transfer of behavioral consequences to the next generation using the behavioral analyses described in Chapter 4.

List of Abbreviations

AFM	Atomic Force Microscopy
Bax	Bcl-2 Associated X protein
Bcl-2	B-cell lymphoma 2
DDM	n-Dodecyl β D-Maltoside
GSK-3β	Glycogen Synthase Kinase 3 β
HI	Hypoxia-Ischemia
JNK	c-Jun N-terminal Kinase (MAPK8)

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Vita

Smitha Krishnan Infante was born in 1984 in Bangalore, India. At 8 years of age, she, her parents, and her brother relocated to the United States. In August 2002, Smitha joined Georgia State University to obtain her Bachelors degree and pursue a dental degree. Her interest for neuroscience research was born from her student laboratory position in the lab of Dr. Donald Edwards, under the supervision of Dr. Brian Antonsen in 2004. In May of 2006, Smitha obtained her BS and began work as a research assistant in Dr. Krishna Bhat's lab, studying neurodevelopment in drosophila. She then relocated with Dr. Bhat to Galveston, Texas, where she eventually joined graduate school at the University of Texas Medical Branch. While at UTMB, Smitha pursued a graduate degree in Cell Biology and was awarded the NIEHS Predoctoral Training Grant for two years. The training grant, as well as opportunities at UTMB, led Smitha to participate in a number of extracurricular activities and committees. She was also able to teach an undergraduate course in toxicology for four years. During her stay in Galveston, Smitha met the love of her life, Ernesto Infante Jr. In January of 2011, they were married in a proper South Indian wedding ceremony set in her birthplace, and once again in May 2011 in Brownsville, TX. Smitha plans to pursue a career in risk assessment and toxicology.

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Education

Bachelor of Science – Biological Science, 2002, Georgia State University

Publications

Bhat KM, Gaziova I, and **Krishnan S**. Regulation of axon guidance by slit and netrin signaling in the Drosophila ventral nerve cord. Genetics. 2007 Aug; 176(4):2235-46.

Abstracts

Mar 2012	**Society of Toxicology**; San Francisco, CA "Bax phosphorylation and multi-organelle translocation affects cell death in the brain after neonatal hypoxia-ischemia"
Jan 2012	**International Journal of Developmental Neuroscience**; Mumbai, India "Genotoxic consequences of hypoxia ischemia and oxygen resuscitation in a rat model of perinatal ischemia"
Dec 2011	**Mission Connect Symposium**; Houston, TX "Bax phosphorylation and multi-organelle translocation affects cell death in the brain after neonatal hypoxia-ischemia"
Oct 2011	**Society for Neuroscience, Galveston Chapter Symposium**; Galveston, TX "Neonatal Hypoxia-ischemia Triggers Bax Phosphorylation and Translocation"
Mar 2011	**Society of Toxicology**; Washington, DC "Hypoxia-Ischemia Triggers Bax Phosphorylation, Translocation, and Resultant Cell Death Phenotype"
Mar 2010	**Society of Toxicology**; Salt Lake City, UT "Intracellular Bax Trafficking: A Determinant of Cell Death?"
Dec 2009	; "Intracellular Bax Trafficking: A Determinant of Cell Death?" **Mission Connect Symposium** Houston, TX

This dissertation was typed by Smitha Krishnan Infante