DISSERTATION

GLUCOCORTICOIDS EXACERBATE HYPOXIA INDUCED NEURONAL DEATH IN THE DEVELOPING RAT CORTEX THROUGH REGULATION OF THE PRO-APOPTOTIC GENE BNIP3

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY URSULA SUSAN SANDAU ENTITILED: GLUCOCORTICOIDS EXACERBATE HYPOXIA INDUCED NEURONAL DEATH IN THE DEVELOPING RAT CORTEX THROUGH REGULATION OF THE PRO-APOPTOTIC GENE BNIP3 BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

GLUCOCORTICOIDS EXACERBATE HYPOXIA INDUCED NEURONAL DEATH IN THE DEVELOPING RAT CORTEX THROUGH REGULATION OF THE PRO-APOPTOTIC GENE BNIP3

Dexamethasone (DEX) is a synthetic glucocorticoid that is prescribed to treat premature infants with bronchopulmonary dysplasia. However, glucocorticoids exacerbate metabolic insults, such as hypoxia-ischemia, in the rat brain. Furthermore, DEX, acting independent of a secondary insult, induces apoptosis in the developing rat cortex and hippocampus. In the adult rat hippocampus, DEX-induced apoptosis is attributed to Bcl-2 family member regulation, but in the developing brain the putative effector proteins for DEX-mediated apoptosis have yet to be identified.

Bnip3 is a pro-apoptotic Bcl-2 family member that is upregulated in the mature rat brain during hypoxia-ischemia. Since Bnip3 is a hypoxic responsive gene, the experiments in this dissertation investigate Bnip3 as a putative effector protein for glucocorticoid exacerbation of hypoxic insults in the developing rat cortex. In chapter 3, I determined Bnip3 localization and mRNA ontogeny in the postnatal rat brain to identify brain regions and ages that may be susceptible to hypoxic insults. Bnip3 mRNA was localized to the neonatal cortex and hippocampus. Furthermore, Bnip3 mRNA levels were found to be greatest at postnatal day 6.5 in the female anterior and

posterior cingulate cortices and hippocampus. Conversely, in the male brain Bnip3 mRNA was only increased in the anterior cingulate cortex and this was at postnatal day 6.5.

In chapter 4, I subsequently investigated glucocorticoid and hypoxia interactions of Bnip3 mRNA regulation. I found that Bnip3 mRNA expression was increased in neonatal rat pups treated with DEX. An increase in Bnip3 mRNA was also measured in primary cortical neurons 72 hours after treatment with RU28362, a glucocorticoid receptor selective agonist. In cortical neurons, hypoxia increased Bnip3 mRNA expression and this was exacerbated with RU28362 treatment. This treatment paradigm also corresponded to decreased cell viability. Glucocorticoid and hypoxia regulation of the Bnip3 promoter was found to be mediated by a glucocorticoid response element and hypoxic response element. Finally, Bnip3 over-expression in cortical neurons increased cell death. This effect was eliminated when the Bnip3 transmembrane domain was removed. Cumulatively, these studies identify a novel pathway in the developing cortex through which glucocorticoids may exacerbate a metabolic insult, such as hypoxia.

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Chapter 1

General Introduction

Infants born prior to 36 weeks of gestation are susceptible to developing the chronic lung disease bronchopulmonary dysplasia. Several lines of research report that hypoxia, which is a symptom of premature birth and bronchopulmonary dysplasia, results in abnormal human brain development. For example, premature infants diagnosed with bronchopulmonary dysplasia exhibit increased levels of cerebral cortical apoptosis and central nervous system (CNS) complications (Hargitai et al., 2001). Furthermore, prematurity and hypoxia-ischemia correlate with a higher incidence of behavioral disorders, such as schizophrenia (Jones and Cannon, 1998; Dalman et al., 1999) and attention deficit disorder (Krageloh-Mann et al., 1999).

Bronchopulmonary dysplasia is commonly treated with the synthetic glucocorticoid Dexamethasone (DEX) (Adams and Cory, 1998; Halliday, 1999). Furthermore, the DEX use in premature infants is correlated with a 34% increase in the incidence of cerebral palsy compared to children treated with saline (Shinwell et al., 2000). Infants that receive glucocorticoid treatment are also predisposed to developing neurological abnormalities, neuromotor dysfunction and schizophrenia in adulthood (Fitzhardinge et al., 1974; Gibson et al., 1993; Bos et al., 1998; Shrivastava et al., 2000; Koenig et al., 2002). Cumulatively, these clinical observations suggest

that glucocorticoids act synergistically with hypoxia to alter normal CNS development.

Studies utilizing both *in vivo* and *in vitro* approaches provide additional evidence that glucocorticoids act as neuroendangering compounds. Glucocorticoids administered to the adult rat causes a decrease in anterior cingulate cortex volume (Cerqueira et al., 2005). Furthermore, in the postnatal rat brain glucocorticoids decrease the number of cortical neurons (Kreider et al., 2006). Glucocorticoids also exacerbate infarcts attributed to secondary metabolic insults such as kainic acid and hypoxia-ischemia (Sapolsky and Pulsinelli, 1985; Sapolsky, 1986b; Roy and Sapolsky, 2003). *In vitro* studies also reveal that glucocorticoids modulate hypoxia-responsive gene expression (Kodama et al, 2003; Leonard et al, 2005)

The neuroendangering aspects of glucocorticoids are mediated by the glucocorticoid receptor (GR). Ligand activated GR predominantly regulates cellular function by either inducing or repressing transcription of glucocorticoid responsive genes (Becker et al., 1986; Wright et al., 1993). For example, activated GR modulates hypoxic responsive gene expression via transactivation of the hypoxia-regulated transcription factor, hypoxia inducible factor-1 (HIF1) (Kodama et al., 2003; Leonard et al., 2005). Studies also reveal that activated GR influences the onset of apoptosis by regulating pro- and anti-apoptotic Bcl-2 family member expression.

Apoptosis is the genetically regulated mechanism employed by the CNS to eliminate cells either during the normal developmental process or in response to injuries, such as hypoxia and glucocorticoid treatment. The Bcl-2 gene family serves as the mediators for apoptosis and is composed of genes that function in either a pro-

or anti-apoptotic fashion. The pro-apoptotic members are further classified into two subfamilies based on the Bcl-2 homology (BH) domains possessed. Bax, Bak, and Bok posses multiple BH domains and characteristically act as the effectors to mitochondria membrane disruption. The remaining pro-apoptotic members are in the Bcl-2 homology domain 3 (BH3)-only subfamily (Adams and Cory, 1998). Typically, the BH3-only members initiate cell death by responding to an apoptotic stimulus and subsequently interacting with either a multi-domain member or anti-apoptotic member (Yin et al., 1994a; Cheng et al., 2001; Wei et al., 2001; Letai et al., 2002).

Bcl-2 and 19 kDa interacting protein-3 (Bnip3) is a unique pro-apoptotic member of the BH3-only subfamily. Bnip3 is classified into this family based on limited shared sequence homology of the BH3 and transmembrane (TM) domains (Boyd et al., 1994; Yasuda et al., 1998). Unlike the other BH3-only members, Bnip3 does not require the BH3 domain to induce apoptosis or to heterodimerize with either Bcl-2 or Bcl-X_L. Conversely, localization to the mitochondria, as well as heterodimerization of Bnip3 with Bcl-2 or Bcl-X_L and subsequent cell death are a result of the NH_2 terminus and TM domain (Chen et al., 1997a; Ray et al., 2000). Furthermore, over-expression of Bcl-2 and Bcl-X_L are unable to circumvent cell death induced by Bnip3 (Ray et al., 2000), suggesting that Bnip3 can act independently to induce apoptosis.

Bnip3 is also unique in that it is directly regulated by hypoxia through HIF1 binding to a hypoxic response element (HRE) within the Bnip3 promoter (Bruick, 2000). Furthermore, studies in the adult rat brain reveal that hypoxia induces cell death by regulating Bnip3 actions. In response to hypoxic-ischemic insult, Bnip3

protein levels are elevated in the adult rat hippocampus, cortex and striatum and this increase in Bnip3 expression closely parallels an increase in hypoxia-induced cell death in these regions (Schmidt-Kastner et al., 2004; Althaus et al., 2006). In the neonatal rat cortex, hypoxia also initiates apoptosis; however, the effector protein that mediates hypoxic-induced cell death in the postnatal brain has yet to be determined.

Bcl-2 family member recruitment and regulation governs the apoptotic process, but varies depending on the cell type, age, or the apoptotic stimulus. For example, studies report that glucocorticoids select for apoptosis in the juvenile and adult rat dentate gyrus by upregulating the pro-apoptotic protein Bax and downregulating the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Almeida et al., 2000; Crochemore et al., 2005). Conversely, DEX administered to the postnatal rat does not alter expression of either Bcl-2 or Bax mRNA and protein in the Ammon's horn pyramidal neurons and dentate gyrus granule cells (Tan et al., 2002) despite reports of DEX-induced cell death (Kreider et al., 2005). These results suggest different mechanisms of glucocorticoid neuroendangerment exist depending on the developmental period.

Based on these reports, I hypothesized that glucocorticoids exacerbate hypoxia induced cortical neuron death, thereby altering normal CNS development. Further, the glucocorticoid enhancement of hypoxia cell death is mediated by GR modulation of hypoxia induced Bnip3 expression. The two research chapters in this dissertation address this hypothesis, with chapter 3 characterizing Bnip3 expression and ontogeny in the developing rat brain and chapter 4 investigating glucocorticoid and hypoxia regulation of Bnip3 and cell death in the cortex.

Postnatal development of the rat CNS is characterized by a period of naturally occurring cell death (NOCD) in the cerebral cortex and hippocampus that peaks during the first postnatal week (Gould et al., 1991; Ferrer et al., 1994b; Spreafico et al., 1995). Despite these reports of NOCD in the postnatal rat brain, limited studies have been performed to determine the developmental ontogeny of potential effector proteins (Castren et al., 1994; Ferrer et al., 1994a; Vekrellis et al., 1997; Hamner et al., 1999; Rickman et al., 1999; Mooney and Miller, 2000; Groc et al., 2001; Vinet et al., 2002). Consequently, the experiments described in the first research chapter (chapter 3) address the localization and developmental ontogeny of Bnip3 mRNA in the anterior and posterior cingulate cortices and hippocampus of the postnatal male and female rat brain. The experiments identified a transient increase in Bnip3 mRNA expression on postnatal day (PND) 6.5 in the male and female anterior cingulate cortex. Furthermore, a transient increase in Bnip3 mRNA expression was also identified on PND 6.5 in the female posterior cingulate cortex and hippocampus, but not in the male. These experiments were performed in order to establish an expression profile for Bnip3 in the developing brain, which also correlates with the period of elevated NOCD. Furthermore, these studies also indicate regions and ages of the developing rat brain which may be susceptible to insults resulting in Bnip3 mediated cell death, such as hypoxia.

The second research chapter (chapter 4) describes studies, which first investigate glucocorticoid upregulation of Bnip3 mRNA expression in the postnatal male and female rat cortex and in primary cortical neurons. The subsequent experiments address the putative glucocorticoid exacerbation of hypoxia induced

Bnip3 mRNA expression in primary cortical neurons. The mechanism(s) governing the glucocorticoid and hypoxia regulation of Bnip3 transcription are also investigated using Bnip3 promoter reporter luciferase reporter constructs. The gene transcription studies indicate that Bnip3 is regulated by GR and hypoxia via a glucocorticoid response element (GRE) and HRE within the Bnip3 promoter, respectively. Glucocorticoids were found to exacerbate hypoxia induced Bnip3 mRNA expression; which also correlates with an increase in cell death. A final set of experiments establish that over-expression of Bnip3 independently induces primary cortical neuron death, which is dependent on the TM domain of the Bnip3 protein. Cumulatively these studies identify a novel mechanism by which glucocorticoids endanger the developing CNS.

Chapter 2

Literature Review

1. Mechanisms of Cell Death: Apoptosis, Autophagy and Necrosis

All living cells must undergo death, either as a result of injury or as a consequence of the natural aging process. However, the mechanisms of cell death vary depending on the dying cell's age and type as well as the initiating stimulus. Three categories of cell death were defined by Schweichel and Merker (1973) based on the morphological changes of the dying cell. Type I cell death, previously identified as apoptosis by Kerr et al., (1972), is characterized by chromatin condensation, DNA fragmentation and apoptotic body formation. Type II cell death or autophagy, has extensive cytoplasmic vacuoles that are lysosomal in nature (Schweichel and Merker, 1973; Yuan et al., 2003). Finally, type III cell death, necrosis, is characterized by the rapid swelling of intracellular organelles, such as the mitochondria, which results in plasma and nuclear membrane disruption (Schweichel and Merker, 1973; Syntichaki and Tavernarakis, 2002).

Unfortunately, the morphological features for the three cell death types are not clearly delineated; for example, nucleus pyknosis and membrane blebbing, both typical apoptosis characteristics, are also associated with end stage autophagy (Yuan et al., 2003). Additionally, cytoplasmic vacuole formation is present in both necrotic and autophagic cells (Yuan et al., 2003; Marino and Lopez-Otin, 2004). Therefore,

when identifying the mechanism of cell death both the morphological features and biochemical process should be considered. The following sections further define the morphological and biochemical properties associated with apoptosis, autophagy and necrosis; as well as the putative roles for each cell death type in the central nervous system (CNS).

Apoptosis

Apoptosis, also referred to as programmed cell death, has been extensively characterized. Apoptosis is a genetically regulated mechanism involving RNA and protein synthesis (Adams and Cory, 1998; Putcha et al., 2001). Activation of catalytic enzymes, such as caspase-3, -8 and-9, also occurs which leads to downstream events including DNA fragmentation and cell death (Reed, 2000). Apoptosis mediated cell death is characterized by morphological features such as chromatin fragmentation, nuclear condensation and pyknosis, swelling and fragmentation of the cytoplasm, apoptotic body formation and cell condensation (Kerr et al., 1972; Schweichel and Merker, 1973). Additionally, apoptotic cells do not burst during the cell death process (Kerr et al., 1972), but are instead phagocytized by neighboring cells (Duvall et al., 1985; Savill et al., 1993). Compared to necrosis, the apoptotic process is slow and potentially reversible. Additionally, apoptosis is an energy demanding process as adenosine triphosphate (ATP) is necessary for nuclear condensation and DNA fragmentation (Leist et al., 1997).

The Bcl-2 gene family: Mediators of apoptosis

There are two distinct apoptosis mechanisms that initiate cell death, the intrinsic and extrinsic pathways. Pro- and anti-apoptotic proteins that are Bcl-2 family

members act as mediators for both pathways. The intrinsic pathway is completely dependent on the Bcl-2 family members, while under certain circumstances the extrinsic pathway may circumvent these proteins. The Bcl-2 family is composed of a group of genes that function in either a pro- or anti-apoptotic fashion. However, all members of the Bcl-2 gene family share homology with at least one of the four conserved Bcl-2 homology (BH) domains (Table 1).

Bcl-2 is an anti-apoptotic Bcl-2 gene family member and encodes all four BH domains (Fig. 1). The other anti-apoptotic family members include the genes Bcl-X_L, Bcl-w, Boo, A1 and MCL-1, each of these genes possess at least BH1, 2 and 3 domains (Tsujimoto et al., 1985; Boise et al., 1993; Gibson et al., 1996; Adams and Cory, 1998, , 2001). The anti-apoptotic members act to stabilize the mitochondrial membrane and prevent the onset of apoptosis in the absence of a stimulus. The pro-apoptotic Bcl-2 family members are further divided into two sub-families based on the number of BH domains they possess. The members of one sub-family posses multiple Bcl-2 homology domains and include Bax, Bak and Bok. These members have the BH1, 2 and 3 domains (Adams and Cory, 2001). The second group possesses only the BH3 domain (hence their designation as BH3-only proteins) and includes members such as Bnip3, Bik, Bim, Bad and Bid (Boyd et al., 1994; Yang et al., 1995; Wang et al., 1996; O'Connor et al., 1998; Oda et al., 2000).

The BH domains possessed by the pro- and anti-apoptotic Bcl-2 gene family members typically mediates the proteins' actions. For a majority of the Bcl-2 family members the BH domains are sites of protein-protein interactions between the BH3-only members and the multi-domain members; as well as between the pro- and anti-

Table 1

Division of the Bcl-2 gene family members into pro- and anti-apoptotic subfamilies

Anti-apoptotic	Pro-apoptotic (multi-domain subfamily)	Pro-apoptotic (BH3-only subfamily)
Bcl-2	Bax	**Bnip3**
Bcl-X_L	Bak	Bim
Bcl-W	Bok	Bid
Boo		Bad
A1		Nix
MCL-1		Bik
		Puma
		Noxa

Pro- and anti-apoptotic Bcl-2 family member characterization. The anti-apoptotic members, which all possess at least BH domains 1, 2 and 3, are listed. The pro-apoptotic members are divided into the two subfamilies. The multi-domain members posses the BH domains 1, 2 and 3; while the BH3-only members posses BH domain 3. Bnip3 is the BH3-only pro-apoptotic Bcl-2 family member depicted in bold font.

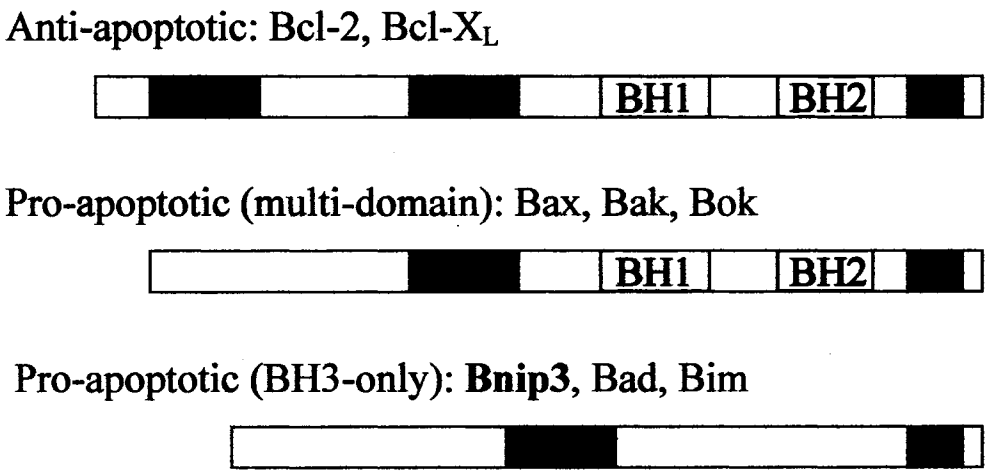


Figure 1. Schematic illustration depicting the Bcl-2 homology (BH) domains of the pro- and anti-apoptotic Bcl-2 family members. Upper illustration depicts the anti-apoptotic proteins, Bcl-2 and Bcl-XL, with the relative locations of the transmembrane (TM) and BH1, 2, 3 and 4 domains. Middle illustration depicts the pro-apoptotic multi-domain proteins Bax, Bak and Bok with the relative locations of the TM and BH1, 2 and 3 domains. Lower illustration depicts the pro-apoptotic BH3-only proteins Bnip3 and Bad with the relative locations of the TM and BH3 domains.

apoptotic Bcl-2 family members (Yin et al., 1994a; Muchmore et al., 1996). Additionally, the pro- or anti-apoptotic properties of the proteins are generally conferred by the BH domains. Even though each family member shares the conserved BH3 domain, the processes by which the pro-apoptotic members initiate cell death are not conserved. One typical mechanism by which apoptosis is initiated results from an interaction between a BH3-only and multi-domain member. During this process, the BH3-only member acts as the intermediate between an apoptotic stimulus and multi-domain member activation (Wei et al., 2001; Letai et al., 2002). The BH3-only member, initiates cell death by inserting the amphipathic α-helix BH3 domain into the hydrophobic cleft formed by the BH1, BH2 and BH3 domains of a multi-domain family member (Yin et al., 1994a; Muchmore et al., 1996; Sattler et al., 1997; Letai et al., 2002). The multi-domain member is subsequently activated, translocates to the mitochondrial membrane, heterodimerizes with an anti-apoptotic member and disrupts mitochondria function (Heimlich et al., 2004). Furthermore, studies indicate apoptosis initiated by a BH3-only member requires the downstream activation multi-domain member (Wei et al., 2001; Zong et al., 2001). For example, the direct interaction of the BH3-only proteins, Bim and Bad, with anti-apoptotic Bcl-2 fails to induce cell death in the absence of the multi-domain members, Bax and Bak (Zong et al., 2001). However, not all cell death mechanisms undergo this process. For example, the multi-domain member Bax can initiate apoptosis by forming a pore in the mitochondria membrane independent of heterodimerization with an anti-apoptotic member (Zha et al., 1996; Ishibashi et al., 1998; Nouraini et al., 2000). Additional studies reveal that the BH3-only members, Bad and Bik, indirectly induce apoptosis

by sequestering the anti-apoptotic Bcl-2 and Bcl-X_L proteins and enhancing a cell's susceptibility to cell death (Letai et al., 2002). Conversely, the anti-apoptotic members are capable of preventing cell death by sequestering the BH3-only members and preventing Bax and Bak activation (Cheng et al., 2001).

The mitochondria and apoptosis

The intrinsic and extrinsic apoptotic pathways are regulated by distinct mechanisms; however, mitochondrial function is disrupted in both. Putative alterations in mitochondrial function, which leads to cell death, include: loss of mitochondrial membrane potential, disruption of oxidative phosphorylation, electron transport chain collapse and generation of reactive oxygen species (Zamzami et al., 1996; Cai and Jones, 1998; Kroemer and Reed, 2000).

Mitochondria are organelles composed of two well defined spaces, the matrix and intermembrane space, which are demarcated by the inner and outer mitochondrial membranes. The inner membrane contains the proteins ATP synthase, adenine nucleotide translocator (ANT) and protein complexes that constitute the electron transport chain. These proteins produce the majority of a cell's ATP supply by shuttling protons across the inner membrane. Normal mitochondrial function is characterized by an electrochemical gradient present between the intermembrane space and matrix. The subsequent flow of protons down the electrochemical gradient mediates ATP synthase's conversion of ADP to ATP. An apoptotic stimulus can disrupt the inner and outer mitochondrial membranes, consequently collapsing the electrochemical gradient, releasing apoptogenic factors and inducing cell death.

Mitochondria membrane permeabilization (MMP) occurs through the disruption of either the outer or both the inner and outer mitochondrial membranes. The disruption of either membrane collapses the electrochemical gradient and releases the pro-apoptotic factors cytochrome c and apoptosis inducing factor. Three putative mechanisms of MMP have been described (Fig. 2). The first mechanism opens the mitochondria permeability transition (PT) pore, which spans both the inner and outer membranes and is permeant to molecules that are up to 1.5kD in size (Kroemer and Reed, 2000). The PT pore forms at places where voltage dependent anion channel (VDAC) and ANT interact, thus bringing the inner and outer membranes into close proximity (Adams and Cory, 2001). However, the PT pore can also open independent ANT (Kokoszka et al., 2004). The exact mechanisms that initiate PT pore opening are not yet clearly identified; however, pro-apoptotic Bcl-2 family member activation, a change in voltage across the electrochemical gradient, ANT ligands, caspases and cyclosporin A have all been implicated (Zamzami et al., 1996; Vander Heiden et al., 1999; Hengartner, 2000). Opening of the PT pore is followed by matrix swelling, electrochemical gradient loss and disintegration of the outer membrane (Kroemer and Reed, 2000). The second and third mechanisms do not directly involve the inner mitochondrial membrane. The second mechanism results from the interaction of pro-apoptotic Bcl-2 family members, such as Bax and Bak, with VDAC (Shimizu et al., 1999). In normal conditions VDAC is permeable to solutes up to 5 KD and is stabilized by the anti-apoptotic Bcl-2. However, Bax interaction with VDAC alters the channels properties and it becomes permeant to apoptotic factors such as cytochrome c. The final mechanism is the direct result of the

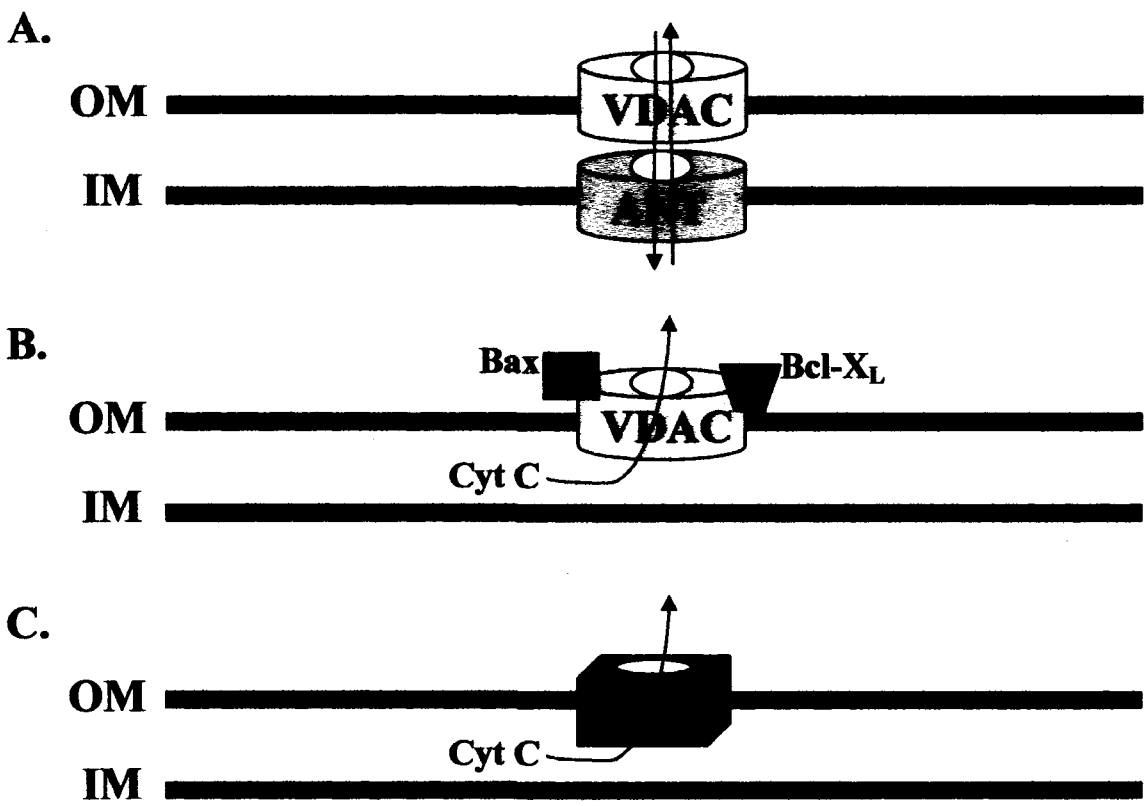


Figure 2. Schematic diagram illustrating the three putative mechanisms of MMP. (A) PT pore opening occurs at contact sites between the inner membrane (IM) and outer membrane (OM) and is mediated by VDAC and ANT interaction. PT pore opening causes matrix swelling and disrupts electrochemical gradient. (B) VDAC is opened by pro-apoptotic proteins such as Bax, which releases apoptosis inducing factors such as cytochrome c (cyt c) from intermembrane space. (C) Pro-apoptotic proteins directly disrupt the OM by forming a pore and releasing cyt c

pro-apoptotic members forming a pore in the outer membrane, causing selective outer membrane permeabilization and cytochrome c release (Eskes et al., 2000). Despite these differences in the mechanisms mitochondrial disruption is a component of apoptosis.

Intrinsic and extrinsic cell death pathways

The intrinsic pathway is initiated by an apoptotic stimulus that activates a pro-apoptotic Bcl-2 family member, disrupts the mitochondrial membrane and subsequently releases apoptogenic proteins from the intermembrane space (Zamzami et al., 1996). The Bcl-2 gene family is responsible for either initiating or preventing mitochondrial membrane disruption. Upon MMP, cytochrome c is released from the mitochondria, which interacts with apoptotic protease-activating factor-1 to form the apoptosome (LI et al, 1997). The apoptosome recruits the initiator caspase, procaspase-9, which is cleaved into active caspase-9. The activated caspase-9 cleaves the effector caspases -3, -6 and -7 to initiate apoptosis (Slee et al., 1999). Caspases are cysteine proteases that exist in the cytoplasm and induce apoptosis by specifically cleaving proteins after every aspartate residue (Hengartner, 2000). The effector caspases mediate downstream biochemical processes that result in the typical morphological features of apoptosis. For example, caspase-3 activates caspase-activated DNase by cleaving the proenzyme inhibitor of caspase-activated DNase. Activated caspase-activated DNase mediates DNA laddering (Enari et al., 1998), the characteristic feature of apoptosis.

An extracellular apoptotic stimulus initiates the extrinsic cell death pathway. Tumor necrosis factor receptor superfamily activation by an extracellular ligand, such

as tumor necrosis factor α, is one mechanism that initiates the pathway. Tumor necrosis factor α activation of tumor necrosis factor receptor type 1, also known as p55, recruits Fas-associated death domain protein (Chinnaiyan et al., 1995). Fas-associated death domain protein subsequently recruits and activates caspase-8, which is an upstream effector caspase that induces cell death either dependent or independent of the Bcl-2 family. Caspase-8 can directly activate the downstream caspases -3,-6, and -7 to induce cell death independent of the Bcl-2 family (Muzio, 1998; Muzio et al., 1998). Caspase-8 can also mediate a convergence of the extrinsic and intrinsic pathways by cleaving Bid to truncated Bid (Li et al., 1998). Bid is a BH3-only pro-apoptotic Bcl-2 family member that once cleaved activates the intrinsic pathway and subsequently induces MMP and cytochrome c release (Luo et al., 1998).

Apoptosis and the CNS

Apoptosis has been extensively studied in the brain and has implications in both normal CNS development and in some neuropathologies that afflict the developing and mature brain. Knockout mice have been used to investigate the putative roles of apoptosis associated proteins during mammalian CNS development. Elimination of select caspases by knockout results in abnormal brain development. For example, the caspase-9 knockout phenotype is embryonic lethal with severe malformations in the cortex (Kuida et al., 1995). Similarly, capsase-3 knockout causes cerebral abnormalities, but the animal lives for approximately 1 month (Kuida et al., 1995). Mice with knockouts of the pro- and anti-apoptotic Bcl-2 family members also have altered CNS development. An example can be demonstrated in Bcl-X_L knockout mice, which are similar to the caspase-9 knockout phenotype in that

both are embryonic lethal with altered CNS development. Additionally, excessive apoptosis occurs within the intermediate zone of the spinal cord and brainstem as well as in the dorsal root ganglion of Bcl-X_L deficient mice (Motoyama et al., 1995; Shindler et al., 1997; Lindsten et al., 2005). Furthermore, Bcl-X_L is an essential component of telencephalic neuron development (Roth et al., 1996; Shindler et al., 1997; Lindsten et al., 2005).

In addition to the role apoptosis plays during CNS development it has also been implicated as a mechanism of cell death associated with various neuropathologies. For example, Bcl-2 family member expression is altered in the hippocampus of epileptic rats, with elevated levels of the pro-apoptotic Bax and decreased levels of the anti-apoptotic Bcl-X_L (Akcali et al., 2005). Beta-amyloid induced neurodegeneration is also associated with apoptosis and the pathology is accompanied decreases expression of the anti-apoptotic protein Bcl-w (Zhu et al., 2004; Yao et al., 2005). Apoptosis has also been extensively studied as a putative mechanism for cell death following growth factor withdrawal (Garcia et al., 1992; Greenlund et al., 1995; Deckwerth et al., 1996; Putcha et al., 2001; Putcha et al., 2002) and hypoxic-ischemic insults to the CNS (Freeland et al., 2001; Gibson et al., 2001; Daval et al., 2004; Schmidt-Kastner et al., 2004; Althaus et al., 2006).

The mechanism of autophagic cell death

Autophagy is an evolutionarily conserved mechanism that is employed by yeast and multicellular organisms to accommodate extracellular stressors, such as hypoxia and nutrient deprivation (Yuan et al., 2003; Marino and Lopez-Otin, 2004; Mizushima et al., 2004). Multicellular organisms also use autophagy for the removal

of damaged organelles and during development (Gorski et al., 2003; Marino and Lopez-Otin, 2004). Autophagy is an active process that requires ATP as an energy source and kinase/phosphatase signaling pathways to induce caspase-independent cell death (Marino and Lopez-Otin, 2004). The formation of a double membrane vesicle, referred to as an autophagosome, initiates autophagy. Cytoplasmic contents, which may include entire organelles, are sequestered into the autophagosome. The autophagosome subsequently fuses with a lysosomal vacuole and is degraded by acid lysosomal/vacuole hydrolases (Marino and Lopez-Otin, 2004; Yoshimori, 2004). This catabolic process prolongs cell survival during adverse conditions by generating energy and destroying damaged organelles; however, autophagy can result in cell death (Lum et al., 2005).

Autophagy and the CNS

An essential component of mammalian CNS development is the induction of naturally occurring cell death (NOCD) following growth factor withdrawal. A surge of recent studies implicate autophagy as a mediator for growth factor withdrawal induced cell death (Xue et al., 1999; Yu et al., 2003; Florez-McClure et al., 2004; Gu et al., 2004). Lum et al, (2005) reported that growth factor induced cell death occur independent of apoptosis via autophagy. Furthermore, cells generated from Bax/Bak deficient mice and subjected to growth factor withdrawal are eliminated by autophagic cell death mechanisms if growth factors are not restored (Lum et al., 2005).

Autophagy is also a putative mediator of numerous neuropathologies. In the neonatal rat hippocampus a hypoxic-ischemic insult induces a novel type of delayed

cell death that is not morphologically similar to either apoptosis or necrosis. Additionally the dying hypoxic hippocampal cells have condensed nuclei, but the chromatin is clumped not fragmented. Cytoplasmic vacuoles are also present; however, the nature of these vacuoles, as to whether or not they are lysosomal, has not been elucidated (Fukuda et al., 1999; Sheldon et al., 2001). Autophagic cell death is also implicated as a process that regulates neurodegenerative diseases. Ultra-structural examination of the substantia nigra of Parkinson's disease patients has identified neurons undergoing autophagy (Anglade et al., 1997). Furthermore, abnormal lysosomal activity, resembling autophagy, occurs throughout the degenerative process of Alzheimer's disease (Cataldo et al., 1994). During Huntington's disease, the presence of accumulated misfolded Huntington protein also induces autophagic cell death (Kegel et al., 2000).

Necrotic cell death

Necrosis in an evolutionarily conserved process that both nematodes and mammals use when challenged with an extreme stressor. However, unlike apoptosis and autophagy, there are no specialized proteins that mediate necrosis (Syntichaki and Tavernarakis, 2002). The morphological characteristics associated with necrosis include mitochondria swelling, formation of cytoplasm vacuoles and dilation of the endoplasmic reticulum. Additionally, the nucleus of necrotic cells eventually disintegrates or undergoes karyolysis (Schweichel and Merker, 1973; Syntichaki and Tavernarakis, 2002). Necrosis culminates with cell lyses and subsequent release of its cytoplasmic contents into the intercellular space, which mounts an inflammation response capable of injuring neighboring cells (Majno and Joris, 1995).

Calcium acts as the common effector stimulus that initiates most types of necrotic cell death. Intracellular increases in Ca2+ result from either extracellular Ca2+ influx through voltage-, ligand- and receptor-gated channels (Sattler and Tymianski, 2000; Sattler et al., 2000) or through Ca2+ release from intracellular stores such as the endoplasmic reticulum (Mattson et al., 2000; Paschen, 2001; Paschen and Frandsen, 2001). The rise in intracellular Ca2+ levels results in a Ca2+ overload, which causes a secondary Ca2+ release from mitochondrial stores. The secondary release of Ca2+ is mediated by both the mitochondrial sodium/calcium exchanger and PT pore opening (Halestrap et al., 1998; Lemasters et al., 1998b; Lemasters et al., 1998a; Zhu et al., 2000).

Necrosis and the CNS

Hypoxia-ischemia and excitotoxicity are two insults that induce necrotic cell death in the CNS. Additionally, intracellular Ca2+ overload resulting in excitotoxicity is the underlying mediator of ischemia-induced necrosis (Ankarcrona et al., 1995; Miyamoto and Auer, 2000). The infarct associated with a hypoxic-ischemic insult results from both apoptosis and necrosis. A hypoxic-ischemic insult initially induces necrosis at the infarct's core. Conversely, in the penumbra delayed cell death or apoptosis occurs. Similarly, cerebellar granule neuron cultures subjected to glutamate over-stimulation have two phases of cell death. The first being a rapid necrotic response, while the second is delayed apoptosis (Ankarcrona et al., 1995). Finally, recent studies question if hypoxia-ischemia induced necrosis results from the hypoxic or ischemic component. Miyamoto and Auer (2000), report that ischemia,

but not hypoxia, induces extensive necrosis in the mature rat brain. However, hypoxia exacerbates the ischemic insult by 8% (Miyamoto and Auer, 2000).

2. Naturally Occurring Cell Death in the Developing CNS

A hallmark of peripheral nervous system (PNS) and CNS development is the genesis of excess undifferentiated progenitor and differentiated neuronal and glial cells than are present in the mature organism (Heumann and Leuba, 1983; Gould et al., 1991; Ferrer et al., 1994b; Ferrer et al., 1994a; Spreafico et al., 1995). It is estimated that during CNS development an extra 20 to 80% of cells are born (Blaschke et al., 1996; Thomaidou et al., 1997). These excess cells are removed by a process of naturally occurring cell death (NOCD) to ensure normal development occurs (Gould et al., 1991; Oppenheim, 1991; Ferrer et al., 1994b; Spreafico et al., 1995). Neurons that fail to make the appropriate synaptic connections are fated to initiate an active mechanism of cell death. Synapse outgrowth and synaptic connections are mediated by neurotrophin actions and their respective tyrosine kinase receptors. The withdrawal of growth factors, as a result of inappropriate connectivity, serves as a death initiating signal (Levi-Montalcini and Booker, 1960; Johnson et al., 1978; Sanes et al., 2000). However, the specific growth factors and downstream death effector proteins responsible for mediating cell death are not conserved across all cell types. Additionally, the elimination of extra cells was historically attributed to apoptosis; however, a few reports insinuate that autophagy may also play a role in this process. Regardless of the intracellular mechanism, tight regulation of NOCD is critical for normal development of the CNS.

Overview of neurogenesis in the developing CNS

Neurons and glia which comprise the mature cortex and hippocampus are derived from undifferentiated progenitor cells in an active state of mitosis. Actively dividing progenitor cells give rise to daughter cells that either remain as progenitor cells or become mature differentiated neurons or glia. The primary site of telencephalic neurogenesis is the ventricular zone. There are also three additional regions of neurogenesis in the rodent brain: the subventricular zone, hippocampal granule cell precursors and external granule layer. The progenitor cells reside in either the ventricular zone or the three secondary sites of neurogenesis. Additionally, the progenitor cells of the subventricular zone or hippocampal granule cell precursors are derived from the ventricular zone progenitor cells. The ventricular and subventricular zones give rise to the neurons and glia of the mature cortex. Additionally, cortical cells generated during early rat embryogenesis (embryonic day (E) 11 to E15) arise from the ventricular zone progenitor cells; while late cortical development is mediated by the subventricular zone progenitor cells (Sanes et al., 2000; Campbell, 2005).

Excess neurogenesis: The removal of progenitor and differentiated cells

Normal CNS development is characterized by the genesis of excess progenitor and differentiated cells across multiple species including the chicken, rat, mouse and ferret (Heumann and Leuba, 1983; Gould et al., 1991; Ferrer et al., 1994b; Price et al., 1994; Spreafico et al., 1995; Johnson and Berman, 1996; Blaschke et al., 1998). The extra cells are eliminated during either embryogenesis or postnatal life in order to achieve normal CNS development.

During late embryogenesis the majority of dying cells in the rat cortex are located in the proliferative zones. The greatest levels of cell death occur from E12 to E18. Further, apoptosis peaks on E14 with death occurring in 70% of progenitor cells (Blaschke et al., 1996; Blaschke et al., 1998). As embryogenesis continues the percent of dying cells decreases within the ventricular zone (Blaschke et al., 1996; Thomaidou et al., 1997). Thomaidou et al., (1997) reports that apoptosis affects 1 in 14 progenitor cells within the ventricular zone of E16 rats, while Blaschke et al., (1996) measured up to 50% rate of cell death. Additionally, during later embryogenesis, E16-18, cell death begins to increase in postmitotic cells localized to the migratory zone, intermediate zone and cortical plate (Blaschke et al., 1996).

Levels of NOCD have also been measured in the postnatal rodent brain with a peak during the first postnatal week in the rat cortex and hippocampus (Gould et al., 1991; Ferrer et al., 1994b; Spreafico et al., 1995; Johnson and Berman, 1996; Thomaidou et al., 1997; Nunez et al., 2001). In the neonatal rat cortex, NOCD peaks at PND 5 in the postmitotic cells of the somatosensory cortex relative to the number of apoptotic cells in PND 1, 8, 14 and adult rats. Further, the transient increase in NOCD on PND 5 is 3 and 30 fold greater than the amount of cell death detected on PND 8 and 1, respectively (Spreafico et al., 1995). In the neonatal rat, high levels of cell death (60%) are also detectable in the subventricular zone progenitor cells (Thomaidou et al., 1997). Similar to the postnatal rat cortex, a transient peak in NOCD on PND 6 has been measured in the dentate gyrus region of the rat hippocampus (Gould et al., 1991). The periods of NOCD in the postnatal brain suggest that cell death is an essential component to normal development.

The sex of the animal also influences the amount and duration of NOCD in the postnatal cortex. Female rats have a prolonged period of NOCD from PND 7-11 in the primary visual cortex. Conversely, male rats have a transient peak of NOCD on PND 7 that rapidly declines. The prolonged period of NOCD in the female results in an overall increase in cell death within the primary visual cortex (Nunez et al., 2001).

Normal brain development is contingent on the removal of extra progenitor and differentiated cells. Preventing NOCD by over-expression of the anti-apoptotic protein Bcl-2 subsequently causes an enlarged brain and optic nerves relative to wild type mice (Porciatti et al., 1999). Additionally, mice deficient in the pro-apoptotic proteins Bax and Bak have increased brain size and develop abnormal behaviors. Also, eliminating these two proteins results in 90% lethality of the animals with this genotype. Bax and Bak deficient mice that do survive into adulthood are unresponsive to auditory stimuli, display circling behavior when exposed to an external stressor and are susceptible to developing seizures (Lindsten et al., 2000).

Apoptosis involvement in NOCD

Apoptosis has been implicated as the primary mechanism that regulates NOCD during CNS development. Extensive studies examining the necessity of the intrinsic and extrinsic pathways for this process have been conducted. The developmental ontogeny for a select few pro- and anti-apoptotic Bcl-2 family members has been established in various brain regions. The time-course of expression appears to indicate that some members act as mediators of NOCD (Castren et al., 1994; Ferrer et al., 1994a; Hamner et al., 1999; Rickman et al., 1999; Mooney and Miller, 2000; Groc et al., 2001; Krajewska et al., 2002).

The ontogenys' of the Bcl-2 family members have been described for rat embryogenesis and postnatal development in order to identify putative mediators of NOCD. Elevated levels of Bcl-2 protein are detectable during late embryogenesis in the rat cortex, with a peak at E16 (Mooney and Miller, 2000). Bcl-2 protein levels remain elevated throughout the first postnatal week then subsequently decline until low adult levels are reached (Ferrer et al., 1994b; Mooney and Miller, 2000). The postnatal hippocampus has a similar expression pattern with elevated Bcl-2 protein levels measured during early postnatal life, which decline during the second postnatal week (Ferrer et al., 1994a). Bcl-X_L expression closely resembles Bcl-2 expression with high levels detectable during late gestation and early postnatal life. Elevated levels of Bcl-X_L subsequently decline to adult levels by PND 6 in both the cortex and hippocampus (Hamner et al., 1999). Multiple studies have also investigated the ontogeny for Bax; however, the data is conflicting. Mooney and Miller, (2000) report no change in Bax protein levels during late embryonic and postnatal cortical development when the data is normalized to total protein content. However, when the data is normalized to DNA content Bax protein levels from PND 0 through adulthood are quadruple the embryonic levels (Mooney and Miller, 2000). Vekrellis et al., (1997) report a different finding with high levels of Bax protein being maintained throughout the second postnatal week, which then decline to low adult levels.

The ontogeny of Bcl-2, Bcl-X_L, Bax, Bak and Bid proteins has also been characterized during both peri- and postnatal mouse CNS development (Krajewska et al., 2002). In the mouse brain, the highest levels of NOCD are at PND 3 in the hippocampus (Resnikov, 1982). However, the ontogeny for the pro-apoptotic

members Bax, Bak and Bid indicate that peak expression is during late embryogenesis from E11 through 15, prior to postnatal NOCD. Similar to the pro-apoptotic members, Bcl-2 expression is also greatest during embryonic development. Conversely, Bcl-X_L expression in cortical and CA3 pyramidal neurons is high in the postnatal and juvenile mouse brain (Krajewska et al., 2002).

Cumulatively, the developmental ontogenys described in the developing rodent brain implicates Bcl-2, Bcl-X_L and Bax as putative mediators of NOCD. This assumption is based on the observed decrease in Bcl-2 and Bcl-X_L expression and increase in Bax expression during the first postnatal week which corresponds with the increase in NOCD. However, these are merely correlative observations. To elucidate the proteins necessary for NOCD, transgenic mice with selective knockouts of the Bcl-2 gene family or caspases have been created (Knudson et al., 1995; Motoyama et al., 1995; Yang et al., 1995; Roth et al., 1996; Shindler et al., 1997; Kuida et al., 1998; Lindsten et al., 2000; Oppenheim et al., 2001; Zaidi et al., 2001; Lindsten et al., 2003).

Targeted deletion studies of the Bcl-2 gene family indicate that Bcl-X_L is the only member that independently has a profound affect on normal CNS development. The phenotype associated with mice deficient in Bcl-X_L (Bcl-X_L-/-) is embryonic lethal with death resulting at E13.5 and accompanied by excessive apoptosis in the CNS. However, the lethality of the mutation cannot be attributed to abnormal brain development alone as extensive apoptosis was also measured in immature haematopoietic cells. In the developing CNS, apoptosis was measured in regions that contained differentiated neurons and not proliferating neurons of the ventricular zones

(Motoyama et al., 1995). Further, telencephalic cultures generated from Bcl-X_L-/- mice do not appropriately mature or survive and have a 30% increase in apoptosis compared to cultures generated from wild type mice (Roth et al., 1996). These data implicate Bcl-X_L as a mediator of NOCD in differentiated cells but not progenitor cells.

Knockouts of the pro-apoptotic Bcl-2 family members have also been generated to determine the effector proteins of NOCD. Observations in mice lacking in the pro-apoptotic Bax, Bak or Bid family members indicate that these proteins do not act independently in the global regulation of CNS development. However, Bax-/- mice do have decreased levels of apoptosis in select cell types during postnatal development. This is most evident in the CA2 and CA3 regions of the hippocampus on PND 3, which is also the peak of NOCD in the mouse brain (White et al., 1998). An increase in cerebellar granule neurons, but not purkinje neurons, has also been observed in Bax -/- mice (Fan et al., 2001). Despite these observations knockout of a single pro-apoptotic member does not globally alter normal brain development.

Analysis of brain development in dual knockout mice indicates that redundancy exist in the pro-apoptotic effector proteins of NOCD. A profound affect on CNS development occurs in mice deficient in both Bax and Bak (Bax-/-Bak-/-). The Bax-/-Bak-/- dual knockout is predominantly lethal; however, mice that survive to adulthood are unresponsive to auditory stimuli, display circling behavior when exposed to an external stressor and are susceptible to developing seizures (Lindsten et al., 2000). Additionally, the brains of Bax-/-Bak-/- mice were larger than wild type, Bax-/-, and Bak-/- brains. Histological analysis of Bax-/-Bak-/- brain tissue also

identified excess neural progenitor cells and differentiated neurons and glia in the periventricular zone compared to wild type, Bax-/- and Bak-/- brains (Lindsten et al., 2000; Lindsten et al., 2003). These data indicate that there is a redundancy in the pro-apoptotic actions of the multi-domain members Bax and Bak during normal brain development.

Since studies reveal that Bax and Bak activity is dependent on the actions of a BH3-only member (Wei et al., 2001; Letai et al., 2002), the possibility exists that during development Bax and Bak respond to different BH3-only members in order to differentially regulate NOCD in select brain regions at select ages. However, disruption of the BH3-only pro-apoptotic members has been limited to Bid, which does not alter CNS development (Yin et al., 1999). Additional studies investigating the putative contribution of other BH3-only members in CNS development are necessary.

Regulation of NOCD by growth factor withdrawal.

The elegant studies of Levi-Montacelli identified diffusible growth factors as mediators for synapse outgrowth and neuron survival. Since then extensive studies have begun to elucidate growth factor regulation of cell death (Levi-Montalcini and Cohen, 1956; Levi-Montalcini and Booker, 1960; Johnson et al., 1978; Sanes et al., 2000). Growth factors can regulate both the activation and transcription of the Bcl-2 gene family through the second messenger phosphatidylinositol-3-OH kinase (PI3K)-Akt (protein kinase A) pathway. The PI3K-Akt pathway is initiated by growth factor recruitment of PI3K to the plasma membrane. PI3K is cleaved into phosphoinositide phosphate (PIP)2 and PIP3, which in turn activates Akt via phosphorylation

(Vanhaesebroeck and Alessi, 2000). PI3K-Akt pathway activation leads to the subsequent phosphorylation of the transcription factors: forkhead box transcription factor, class O (FOXO), p53 and nuclear factor-κB (NF-κB) (Brunet et al., 1999; Ozes et al., 1999; Romashkova and Makarov, 1999; Yamaguchi et al., 2001). FOXO and p53 phosphorylation sequesters the transcription factors to the cytoplasm and prevents their ability to express the pro-death proteins Bim and Bax (Brunet et al., 1999; Dijkers et al., 2000). Conversely, activation of NF-κB leads to gene transcription of the pro-survival factor Bcl-X_L (Bui et al., 2001).

The contributions of the individual pro- and anti-apoptotic Bcl-2 genes during growth factor mediated cell death been investigated. A majority of the studies have been conducted in sympathetic ganglion neurons, either over-expressing or deficient in a Bcl-2 family member, and subjected to nerve growth factor (NGF) withdrawal (Garcia et al., 1992; Greenlund et al., 1995; Deckwerth et al., 1996; Miller et al., 1997; Putcha et al., 2001; Putcha et al., 2002). Over-expression of the anti-apoptotic members Bcl-2 and Bcl-X_L protect sympathetic neurons following NGF removal (Garcia et al., 1992; Frankowski et al., 1995). Additionally, sympathetic ganglion neurons deficient in the multi-domain pro-apoptotic member Bax are resistant to NGF withdrawal (Deckwerth et al., 1996). Conversely, sympathetic neurons generated from mice deficient in the multi-domain pro-apoptotic member Bak are susceptible to growth factor withdrawal (Putcha et al., 2002). Studies have also been performed to examine which BH3-only members are integral to growth factor withdrawal induced cell death. Cells generated from Bad and Bid deficient mice did not confer protection; while Bim deficient cells were protected from NGF withdrawal (Putcha et al., 2001;

Putcha et al., 2002). Thus, these studies indicate that select members of the Bcl-2 gene family are mediators of growth factor withdrawal induced cell death. Therefore, future studies investigating apoptosis following growth factor withdrawal in the CNS should investigate multiple Bcl-2 family members.

Investigation into growth factor regulation of apoptosis has been conducted in the CNS (Gage et al., 1988; Chrysis et al., 2001). In transgenic mice over-expressing insulin-like growth factor-1 (IGF-1) there is an increase in cortical and hippocampal neurons (Popken et al., 2004). A decrease in apoptosis was also measured on PND 7 in the cerebellum of mice that over-express IGF-1. Further, elevated levels of Bcl-2 and Bcl-X_L exist in the cerebellum of mice that over-express IGF-1. Conversely, these animals have no detectable change in either Bax or Bad expression (Chrysis et al., 2001). Regardless of these measured changes in Bcl-2 family expression these studies have not definitively addressed the effector proteins of IGF-1 survival in the CNS.

Autophagy and NOCD

A majority of research investigating growth factor withdrawal induced cell death have focused on apoptosis. However, there is also evidence that autophagy may mediate this process (Fombonne et al., 2004; Lum et al., 2005). Xue et al., (1999) characterized sympathetic neuron death following NGF withdrawal as autophagic. This assessment is based on the presence of numerous cytoplasmic vacuoles and autophagosomes. Additionally, concomitant administration of the autophagy inhibiting factor 3-methyladenine during NGF withdrawal delays cell death (Xue et al., 1999). Additionally, immortalized bone marrow cells derived from Bax-/-Bak-/-

mice succumb to autophagic cell death following growth factor withdrawal (Lum et al., 2005).

3. Hypoxia and the CNS

Oxygen is an essential component of mammalian life as it serves as the final electron acceptor in the electron transport chain. A cellular environment void of oxygen prevents oxidative phosphorylation and the subsequent synthesis of 38moles of ATP per 1mole of D-glucose. The inability to produce ATP generated during aerobic respiration metabolically endangers a cell (Devlin, 1997). However, during an acute hypoxic insult a cell can accommodate low oxygen levels by generating ATP through anaerobic glycolysis (Vannucci et al., 2005). During an acute hypoxic insult the expression levels of glycolytic enzymes and glucose transporters are also upregulated to restore oxygen homeostasis (Xia et al., 1995; Royer et al., 2000; Greijer et al., 2005; Vannucci et al., 2005; Mense et al., 2006; Vega et al., 2006). However, if hypoxia is prolonged and the metabolic insult is too great for the cell; death will occur. The cellular response to chronic hypoxia is predominantly mediated by hypoxia inducible factor 1(HIF1) (Greijer et al., 2005). Neurons are especially susceptible to injury associated with hypoxic insults as large amounts of ATP are necessary to sustain cell function and viability. As a result an extensive body of research investigating hypoxia-induced cell death in the adult and developing CNS has been conducted.

HIF1 mediates the cellular response to hypoxia

A hypoxic insult initiates a series of events that either restores oxygen homeostasis or induces cell death (Greijer et al., 2005). A major mediator for the

cellular response to hypoxia is the HIF1 protein, a transcription factor in the basic helix-loop-helix (bHLH)-containing Per/ARNT/Sim (PAS) domain family (Wang et al., 1995). HIF1 is a heterodimeric protein that is composed of the two subunits HIF-1α and HIF-1β (Wang and Semenza, 1995). HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), can also bind aryl hydrocarbon receptor (AHR) (Reyes et al., 1992). HIF-1α is a novel protein, but is similar in function and structure to the drosophila transcription factor single-minded (Sim) and AHR (Wang et al., 1995).

Expression levels of HIF-1α are oxygen dependent; while ARNT is constitutively expressed and non-responsive to changes in oxygen levels (Fig. 3). Typically HIF-1α expression levels are regulated at the protein level; however, increases in HIF-1α mRNA have also been reported (Wang et al., 1995).Under normal air conditions, or normoxia, the HIF-1α protein is ubiquitinated in an oxygen dependent manner and is targeted for rapid proteasome degradation (Kamura et al., 2000; Ohh et al., 2000; Bruick and McKnight, 2001; Ivan and Kaelin, 2001; Jaakkola et al., 2001; Yu et al., 2001). Conversely, during hypoxia, HIF-1α protein is stabilized and translocated to the nucleus where it heterodimerizes with ARNT to form HIF1 (Wang and Semenza, 1995). HIF1 subsequently regulates transcription of hypoxic responsive genes through a hypoxic response element (HRE), which is an 8 basepair consensus sequence of 5'-TACGTGCT-3' (Semenza and Wang, 1992; Wang and Semenza, 1993a, 1993b). HIF1 transcriptional activity can also be modulated by transactivation with the transcription factors cAMP response element binding protein, steroid receptor coactivator-1 and GR (Kodama et al., 2003; Ruas et al., 2005). HIF1

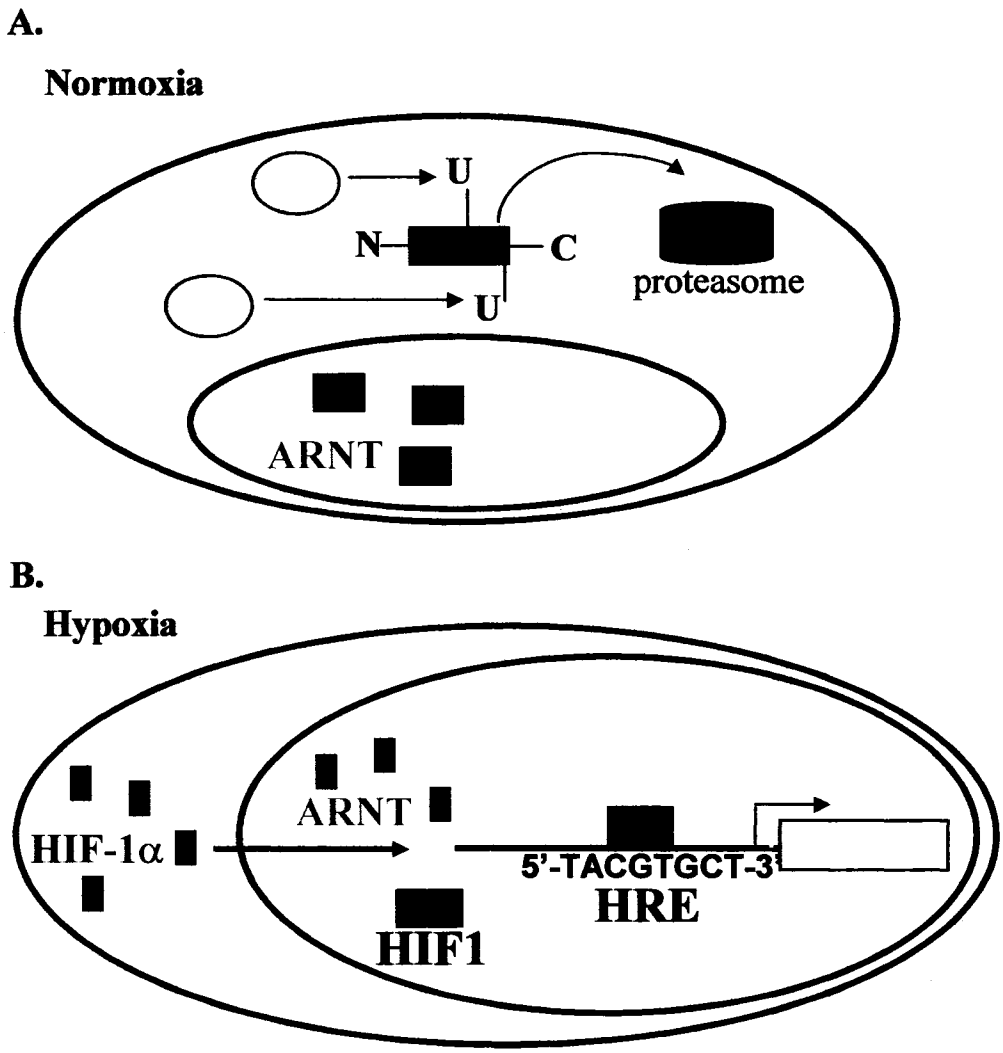


Figure 3. Schematic diagram illustrating hypoxic-regulation of HIF-1α. (A) During normoxia HIF-1α is targeted for degradation and ARNT is constitutively expressed. HIF-1α is ubiquitylation by HIF-1α prolyl hydroxylase (blue) and asparaginyl hydroxylase (yellow). The HIF-1α prolyl hydroxylase ubiquitylation targets HIF-1α for proteasome degradation and the asparaginyl hydroxylase ubiquitylation prevents transactivation. (B) During hypoxia HIF-1α is stabilized, translocates to the nucleus and heterodimerizes with ARNT to form HIF1. HIF1 regulates gene expression at HRE consensus sequences within the 5' UTR of hypoxic responsive genes.

transactivation is also oxygen dependent and mediated by two transactivation domains in the HIF-1α subunit. Normoxic oxygen levels suppress HIF1 transactivation and prevent coactivator interaction (Jiang et al., 1997; Pugh et al., 1997; Dann et al., 2002).

Hypoxia and cell survival

During a hypoxic insult, a cell initiates a series of events that either prolong survival or induce cell death. The survival response generates ATP under anaerobic conditions and also postpones the onset of apoptosis until oxygen homeostasis is returned. In order to generate ATP by glycolysis, an increase in expression of glycolytic enzymes, such as lactate dehydrogenase, and glucose transporters have been reported in the hypoxic brain (Zovein et al., 2004; Greijer et al., 2005; Vannucci et al., 2005; Mense et al., 2006; Vega et al., 2006).

Hypoxia-induced apoptosis is prevented by upregulating the anti-apoptotic protein Inhibitor of Apoptosis-2 (IAP-2). IAP-2 prevents Bax accumulation at the mitochondrial membrane and caspase activation (Dong et al., 2001; Dong et al., 2003). The actions of IAP-2 are presumed to be mediated by the second messenger pathway PI3K-Akt. Studies have shown that a hypoxic stressor activates the PI3K-Akt pathway, which prolongs cell survival (Alvarez-Tejado et al., 2001). Additionally, the transcription factors CREB and NF-κB are implicated as downstream targets of Akt mediated gene transcription (Du and Montminy, 1998; Beraud et al., 1999). Further, the IAP-2 promoter contains a functional CREB response element (CRE) that mediates hypoxia-induced IAP-2 gene transcription (Dong et al., 2001; Dong et al., 2002). IAP-2 is also upregulated by NF-κB (Stehlik et

al., 1998). Cumulatively, these studies implicate IAP-2 as a putative downstream target for PI3K-Akt mediated protection during hypoxia. However, the link between Akt activation and induction of IAP-2 transcription has yet to be established.

Hypoxia- induced cell death in the CNS

In the rat a majority of *in vivo* and *in vitro* studies focus on neuropathologies associated with oxygen-glucose deprivation. The predominant approach utilized for rodent *in vivo* studies is hypoxia-ischemia, a dual insult that results in both low oxygen and glucose levels (Towfighi et al., 1997). Hypoxia-ischemia closely resembles either a human stroke or birth asphyxia in the mature or developing CNS, respectively (Johnston, 1997). Hypoxia-ischemia causes a neural infarct with a necrotic core and apoptotic penumbra (Scott and Hegyi, 1997). However, additional studies reveal that autophagy is a component of hypoxic-ischemic-induced pathologies (Fukuda et al., 1999; Sheldon et al., 2001). Regardless of the cell death type, neonatal rats subjected to hypoxia-ischemia have extensive cell death within the hippocampus and cortex (Nakajima et al., 2000).

Hypoxic-ischemic-induced infarcts afflict multiple brain regions; however, in regard to the time it takes for the injury to occur, Wako et al., (2000) showed that the period of cell death varies between brain regions. In the neonatal hippocampus, cell death occurs for 72 hours following a hypoxic-ischemic insult; while cell death extends up to 7 days in the cortex. Additionally, a particular cell type within a brain region may also be more susceptible to hypoxic-ischemic insults. For example, in the postnatal neocortex a third of the apoptotic cells are oligodendrocyte progenitors

(Rothstein and Levison, 2005). These studies indicate that particular brain regions and cell populations can respond differently to the same insult.

Some studies have investigated hypoxia alone either *in vivo* or *in vitro* (Copin et al., 1996; Tamatani et al., 1998; Gozal et al., 2001; Schwartz et al., 2004). These have shown that a chronic hypoxic insult to the neonatal rat brain most closely resembles the hypoxic environment of the preterm human infant (Stewart et al., 1997; Ment et al., 1998). The *in vivo* model of chronic hypoxia entails postnatal rearing of rat pups from either PND 3 to 13 or PND 3 to 33 in 9.5% O_2. Rat pups reared in hypoxic conditions for either 10 or 30 days have decreased corpus collosum and cortical and subcortical white matter volumes (Stewart et al., 1997; Ment et al., 1998). The decrease in cortex volume also coincides with a decrease in cortical cell numbers. After either 10 or 30 days of hypoxia there is a 31-41% reduction in the total number of cortical glia. There is also a decrease in cortical neuron numbers by 14% after 30 days of hypoxic exposure (Schwartz et al., 2004). Additionally, pups reared in hypoxic conditions for 30 days have an overall decrease in brain growth and increase in ventricle size (Ment et al, 1998).

In vivo studies have also been conducted examining the affects of chronic and acute prenatal hypoxia on adult rat behavior. An acute prenatal hypoxic-insult decreases both the postnatal testosterone surge and corticosterone levels in male rat pups. Despite testosterone attenuation, an accompanied change in adult sexually dimorphic sex behaviors was not observed (Hermans et al., 1994). A separate study also found that acute prenatal hypoxia transiently increases ornithine decarboxylase levels in the developing rat cortex, hippocampus and cerebellum (Hermans et al.,

1992). The hypoxia-induced increases in ornithine decarboxylase levels result from free radicals generated during the insult (Saito et al., 1997). During normal brain development increased ornithine decarboxylase levels parallel brain growth and maturation (Bell and Slotkin, 1988). The authors also suggest that increases in ornithine decarboxylase may protect the brain from acute hypoxic-insults (Gilad and Gilad, 1991; Hermans et al., 1994). In contrast to an acute hypoxic-insult, a chronic prenatal hypoxic-insult does not change perinatal testosterone levels, but permanently alters male adult behavior. Furthermore, the change in behavior was specific to male sexual behaviors (Hermans et al., 1993).

In vitro models are also used to investigate hypoxia mediated cell death (Copin et al., 1996; Tamatani et al., 1998; Freeland et al., 2001; Jin et al., 2002). Hypoxic primary cortical neurons have increased levels of apoptosis levels compared to cultures maintained in ambient conditions (Copin et al., 1996; Tamatani et al., 1998; Jin et al., 2002). The increase in cell death can be seen within 6 hours after the insult by measuring lactate dehydrogenase (LDH). This increase in cell death is also accompanied by an increase in caspase-1 and caspase-3 activity at 12 and 18 hours, respectively. Furthermore, DNA fragmentation was detected at 12 and 18 hours. These data suggest that the early (<12 h) cell death is likely attributed to a non-apoptotic mechanism since caspases and DNA fragmentation are not involved. Conversely, later cell death (>12h) is likely apoptotic in nature (Tamatani et al., 1998). A separate study also shows that hypoxic primary cortical neurons, but not oxygen-glucose deprived neurons, have increased levels of DNA laddering. However, if cultures are treated concomitantly with hypoxia and cycloheximide or actinomycin-

D, DNA fragmentation, but not cell death, is prevented (Copin et al., 1996), indicating that autophagy can also be invoked.

Taken together, these various hypoxic-insult models indicate that hypoxia acting either independently or in conjunction with glucose deprivation injures the developing CNS. Furthermore, the hypoxia-induced increases in developmental CNS cell death are accompanied by behavioral disorders that extend into adulthood. Despite these observations studies have not determined the effector protein that mediates hypoxia-induced cell death in the developing brain.

The Bcl-2 gene family and CNS hypoxia

A sustained hypoxic insult that depletes ATP supplies induces apoptosis (Saikumar et al., 1998). The Bcl-2 gene family, mitochondrial dysfunction and caspase activation have all been extensively studied as putative mediators of hypoxia-induced apoptosis (Bruick, 2000; Gibson et al., 2001; Birse-Archbold et al., 2005; Althaus et al., 2006). Studies reveal that multiple pro-apoptotic members of the Bcl-2 family are involved in hypoxia-induced CNS apoptosis. The implicated pro-apoptotic members are Bax, Nix, Bad and Bnip3 (Saikumar et al., 1998; Gibson et al., 2001; Jin et al., 2002; Schmidt-Kastner et al., 2004; Birse-Archbold et al., 2005; Althaus et al., 2006).

Studies investigating the multi-domain member Bax, suggest that this protein has a definitive, yet potentially redundant role in hypoxia-ischemia induced apoptosis. Bax-/- mice subjected to hypoxia-ischemia are partially protected from the insult, but increased apoptosis is still detectable within the hippocampus when compared to wild type controls (Gibson et al., 2001). Despite these findings, the contributions of Bax

during a hypoxia-only insult are not yet clearly defined. Schwartz et al., (2004) found that in the neonatal rat, chronic hypoxia decreases cortical cell numbers with a corresponding increase in Bax expression levels. However, this study did not determine if the increase in Bax expression is directly responsible for the changes in cell number (Schwartz et al., 2004). Conversely, using an *in vitro* model, Tamatani et al., (1998) report that hypoxia increases primary cortical neuron apoptosis without an accompanied change in Bax expression levels.

Similarly the relative contributions of the BH3-only pro-apoptotic members during hypoxia-induced cell death are unclear. Hypoxia regulation of the BH3-only members has been conducted primarily using the oxygen-glucose deprivation model. Furthermore, the information generated is typically correlative, with exception to one study that showed Bnip3 directly mediates hypoxia-induced oligodendroglial death (Burton et al., 2006). In this study, transfection of a mutant Bnip3 expression plasmid, which acts as a dominant negative, prevents hypoxia-induced oligodendroglial cell death. However, the mutant Bnip3 protein only partially attenuates the increase in hypoxic cell death (Burton et al, 2006). These reports suggest that Bnip3 mediates a component of hypoxia induced oligodendroglial death; however, additional proteins may also contribute. Regardless, based on the design of these studies it is difficult to determine the putative role for each member during a purely hypoxic insult.

4. Bnip3: Structure, Function and Regulation

Bnip3 is a pro-apoptotic BH3-only member of the Bcl-2 gene family (Boyd et al., 1994; Yasuda et al., 1998). However, Bnip3 is unique since it does not require Bcl-2 homology (BH) domain interactions to induce cell death. Additionally, over-

expression of the anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L, does not prevent Bnip3-mediated death (Chen et al., 1997a; Ray et al., 2000). The mechanism that Bnip3 employs to kill cells is also a unique caspase-independent autophagic process (Vande Velde et al., 2000; Kanzawa et al., 2005). Furthermore, unlike other Bcl-2 family members, the carboxyl terminal transmembrane (TM) domain of Bnip3 mediates its killing activity (Chen et al., 1997a; Ray et al., 2000).

The Bnip3 protein is expressed in a number of peripheral tissue types including: spleen, lung, ovary and heart. Bnip3 is also expressed in differentiating and mature oligodendroglial cells, and in the mature rat brain (Chen et al., 1997a; Itoh et al., 2003; Schmidt-Kastner et al., 2004; Althaus et al., 2006; Burton et al., 2006). Bnip3 regulation has been extensively studied during chronic hypoxia and shown to be upregulated by hypoxia-ischemia in the mature brain (Schmidt-Kastner et al., 2004; Althaus et al., 2006; Burton et al., 2006).

Bnip3 protein structure and function

Bnip3 was first identified in a yeast two-hybrid screen using E1B 19kDa protein as bait. E1B 19kDA is a functional substitute for Bcl-2. The screen identified three proteins, Bnip1, Bnip2 and Bnip3 (Boyd et al., 1994). Similar to the other BH3-only members, the Bnip3 protein directly interacts with the anti-apoptotic members Bcl-2 and Bcl-X_L (Boyd et al., 1994; Ray et al., 2000). A number of genes that share a high degree of homology with Bnip3 have also been identified. The human homologues identified include: Bnip3α, Bnip3-like (Bnip3L), *Homo sapiens* Bnip3h (Bnip3h) and Nix (Matsushima et al., 1998; Chen et al., 1999; Yasuda et al., 1999; Farooq et al., 2001). Additionally, a Bnip3 homologue also exists in *Caenorhabditis*

elegans (ceBnip3) indicating that the protein is evolutionarily conserved (Cizeau et al., 2000).

Bnip3 is classified into the BH3-only pro-apoptotic subfamily of the Bcl-2 gene family (Fig. 4) (Yasuda et al., 1998). However, the Bnip3 BH3 domain does not mediate protein-protein interactions with either the pro- or anti-apoptotic Bcl-2 family members. Moreover, the BH3 domain does not confer Bnip3 killing activity. Instead, the protein's carboxyl terminal TM domain is characterized as essential for Bnip3 protein function (Chen et al., 1997a; Ray et al., 2000; Kim et al., 2002; Sulistijo et al., 2003). Furthermore, Bnip3 TM domain properties are conserved throughout evolution as the TM domain is also essential for ceBnip3 protein function (Cizeau et al., 2000).

The TM domain mediates Bnip3 protein homo- and heterodimerization (Fig. 4). Bnip3 homodimerization is inhibited by protein truncation proximal to the TM domain, but not through BH3 domain elimination (Chen et al., 1997a). Bnip3 also heterodimerizes with the anti-apoptotic proteins Bcl-2, adenovirus E1B19K-binding protein (B5) and Bcl-X_L, which is dependent on the TM domain and amino (N) terminal of the Bnip3 protein. Further, removal of the TM domain negates Bnip3 protein-protein interactions with Bcl-2 and B5 (Ohi et al., 1999; Ray et al., 2000). Conversely, Bnip3 interaction with Bcl-X_L is mediated by either the TM domain or the N-terminal. The TM domain is also essential for Bnip3 induced cell death (Fig. 3); since removal of the TM domain, but not BH3 domain, obliterates the protein's killing activity (Chen et al., 1997a; Ray et al., 2000). The TM domain also targets Bnip3 to the mitochondria membrane. Consequently, an apoptotic stimulus fully

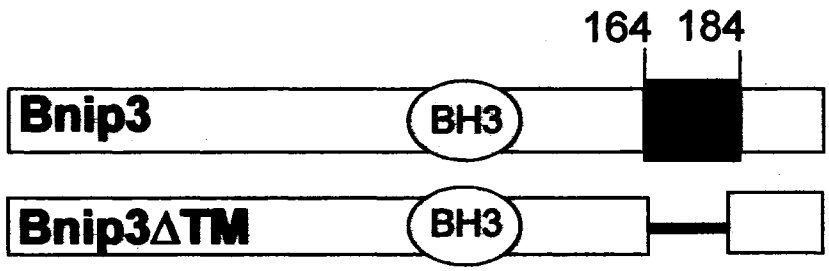


Figure 4. Schematic diagram illustrating the Bnip3 protein structure with the relative locations of the Bcl-2 homology domain-3 (BH3) and transmembrane (TM) domains (upper illustration). The lower illustration depicts the mutant Bnip3 protein with the endogenous TM domain eliminated (Bnip3ΔTM).

integrates the Bnip3 protein into the outer mitochondrial membrane (Yasuda et al., 1998; Ray et al., 2000; Vande Velde et al., 2000; Sulistijo et al., 2003).

Bnip3 protein-protein interactions with the Bcl-2 gene family are also unique. For example, Bcl-2 and Bcl-X_L over-expression merely delays, but does not prevent Bnip3-mediated cell death (Chen et al., 1997a; Ray et al., 2000). Thus suggesting Bnip3-induced cell death is independent of Bcl-2 and Bcl-X_L activity. A recent study also indicates that Bnip3 can induce cell death via interactions with a pro-apoptotic multi-domain Bcl-2 family member. During hypoxia both Bnip3 and the human homolog of the multi-domain member Bok (hBok) are upregulated. Furthermore, Bnip3 directly interacts with hBok, induces hBok oligomerization, and sensitizes cells to hBok mediated cell death (Gao et al., 2005). Taken together, these reports suggest that Bnip3 can act through multiple mechanisms to induce cell death. First, the homodimerization and transmembrane properties of Bnip3 suggest that the protein can disrupt mitochondrial function by directly forming a pore in the outer membrane. Second, the inability of Bcl-2 and Bcl-X_L to prevent Bnip3 mediated cell death suggests that upregulation and activation of Bnip3 alone can suffice to induce cell death. Finally, Bnip3 may serve as a mediator between an apoptotic stimulus, such as hypoxia, and the activation of a multi-domain member to induce cell death.

Regulation of Bnip3 expression.

Bnip3 expression has been predominantly studied in hypoxic peripheral tissue types (Bruick, 2000; Guo et al., 2001; Webster et al., 2005). However, recent studies have also examined hypoxia regulation of Bnip3 in the brain (Schmidt-Kastner et al., 2004; Althaus et al., 2006; Burton et al., 2006). Bruick (2000) first identified the

hypoxic regulation of Bnip3 mRNA and protein expression in numerous cell culture lines including: Chinese hamster ovary, rat fibroblast (Rat-1), human hepatocellular carcinoma, human bladder carcinoma, monkey kidney (CV-1) and human epithelium. Hypoxia-responsive Bnip3 expression also occurs in primary cardiac myocyte cells, differentiated oligodendrocytes and in the adult rat hippocampus, cortex and striatum (Guo et al., 2001; Schmidt-Kastner et al., 2004; Webster et al., 2005; Althaus et al., 2006; Burton et al., 2006).

The induction or silencing of Bnip3 gene transcription is regulated by the methylation state of the Bnip3 promoter. Bnip3 gene transcription, even under hypoxic conditions, is silenced by DNA methylation of the CpG island nearest the transcription start site of the promoter. Conversely, DNA methyltransferase inhibition restores hypoxia-induced Bnip3 transcription (Abe et al., 2005). The Bnip3 promoter also contains a functional HRE that is directly regulated by HIF1 (Fig. 5A). Chinese hamster ovary cells that over-express HIF-1α and are transiently transfected with a Bnip3 promoter luciferase reporter have enhanced Bnip3 promoter activity in comparison to cells that do not express HIF-1α. Furthermore, site directed mutagenesis of the Bnip3 HRE attenuates the HIF-1α-mediated increase in promoter activity (Bruick, 2000).

Studies examining Bnip3 promoter regulation have been followed by both *in vitro* and *in vivo* experiments measuring Bnip3 mRNA and protein expression during hypoxia. In all cell types tested, hypoxia induces an increase in Bnip3 mRNA and protein expression levels. Furthermore, hypoxic-Bnip3 mRNA expression levels are up to 12 fold greater than levels measured in normal air conditions. The increase in

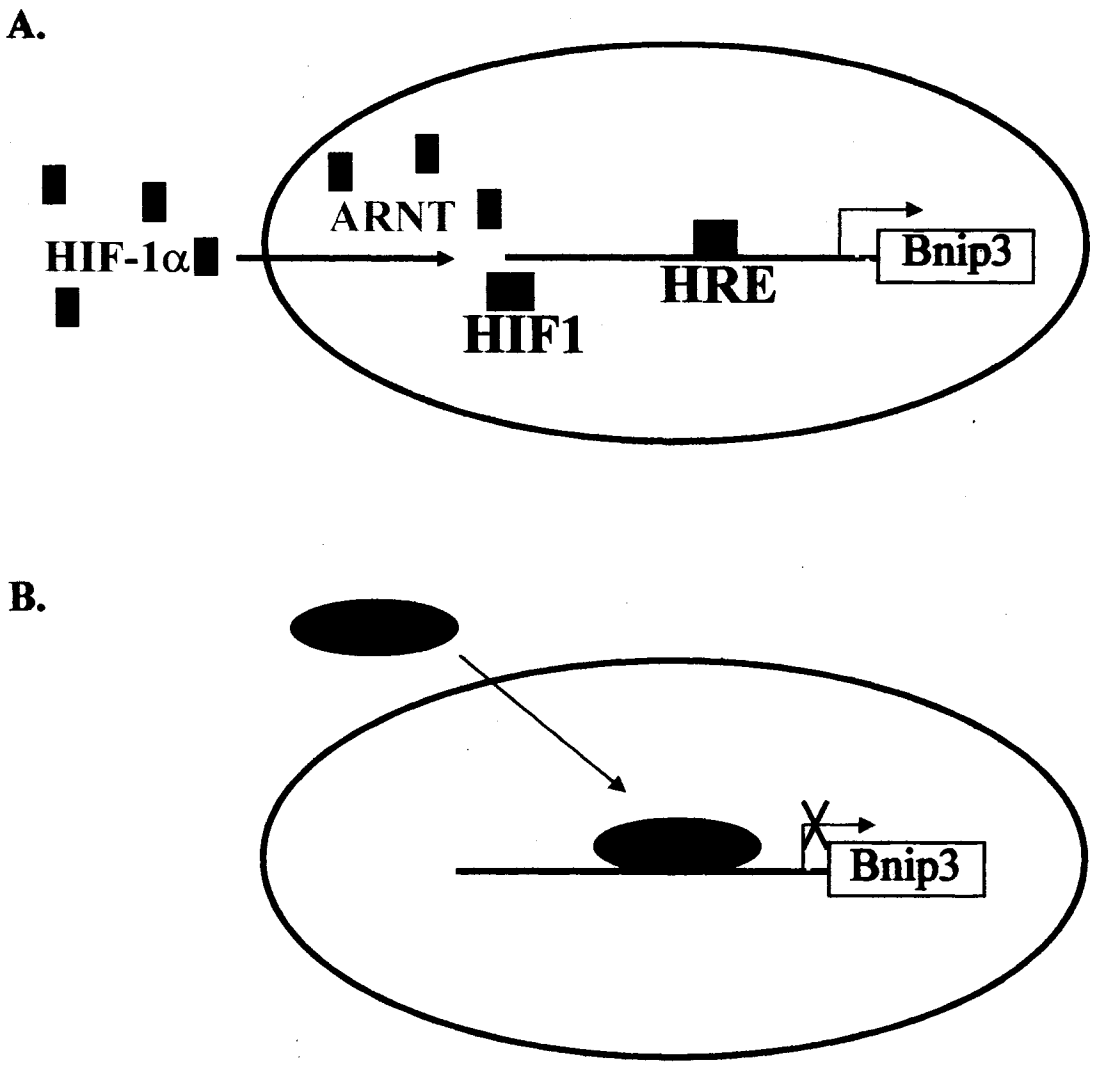


Figure 5. Schematic diagram illustrating the known mechanisms of Bnip3 gene transcription. (A) Hypoxia stabilized HIF-1α translocates into the nucleus and heterodimerizes with ARNT to form the HIF1 transcription factor. HIF1 induces Bnip3 gene transcription at an HRE in the 5' UTR of Bnip3 gene. (B) The NF-κB transcription factor suppresses basal and inducible Bnip3 gene transcription at an NF-κB element in the 5' UTR of the Bnip3 gene.

Bnip3 mRNA expression is also rapid with elevated levels reported as early as 5 hours after hypoxic exposure. Conversely, the increase in protein levels is delayed with elevated levels between 48 and 72 hours after the onset of hypoxia (Bruick, 2000). Additionally, the Bnip3 protein contains a PEST domain that targets it for rapid proteasome degradation (Chen et al., 1999). Rapid protein turnover is a typical characteristic of proteins with PEST domains (Rogers et al., 1986). Thus, the observed delay in hypoxia induced Bnip3 protein levels might be indicative of post-transcription regulation at the PEST domain. However, a putative mechanism by which the Bnip3 protein might be stabilized during chronic hypoxia has yet to be elucidated.

Hypoxia-induced Bnip3 expression closely correlates with an increase in hypoxia-induced cell death in peripheral cell types, oligodendroglial cells and the adult rat cortex and hippocampus (Bruick, 2000; Schmidt-Kastner et al., 2004; Althaus et al., 2006). In a majority of these studies hypoxia-induced cell death has not been directly attributed to Bnip3 activity. However, hypoxia-mediated oligodendroglial cell death has been directly attributed to elevated Bnip3 levels. Burton et al., (2006) utilized a Bnip3 dominant negative to show Bnip3 regulates hypoxia-induced oligodendroglial death. An expression plasmid that encodes a Bnip3 protein minus the TM domain (Bnip3ΔTM) served as the dominant negative to the endogenous Bnip3 protein. Oligodendroglial cells transfected with Bnip3ΔTM and maintained in hypoxic conditions were shown to have attenuated cell death levels compared to non-transfected hypoxic cultures. However, the dominant negative Bnip3 protein did not confer complete protection against the hypoxic insult (Burton et

al., 2006). Suggesting that although Bnip3 mediates a component of hypoxia-induced death additional proteins might also participate.

In addition to hypoxic regulation of Bnip3 expression, Bnip3 gene transcription is regulated by NF-κB (Fig. 5B). However, unlike hypoxia, NF-κB confers protection as it suppresses both basal and inducible Bnip3 gene transcription at the level of the promoter sequence (Baetz et al., 2005). Finally, studies have investigated the putative role p53 plays in hypoxia-mediated Bnip3 expression. p53 was found to be necessary during hypoxic-dependent upregulation of Bnip3L, but not Bnip3 (Fei et al., 2004).

Bnip3 expression and regulation have also been investigated during development, in response to growth factors and nitric oxide. Itoh et al., (2003) measured Bcl-2 family member expression during oligodendrocyte differentiation. Throughout oligodendrocyte differentiation the multi-domain members Bax and Bak are expressed. Additionally, Bnip3 and the BH3-only member Bmf, but not Bim, Biklk, DP5/Hrk, Bad, Bid, Noxa, Luma/Bbc3 and Bnip3L, are highly expressed during differentiation (Itoh et al., 2003). Bnip3 protein activity is also regulated by epidermal-like growth factor (EGF) and insulin-like growth factor (IGF) in MCF-7 breast cancer cells. EGF and IGF attenuate Bnip3-induced cell death; however, these growth factors do not alter Bnip3 expression levels (Kothari et al., 2003). Finally, Bnip3 expression levels are regulated by nitric oxide (NO). However, the induction or suppression of Bnip3 in response to NO is dependent on the cell type. NO attenuates cell death in hepatocytes exposed to tumor necrosis factor-α (Billiar et al., 1992), and correspondingly, Bnip3 mRNA expression is also suppressed by NO (Zamora et al.,

2005). Conversely, NO induces both Bnip3 expression and cell death in macrophages (Yook et al., 2004). These varied mechanisms regulating Bnip3 transcription suggest that multiple factors might simultaneously influence cell survival through Bnip3's regulation and actions.

Bnip3 and the mitochondria

Bnip3 induces cell death by opening the mitochondrial permeability transition (PT) pore; however, the subsequent steps can differ depending on the cell type. Kim et al., (2002) report that Bnip3 protein, over-expressed in isolated mitochondria, induces PT pore opening followed by cytochrome c release. Conversely, Vande Velde et al., (2000) found that Bnip3 over-expressed in MCF-7 and HeLa cells induces cell death that is dependent on PT pore opening, but independent on cytochrome c release. Cell death was also independent of apoptosome formation, caspase activity and translocation of apoptosis inducing factor (Vande Velde et al., 2000). Vande Velde et al., (2000) also report that the morphological features of Bnip3-induced cell death are unique with plasma membrane permeabilization, extensive cytoplasmic vacuole formation, mitochondrial autophagy and low levels of chromatin condensation. Mitochondrial membrane disruption and the described morphological features are more indicative of autophagy rather than apoptosis. Finally, in human malignant glioma cells Bnip3-mediated cell death has been characterized as autophagic (Kanzawa et al., 2005).

A majority of studies reveal that hypoxia causes Bnip3 protein to associate with the mitochondrial membrane, which is followed by PT pore opening. However, Schmidt-Kastner et al., (2003) and Althaus et al., (2006) have both reported that

Bnip3 protein is translocated to the nucleus in the adult rat hippocampus and cortex in response to a hypoxic-ischemic insult. Unfortunately, these studies do not prove that nuclear localization of Bnip3 is responsible or capable of mediating cell death associated with hypoxia-ischemia. Conversely, Burton et al., (2006) found that Bnip3 must be localized to the mitochondrial membrane to induce oligodendroglial cell death. Despite these differences, in each of the cell types tested hypoxia elevates Bnip3 protein levels which closely correlates with an increase in hypoxia induced CNS cell death (Schmidt-Kastner et al., 2004; Althaus et al., 2006). Cumulatively, these studies also indicate that the cell death mechanism employed by Bnip3 has yet to be fully elucidated. Additionally, the possibility exist that the cell death mechanism is varied depending on the cell type.

5. Glucocorticoid Receptor Localization, Structure and Function

Endogenous and exogenous glucocorticoids regulate numerous functions throughout the body, including the CNS. The actions of glucocorticoids are mediated by two receptors, the GR and mineralocorticoid receptor (MR), which are both expressed in the developing and mature rat brain (Ahima and Harlan, 1990; Lawson et al., 1991; McGimsey et al., 1991; van Eekelen et al., 1991; Cintra et al., 1994; Yi et al., 1994; Morimoto et al., 1996). GR and MR are also both active participants in glucocorticoid-induced cell death; however, in regards to apoptosis the receptors actions are typically opposite in nature. For example GR activation induces a neuroendangering phenotype; while MR activation is neuroprotective (Almeida et al., 2000; Crochemore et al., 2005). Typical of all the nuclear receptor superfamily members, the GR and MR predominantly employ regulation of gene transcription to

alter cellular physiology. Since the receptors share common ligands, the relative binding affinities for the various natural and synthetic glucocorticoids confer a portion of receptor specificity for the initiation of downstream events (Coirini et al., 1985; Sutanto and De Kloet, 1987).

GR structure and gene transcription

GR is a member of the class III family of proteins within the nuclear receptor superfamily. This class also includes MR, androgen receptor, estrogen receptor and progesterone receptor. The protein structure for the steroid hormone receptors consist of the three major domains: the N-terminal domain, DNA binding domain and ligand binding domain (Fig. 6A) (Danielsen et al., 1986; Aranda and Pascual, 2001). The three domains mediate both unoccupied and occupied receptor function. The unoccupied GR resides within the cytoplasm in a conformation that prevents GR mediated gene transcription (Mendel et al., 1986). However, ligand binding causes a conformational change, which allows GR translocation to the nucleus, shedding of chaperone proteins such as heat shock protein (HSP)-90, receptor dimerization, DNA interaction and subsequent induction of gene transcription (Fig. 6B) (Picard and Yamamoto, 1987; Tsai et al., 1988; Wrange et al., 1989; Cairns et al., 1991a; Cairns et al., 1991b). The GR DNA binding and ligand binding domains are both involved in ligand mediated gene transcription; while the N-terminal domain, which contains the activation function (AF) 1 domain, is involved in both ligand dependent and independent transcription (Cho et al., 2005; Kumar and Thompson, 2005). The GR regions also confer specificity in terms of ligand interaction and gene transcription. These are described individually below.

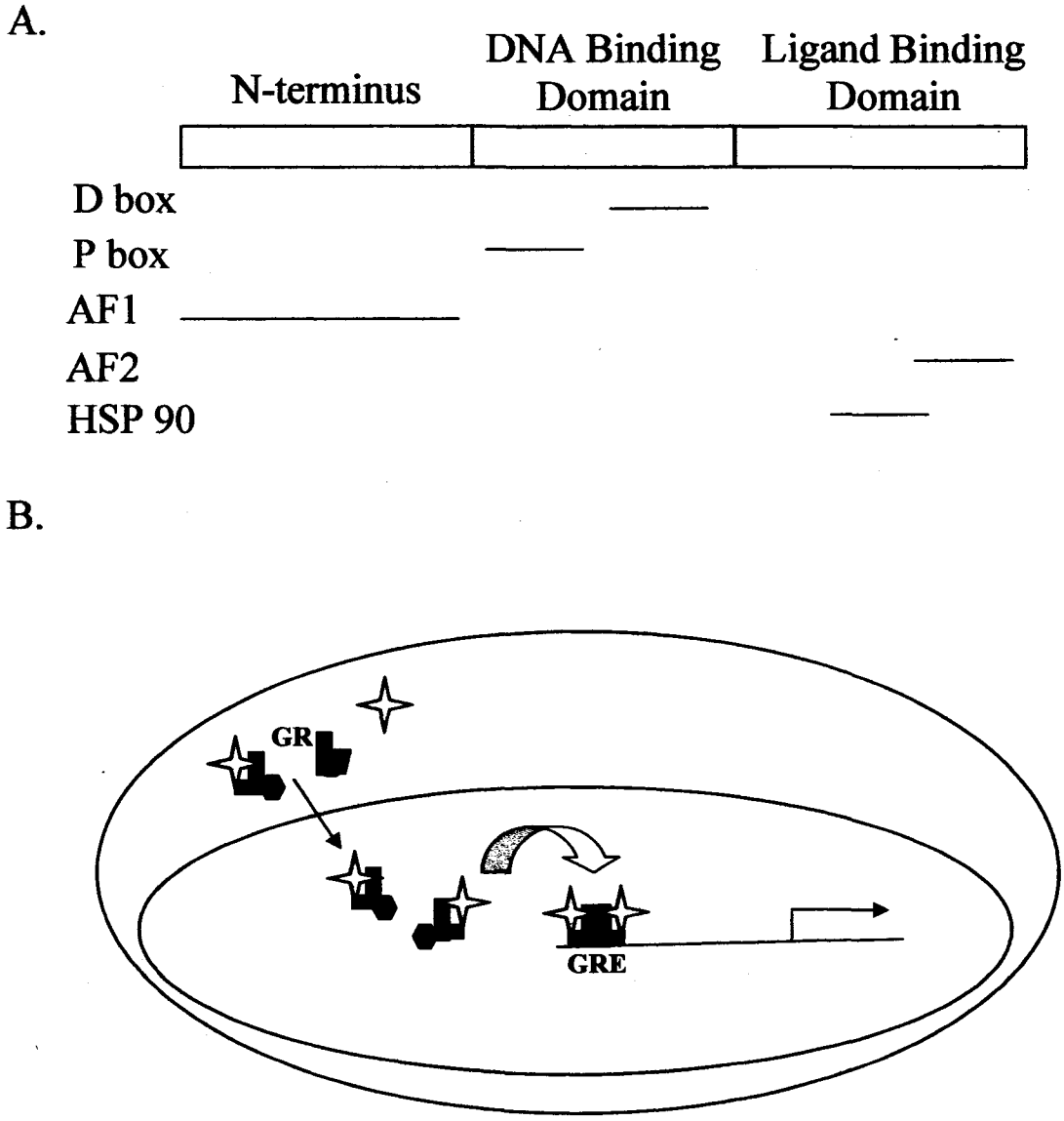


Figure 6. Schematic illustration depicting the protein structure of the GR and mechanisms of GR mediated gene transcription. (A) An illustration of the GR protein and relative locations of the N-terminal, ligand binding and DNA binding domains. The relative GR sub-region locations are also depicted (P Box, D Box, AF1, AF2 and HSP-90 interaction site). (B) Schematic depicting GR regulation of gene transcription. Unoccupied GR (blue) resides in the cytoplasm in multi-protein complex that includes HSP-90 (orange). GR ligand (yellow star) binds GR, induces a conformational change that promotes receptor dimerization and nucleotide interaction. HSP-90 chaperones ligand bound GR to the nucleus, where it homodimerizes and interacts with a GRE to alter gene transcription.

GR DNA binding domain

The GR DNA binding domain is composed of two highly conserved zinc finger motifs that mediate receptor homodimerization and DNA binding. The two zinc fingers each contain four cysteine residues that anchor a zinc ion (Freedman et al., 1988). GR dimerization is conferred by the D box within the second zinc finger that is located near the DNA binding domain carboxyl terminal (Dahlman-Wright et al., 1991; Luisi et al., 1991). A five amino acid sequence within the D box regions of two GR monomers confers receptor homodimerization (Dahlman-Wright et al., 1991). Further, an aspartate arginine salt bridge forms between the D box regions of two activated GR monomers during homodimerization (Luisi et al., 1991; Liu et al., 1995). D box mediated homodimerization subsequently aligns the two GRs head to head, which positions the two receptors for DNA interaction (Tsai et al., 1988; Luisi et al., 1991).

The first zinc finger contains the P box, which is the domain that interacts directly with DNA. The P box domains of the dimerized GRs align to mediate receptor-DNA interactions with conserved sequences, termed glucocorticoid response element (GRE), that are found in a gene's promoter region (Tsai et al., 1988; Danielsen et al., 1989). The GRE is a 15 nucleotide sequence composed of two 6 nucleotide imperfect palindrome sequences separated by three variable nucleotides (GRE consensus = GGTACAnnnTGTTCT) (Nordeen et al., 1990). Binding of the receptor homodimer to the GRE subsequently initiates gene transcription.

The GR DNA binding domain specificity is also shared with other nuclear receptor superfamily members, including androgen receptor, progesterone receptor

and MR, which all interact with similar consensus sequences (Nordeen et al., 1990; Roche et al., 1992; Lieberman et al., 1993). However, variations in the nucleotide sequence subtly alter preferential binding affinities for the various steroid hormone receptors. An example of this can be seen when examining GR. Ligand bound GR preferentially binds the consensus sequence GGTACAnnnTGTTCT; while the consensus sequence of GGAACTnnnTGTTCT is selective for the androgen receptor (Nelson et al., 1999). Further, amino acid substitutions in the first zinc finger of the GR also alter hormone response element selectivity. For example, substitution of two amino acids located between the cysteine residues at positions 445 and 448 of the first zinc finger changes GR selectivity from a GRE to an estrogen response element (Danielsen et al., 1989).

Activated GR also has the capability to regulate gene transcription as either a monomer or heterodimer with MR (Drouin et al., 1993; Liu et al., 1995; Radoja et al., 2000). Studies indicate that GR monomers act to repress gene transcription at promoter sites away from a classical GRE at negative GRE sites (Drouin et al., 1993; Radoja et al., 2000). The gene repression actions of a GR monomer are also independent of the second zinc finger and dimerization. Furthermore, the carboxyl terminal of the DNA binding domain, which contains an A and T box mediate monomer interaction with DNA (Aranda and Pascual, 2001). Conversely, the GR:MR heterodimers are formed in the same manner as GR homodimers with interaction mediated through aspartate arginine salt bridges between the D box regions (Liu et al., 1995).

GR ligand binding domain

Agonists to the GR bind at the ligand binding domain, a hydrophobic pocket that attracts ligands (Danielsen et al., 1986; Giguere et al., 1986; Dey et al., 2001). Prior to hormone binding, the ligand binding domain acts as a silencer of activity by inducing a protein conformation that prevents steroid hormone receptor interaction with either coregulatory elements or nucleotides (Danielsen et al., 1987; Godowski et al., 1987; Hollenberg et al., 1987). Furthermore, unoccupied GR resides in the cytoplasm in a multiprotein complex that includes HSP-90, which prevents receptor-DNA interaction (Mendel et al., 1986; Howard and Distelhorst, 1988; Giannoukos et al., 1999). The carboxyl terminal of the GR ligand binding domain mediates this interaction with HSP-90 (Howard et al., 1990). Agonist binding of the GR initiates a conformational change that releases the receptor from the multiprotein complex and exposes the dimerization motif in the DNA binding domain (Dittmar et al., 1997). The activated GR is subsequently chaperoned to the nucleus by HSP-90 (Govindan, 1979; Papamichail et al., 1981; Dittmar et al., 1997).

The ligand binding domain also contains the GR AF2 domain, which is located at the protein's carboxyl terminal (Webster et al., 1988). The AF2 domain mediates interaction with coregulatory proteins in a ligand dependent fashion. For example, ligand bound GR interacts with the p160 coactivator family members as well as Creb binding protein (Almlof et al., 1998; Windahl et al., 1999; Ko et al., 2002; Vottero et al., 2002). Conversely, unoccupied GR is maintained in a conformational state that prevents interaction with coactivator proteins. Upon ligand binding, the GR changes conformation which exposes a coactivator interaction site in

helix 12 of the ligand binding domain (Kauppi et al., 2003). This interaction between coactivator proteins and GR is mediated by an LXXLL domain that is composed of three highly conserved LXXLL motifs. The L represents a leucine and the X any amino acid (Ding et al., 1998; Giannoukos et al., 1999).

The coactivator proteins induce gene transcription by altering the acetylation state of histone complexes. A histone complex consists of eight proteins that wrap chromatin and regulate gene transcription (Fig. 7). The coactivator proteins, including the p160 family members and Creb binding protein, posses intrinsic histone acetyltransferase activity. This activity acetylates the lysine residues located at the histone ends (Chen et al., 1997b; Jenster et al., 1997; Spencer et al., 1997). By doing so, the positive charge on the lysine residues is eliminated, which negates histone affinity for DNA and subsequently induces chromatin uncoiling (Rhodes, 1997). Conversely, activated steroid hormone receptor recruitment of corepressor proteins prevents gene transcription by also modifying histone acetylation. However, the corepresor proteins do not posses intrinsic histone deacetylase activity, but instead recruit additional proteins that deacetylate histone complexes and promote DNA coiling (Aranda and Pascual, 2001).

N-terminal domain

The N-terminal domain exhibits the greatest amount of variability between the different steroid hormone receptors. The N-terminal domain also contains the AF1 domain (Hollenberg and Evans, 1988), which controls the majority of GR's transcriptional activity. The GR AF1 domain was originally characterized as the region that mediates ligand independent transcription (Kumar and Thompson, 2005).

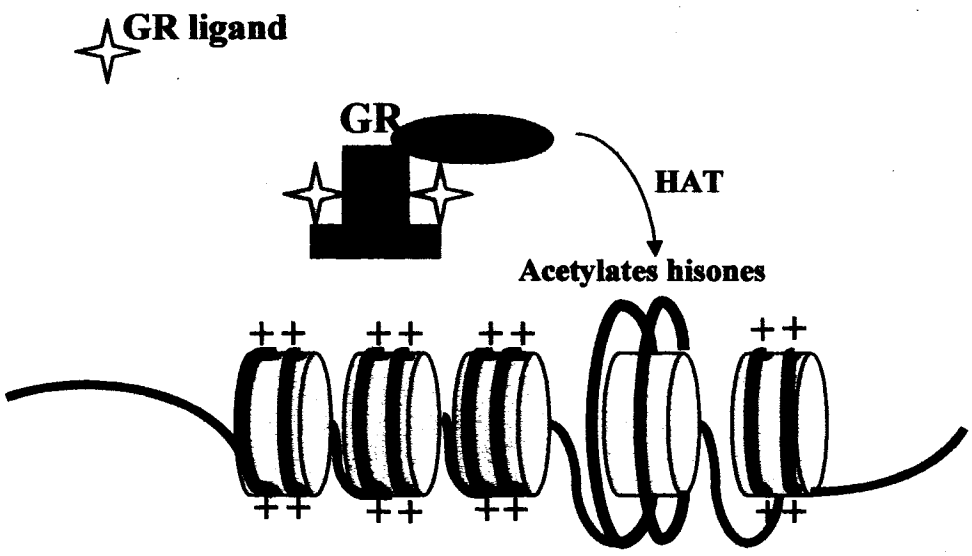


Figure 7. Ligand bound steroid receptors induce gene transcription via recruitment of coregulatory proteins that alter histone acetylation state and subsequently chromatin coiling. Activated GR recruits a p160 family member that has intrinsic histone acetyltransferase (HAT) activity. The p160 coactivator family member acetylates lysines within histone complexes. Acetylation eliminates the positive charge on the lysine residues. The DNA loses affinity for the histone and the chromatin uncoils allowing for gene transcription.

However, more recent studies indicate that the AF1 domain also mediates ligand dependent transcription. GR binding to DNA induces a conformational change in the AF1 domain that promotes interaction with coactivator proteins including the p160 family (Cho et al., 2005). Furthermore, other data reveal that the AF1 and AF2 domains can simultaneously interact with coregulatory proteins (Kumar and Thompson, 2005).

GR agonists

The GR and MR have multiple agonists, including both natural and synthetic ligands. However, the various ligands have different binding affinities for the GR and MR. The GR and MR agonists include the natural glucocorticoids, cortisol and CORT as well as the synthetic glucocorticoids, DEX and RU28362. The endogenous glucocorticoids both preferentially bind MR to GR. The dissociation constants (KD) of cortisol and CORT for the MR are 2.2 and 0.5nM, respectively. The binding affinities of cortisol (KD=20.1nM) and CORT (KD=2.5-5.0nM) for GR are 4 to 10 times less than that of MR (Reul and de Kloet, 1985; Sutanto and De Kloet, 1987). The GR and MR also bind the synthetic glucocorticoid DEX. However, unlike the natural glucocorticoids, DEX has approximately a 2-3 fold preferential binding for the GR over the MR. The KD of DEX for the GR and MR are 0.7nM and 1.7nM, respectively (Handa et al., 1994). RU28362 is another synthetic glucocorticoid, but it is a selective agonist for the GR, with no apparent binding affinity for the MR (Coirini et al., 1985). The KD of RU28362 for the GR is 1.0nM.

Synthetic and endogenous glucocorticoids are lipophilic molecules, which are able to passively cross plasma membranes and subsequently bind cytoplasmic

glucocorticoid receptors. Ligand interaction with a steroid receptor activates the receptor, which mediates downstream events such as gene transcription. The milieu of GR and MR agonists allow for selective receptor activation and subsequent investigation of their downstream actions. However, numerous lines of research indicate that the typical steroid receptor response to a ligand is not linear but instead biphasic, which may complicate result interpretation (Calabrese and Baldwin, 2001b, 2001a).

Glucocorticoid receptor localization

Numerous studies have characterized GR and MR mRNA and protein localization in the developing and mature rat brain. These characterization studies indicate the brain regions, cell types and developmental periods where glucocorticoids acting through MR and GR can directly modulate gene transcription.

Autoradiographic, in situ hybridization (ISH) and immunohistochemical analysis studies have identified GR mRNA and protein expression throughout the adult and developing rat brain. In the adult rat brain both GR mRNA and protein are expressed in the cortex, hippocampus, thalamus, hypothalamus, amygdala, septum, striatum and cerebellum (Ahima and Harlan, 1990; McGimsey et al., 1991; Cintra et al., 1994; Morimoto et al., 1996). The cortex and hippocampus express high levels of GR mRNA and protein. Within the adult rat cortex, GR mRNA and protein are expressed at highest levels in the anterior cingulate cortex and retrosplenial cortex (also known as the posterior cingulate cortex). The cingulate cortex laminar layers II, III, and VI have been reported to have the greatest concentration of GR with more than 70% of the cells expressing either the mRNA or protein. These studies also

reveal that GR is expressed, but in a smaller percentage of cells, in layers I and V (Aronsson et al., 1988; Ahima and Harlan, 1990; Cintra et al., 1994; Morimoto et al., 1996). In contrast, McGimsey et al., (1991) report that highest GR protein expression is found in laminar layers IV and V. The discrepancy between these two studies may be due to the use of different fixation protocols or GR antibodies. In the adult rat hippocampus, however, there is good agreement as to the expression pattern for GR mRNA and protein. GR is expressed the highest in the hippocampal regions CA1 and CA2 and to a lesser extent in the granule cell layer of the dentate gyrus, with lowest levels in CA3 (Aronsson et al., 1988; Ahima and Harlan, 1990; McGimsey et al., 1991; Cintra et al., 1994; Morimoto et al., 1996).

In comparison to the mature rat brain a very similar localization pattern has been found in the developing brain. GR mRNA and protein are highly expressed within the postnatal rat hippocampus regions CA1 and CA2 and to a lesser extent in the CA3 region and dentate gyrus granule cell layer (Rosenfeld et al., 1988; Lawson et al., 1991; van Eekelen et al., 1991; Yi et al., 1994). Furthermore, in cortex, GR mRNA and protein are expressed within the cingulate cortex and expression is greatest in laminar layer II, a pattern that is similar to the adult (Rosenfeld et al., 1988; van Eekelen et al., 1991; Yi et al., 1994).

The ontogeny of GR mRNA expression has also been determined throughout CNS development. During postnatal development a transient peak in GR mRNA levels is detectable between PND 6 and 10 in the cingulate cortex (Yi et al., 1994). Conversely, in the hippocampus, GR mRNA steadily increases until PND 10 and then remains elevated in the CA1 region. In the CA2 region, GR mRNA expression

increases until PND 16 and then remains elevated to adulthood (Yi et al., 1994). Predictably, the GR protein ontogeny in the hippocampus closely resembles the mRNA ontogeny. Low GR protein levels are measured during the second postnatal week (PND 10-12) and a subsequent increase in expression to adult levels occurs by the end of the third postnatal week (PND 15-20) (Rosenfeld et al., 1988; Lawson et al., 1991).

GR protein subcellular localization has also been established in the rat brain. GR predominantly localizes to the nucleus with weak cytoplasmic localization in the normal postnatal and adult animal (Ahima and Harlan, 1991; Lawson et al., 1991; Morimoto et al., 1996). However, after adrenalectomy GR is predominantly localized to the cytoplasm (Ahima and Harlan, 1991; Morimoto et al., 1996). These data indicate that CORT levels in the intact animal induce ligand-bound GR translocation to the nucleus. In the absence of ligand, as found in the adrenalectomized animal, the unbound GR is localized to cytoplasm. Several studies have also localized GR to other intracellular organelles such as the mitochondria and synaptosome (Demonacos et al., 1993; Moutsatsou et al., 2001).

The distribution of MR mRNA and protein in the developing and adult rat brain has some overlap with GR expression (Ahima et al., 1991; Lawson et al., 1991; van Eekelen et al., 1991). *In vivo* binding studies using tritiated aldosterone (ALDO), an MR selective ligand, reveal that a majority of MR binding sites in the adult rat brain are within the hippocampus (Coirini et al., 1985). Immunohistochemical detection of MR protein reveals that is highly expressed in the adult hippocampal CA1 and CA2 regions and to a much lesser extent in the CA3 and dentate gyrus.

Furthermore, MR protein is expressed at moderate levels in the cortex, including the cingulate cortex, the hypothalamus and subcortical regions. Despite the apparent complete overlap in MR and GR protein in the cingulate cortex, studies have shown that MR protein is confined to only the region of the cingulate cortex located dorsal to the triangular septal nucleus (Ahima et al., 1991). MR mRNA expression in the neonatal rat brain is found throughout the hippocampus and in laminar layer II of the cingulate cortex (van Eekelen et al., 1991). Furthermore, MR immunoreactive cells were also found in the hippocampus with greatest levels in the CA1 and CA2 regions (Lawson et al., 1991).

In addition to the overlap in GR and MR expression within hippocampus and cortex, GR and MR can be co-expressed within an individual cell (van Steensel et al., 1996). These studies found that brain regions or cells expressing both receptor types can mediate varied responses to the endogenous glucocorticoid. Finally, MR and GR expression levels are autologously regulated. For example, CORT removal by adrenalectomy increases both MR and GR mRNA expression in the rat hippocampal CA1 and dentate gyrus regions. The adrenalectomy induced receptor expression can subsequently be negated by concomitant treatment with the MR selective agonist ALDO in both the CA1 and dentate gyrus. Additionally, GR activation by RU28362 also attenuates the adrenalectomy induced expression of GR and MR mRNA in the CA1 region, but not the dentate gyrus (Chao et al., 1998).

GR modulation of additional transcription factors

Studies reveal that GR can alter the activity of additional transcription factors. For example, multiple studies have found that ligand-bound GR either enhances or

represses hypoxic responsive gene expression through HIF1 transactivation (Fig. 8A) (Leonard et al, 2005; Kodama et al, 2003). Kodama et al., (2003) determined that during hypoxia the GR ligand binding domain interacts with the HIF-1α carboxyl terminal to enhance HIF1 mediated gene transcription at a HRE, but not at a GRE. Furthermore, HIF1 and activated GR co-localize within the nucleus of hypoxic cells (Kodama et al, 2003). Such results suggest that GR directly transactivates HIF1 via protein:protein interactions to either enhance or repress hypoxia induced gene transcription. However, Kodama et al., (2003) have also reported that gel shift pull-down assay does provide evidence of a direct interaction between the GR and HIF1 during a hypoxic event. In opposition to these studies, Leonard et al., (2005) have reported that ligand-bound GR potentiates HIF1 activity through a GRE. A potential explanation for these differences may be the use of different cell culture lines. The studies conducted by Kodama et al., (2003) were performed in HeLa and COS7 cells; while Leonard et al., (2005) used HK-2 cells (derived from human proximal tubular epithelial cells). Additional support that the difference is a result of the cell line used is that in the HK-2 cells hypoxia directly induces GR expression (Leonard et al., 2003; Leonard et al., 2005). Conversely, in the HeLa and COS7 cells hypoxia did not induce GR expression (Kodama et al., 2003). Despite these reported differences, the data indicate that in peripheral cell types glucocorticoids can modulate hypoxic responsive gene; therefore, the possibility exists that in the CNS glucocorticoids can also act in this capacity.

GR also suppresses NF-κB transcriptional activity, which is typically neuroprotective. Activated NF-κB promotes cell survival by increasing expression of

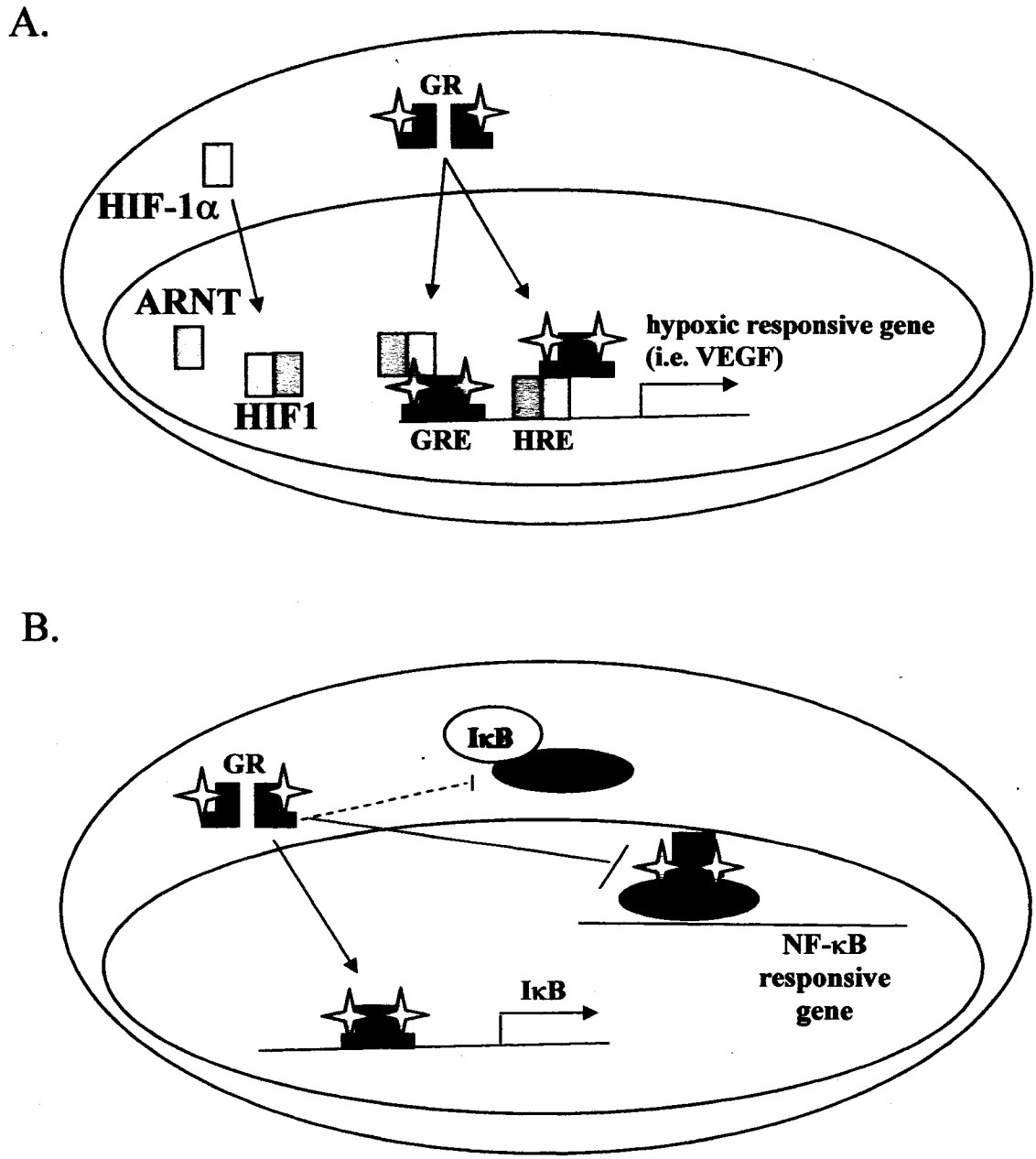


Figure 8. GR mediated transcription is altered by additional transcription factors. (A) Ligand-bound GR modulates hypoxia induced gene transcription by transactivation of the HIF-1α subunit of the HIF1 transcription factor. The GR:HIF1 transcriptional complex is postulated to regulate gene transcription at either a GRE or HRE. (B) GR suppresses NF-κB mediated gene transcription. GR directly inhibits NF-κB actions at the promoter level. GR also indirectly (represented by dashed line) inhibits NF-κB translocation to the nucleus via upregulating IκB expression levels. IκB works in the capacity to sequester NF-κB in the cytoplasm.

Bcl-X_L (Bui et al., 2001). Additionally, NF-κB suppresses both basal and inducible levels of Bnip3 (Baetz et al., 2005). GR prevents the activities of NF-κB by inhibiting either NF-κB translocation to the nucleus or actions at an NK-κB element in a genes promoter (Fig 8B). NF-κB translocation is inhibited by GR through inducing expression of IκB, which functions to sequester NF-κB in the cytoplasm (Auphan et al., 1995; Scheinman et al., 1995; Ramdas and Harmon, 1998). Studies also indicate that GR directly inhibits NF-κB mediated gene transcription at NF-κB element sites within a promoter by squelching transcription factors and blocking basal transcriptional machinery and transactivation domains (De Bosscher et al., 2003).

GR regulation of Bcl-2 gene family expression

In regards to cell death, GR and MR regulation of gene transcription are typically opposite in nature with GR activation being neuroendangering and MR activation being neuroprotective. For example, MR prevents apoptosis by downregulating pro-apoptotic proteins and upregulating anti-apoptotic proteins. Activated GR also alters pro- and anti-apoptotic Bcl-2 family member expression; but in contrast to MR, the change in protein levels selects for apoptosis (Almeida et al., 2000).

Both *in vivo* and *in vitro* studies have been conducted to investigate the underlying mechanism of DEX-induced cell death. DEX treatment of either adult or juvenile rats increases cell death levels in the hippocampal dentate gyrus region (Hassan et al., 1996; Almeida et al., 2000; Haynes et al., 2001). Almeida et al., (2000) subsequently investigated a putative mechanism of DEX-induced apoptosis and reported that DEX administered to young and adult rats elevates Bax mRNA and

protein levels. Furthermore, these authors suggest that in the adult mouse hippocampus DEX-mediated apoptosis is dependent on Bax; since DEX-treated Bax deficient mice have similar levels of dentate gyrus apoptosis as vehicle-treated wild type controls (Almeida et al., 2000). Bax mRNA expression levels also increase following DEX treatment in a hippocampal cell line. However, a corresponding change in Bax protein levels was not observed (Crochemore et al., 2002). In addition to altering pro-apoptotic expression levels, DEX treatment decreases expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L. Bcl-2 levels are altered similarly in the young and old rat; while Bcl-X_L expression levels predominantly changed in the juvenile rat (Almeida et al., 2000). These studies reveal that DEX treatment preferentially alters the pro- to anti-apoptotic protein ratio in favor of apoptosis. Furthermore, the pro-apoptotic protein Bax is implicated as an effector protein of DEX-induced apoptosis in the adult rodent hippocampus.

In the postnatal rat brain DEX is also neuroendangering; however, the potential mechanism has yet to be elucidated. Postnatal rats treated with DEX have elevated levels of CNS cell death. However, in contrast to the data reported in the juvenile and adult rat hippocampus, DEX-treated postnatal rats do not have altered hippocampal Bcl-2 or Bax expression levels (Tan et al., 2002). Taken together, these data indicate that GR induces cell death in the developing and mature CNS. Furthermore, the Bcl-2 gene family members have been implicated as putative effector proteins for DEX-induced apoptosis in the mature rat brain. However, the effector proteins of DEX-induced death in the developing rat brain have yet to be identified.

6. Glucocorticoid Neuroendangerment

Glucocorticoids are endogenously secreted hormones with basal circulating levels maintained less than 10 μg/DL. However, a stressful situation can result in a surge of circulating glucocorticoids that can reach levels of 50-100 μg/DL (Lund et al., 2006). The stress-responsive CORT secretion is used to regulate a variety of events that alter a cell's metabolic state. These include gluconeogenesis, lipolysis of adipose tissue and glucose transport (Buckingham, 2000). Additionally, acute elevations in glucocorticoid levels may positively modulate learning and memory by increasing glucose supply to the brain, and enhancing long term potentiation (Kamphuis et al., 2003; Korz and Frey, 2003). Despite the positive affects that glucocorticoids initiate following an acutely applied stressor, chronically elevated glucocorticoid levels can be neuroendangering to both the mature and developing rat brain (Roy and Sapolsky, 2003; Kamphuis et al., 2004; Neal et al., 2004; Burlet et al., 2005).

The hypothalamo-pituitary adrenal axis

The hypothalamo-pituitary adrenal (HPA) axis is a neuroendocrine system that regulates plasma glucocorticoid levels. The HPA axis is typically activated in response to stress. Humans (and hamsters) respond to stress by secreting the endogenous glucocorticoid, cortisol; while rats and mice secrete CORT. A real or perceived stressor causes the release of corticotrophin releasing hormone (CRH) or arginine vasopressin (AVP) from neurons found in the paraventricular nucleus of the hypothalamus. CRH is released into the hypothalamo-hypophyseal portal vessels at the level of the median eminence and subsequently acts on the anterior pituitary

corticotropes to induce adrenocorticotropic hormone (ACTH) secretion into the general circulation. ACTH induces the synthesis and secretion of glucocorticoids from the adrenal cortex (Dallman et al., 2000; Watts, 2000). Circulating levels of CORT can act to control additional HPA axis activity via a negative feedback loop acting predominantly through the anterior pituitary, hypothalamus, and hippocampus (Sapolsky et al., 1984a; Akana et al., 1986; Bradbury et al., 1994).

Circulating glucocorticoids bind either GR or MR, which have both been implicated in the negative feedback regulation of the HPA axis. MR has a high affinity (0.5nM) for the endogenous glucocorticoid and is predominantly bound (80%) at baseline CORT levels (Reul and de Kloet, 1985; Reul et al., 1990). This indicates that MR regulates negative feedback to control HPA axis activity during the non-stressed state. This is supported by studies showing that adrenalectomy of adult rats increases basal CRH and ACTH levels (Dallman et al., 1987), whereas CORT replacement at doses that selectively bind MR returns ACTH levels, but not necessarily CRH levels to normal (Bradbury et al., 1994). A majority of MR is expressed within the hippocampus (Coirini et al., 1985), which indicates that this is a predominant site for HPA axis regulation during the non-stress state. Consistent with this hypothesis, MR antagonists administered directly to the hippocampus elevate basal ACTH and CORT levels (van Haarst et al., 1997).

The GR, which has a lower affinity (2.0-5.0nM) for CORT, is unoccupied during low basal CORT levels, but becomes occupied by stress induced levels of CORT (Reul and de Kloet, 1985; Reul et al., 1990). GR activation is hypothesized to regulate the return of HPA axis activity to baseline following a stressor. A majority of

the GR is localized to the hippocampus and to a lesser extent the anterior pituitary and hypothalamus (Reul and de Kloet, 1986; Ahima and Harlan, 1990; McGimsey et al., 1991; Cintra et al., 1994; Morimoto et al., 1996). An acute stressor causes a surge of CORT secretion that acts predominantly upon the AVP neurons of the paraventriculer nucleus to inhibit additional HPA axis output (Kovacs et al., 2000). HPA axis regulation during a stress response is also mediated through GR within the hippocampus (Sapolsky et al., 1984a).

The HPA axis can become dysregulated and studies indicate this results from chronic stress and altered GR regulation and function. In this "glucocorticoid cascade hypothesis" a chronic stressor would cause long term elevations in circulating CORT levels, and subsequently downregulate GR expression within the hypothalamus and the hippocampus (Sapolsky et al., 1984b; Herman et al., 1995). The resulting low GR numbers may subsequently prevent appropriate negative feedback signaling for the HPA axis (Sapolsky et al., 1984b) thereby further elevating CORT levels.

The catabolic nature of glucocorticoids

Glucocorticoids have been shown to be catabolic in nature due to their effects on the synthesis of more readily utilizable sources of energy. An increase in circulating CORT results in the catabolism of glycogen and proteins into glucose, which is delivered to the brain and used as a beneficial energy supply (Buckingham, 2000). However, a chronic stressor, which results in an increased and sustained glucocorticoid secretion, metabolically endangers CNS cells (Sapolsky, 1985, , 1986b; Horner et al., 1990; Virgin et al., 1991; Booth et al., 1998). The putative neuroendangering aspects of chronic elevated CORT could be the result of GR

mediated inhibition of glucose uptake, as has been demonstrated in primary hippocampal neurons and glia (Horner et al., 1990; Virgin et al., 1991; Booth et al., 1998). The resulting decreased glucose stores subsequently leave a neuron in a depleted energy state which may result in neuronal death when faced with a secondary metabolic insult (Sapolsky and Pulsinelli, 1985; Sapolsky, 1986b). In further support of this hypothesis, an increase in hippocampal lesion size was measured in adult rats treated with 5mg/day CORT in conjunction with either a kainic acid, 3-acetylpyridine (Sapolsky, 1986b) or hypoxic-ischemic insult (Sapolsky and Pulsinelli, 1985). The primary insult for each treatment paradigm either prevents additional energy synthesis (hypoxia-ischemia and 3-acetylpyridine) or energy uptake (kainic acid). Furthermore, supplements of glucose, mannose, and β-hydroxybutyrate attenuate CORT exacerbation of both 3-acetylpyridine and kainic acid insults (Sapolsky, 1986a). Cumulatively, these reports indicate that glucocorticoids can metabolically neuroendanger cells and subsequently potentiate primary metabolic insults.

Glucocorticoids and the adult rat brain

The potentially neuroendangering aspect of glucocorticoids was first examined using the aged rat as the primary model. With advanced age, rodents exhibit elevated basal CORT levels and are unable to return CORT levels to baseline following a stressful stimulus. Both of these changes are indicative of HPA axis negative feedback dysregulation (Sapolsky et al., 1983b). The aged rat also has decreased GR expression in the hippocampus, which provides further support for HPA axis insensitivity to negative feedback regulation (Sapolsky et al., 1983a). In

addition to abnormal HPA axis output, a decrease in hippocampal neuron number also accompanies the increased CORT secretion (Sapolsky et al., 1984c; Sapolsky et al., 1986). However, if a rat is adrenalectomized in early life neuronal loss is attenuated in the aged animal (Landfield et al., 1981). Separate studies have reproduced the aged rat phenotype in the immature rat by maintaining chronically elevated glucocorticoid levels through daily CORT injections. Chronic glucocorticoid exposure results in an accelerated rate of neuronal loss in the hippocampal CA3 region and further downregulates hippocampal GR expression (Sapolsky et al., 1985). Taken together, the results of these studies indicate that cumulative glucocorticoid exposure or the heightened circulating CORT levels following chronic stressors accelerate hippocampal cell loss in the aging brain.

Glucocorticoids also induce cell death in multiple regions of the CNS. A recent study demonstrated that DEX activation of GR in adrenalectomized adult rats results in a decrease in anterior cingulate cortex volume compared to either adrenalectomized rats replaced with low dose CORT or adrenalectomized rats without steroid replacement. Additionally, GR mediated reduction in anterior cingulate cortex volume was specific to that brain region since no treatment effect was observed in the posterior cingulate cortex (Cerqueira et al., 2005). Adult rats administered DEX also show increased apoptosis in the hippocampus. Hassen et al., (1996) demonstrated that in rats ranging in age from 6 to 36 months a single DEX injection (60μg/kg, intraperitoneal) induced apoptosis in the dentate gyrus hilar region and granule cell layer, but not the CA1-CA3 region. Furthermore, MR activation by ALDO negates the GR mediated increase in dentate gyrus cell death

(Hassan et al., 1996). Haynes et al., (2001) report a similar finding, in that a single DEX injection induces hippocampal apoptosis with cell death in both the dentate gyrus and CA1-CA3 regions. Recent studies have also established that glucocorticoids specifically target hippocampal neurons for apoptosis (Haynes et al., 2001). Consistent with this, Crochemore et al., (2005) has reported that primary hippocampal neurons treated with DEX have increased levels of apoptosis; while concurrent MR activation negates this affect. Additionally, MR antagonism with spironolactone potentiates GR induced hippocampal cell death (Crochemore et al., 2005). DEX treatment also induces apoptosis in the adult straitum (Haynes et al., 2001). Taken together, these studies provide evidence that ligand-bound GR is capable of inducing cortical and hippocampal cell death independent of an additional insult.

Despite reports that DEX treatment either *in vivo* or *in vitro* induces hippocampal apoptosis, Sapolsky and colleagues suggest that GR mediated cell death is excitotoxic (Roy and Sapolsky, 2003). Glucocorticoids were found to induce primary hippocampal cell death independent of caspase-3 activation (Roy and Sapolsky, 2003) and DNA fragmentation (Masters et al., 1989) both hallmarks of apoptosis. Furthermore, inhibition of poly (ADP-ribose) synthase and subsequently DNA repair does not alter the survival rate of glucocorticoid treated hippocampal neurons (Masters et al., 1989). The potential discrepancy between these studies may be the result of differences in the treatment paradigms. First, the studies conducted in the Sapolsky laboratory were investigating glucocorticoid exacerbation of cell death in primary hippocampal cultures treated concurrently with kainic acid (Masters et al.,

1989; Roy and Sapolsky, 2003), whereas the studies which report apoptosis as the cell death mechanism were investigating *in vivo* GR activation as the primary insult in the hippocampus (Hassan et al., 1996; Haynes et al., 2001). Moreover, the Sapolsky laboratory utilized high levels of CORT as the GR agonist; however, under this treatment paradigm both GR and MR are activated. Conversely, the studies that showed increases in apoptosis used DEX (60-70μg/kg) to selectively activate the GR (Hassan et al., 1996; Haynes et al., 2001). However, the possibility exists that the DEX dose administered can also bind and activate MR. Regardless of the cell death mechanism these studies indicate that glucocorticoids acting via the GR induce CNS death.

The stress hyporesponsive period

Studies reveal that inappropriate glucocorticoid exposure during development results in the onset of pathologies which emerge in juvenile life and persist into adulthood. Elevated glucocorticoid levels in the neonate induce growth retardation and impaired CNS development. Additionally, excess glucocorticoid exposure during embryonic life potentiates the occurrence of CNS cell death, cognitive and behavioral disorders and altered HPA axis regulation and these may persist into adulthood (Flagel et al., 2002; Kamphuis et al., 2004; Neal et al., 2004).

The stress hyporesponsive period (SHRP) in the neonatal rat protects it from the potentially harmful effects of endogenous glucocorticoids. During the SHRP, which extends from PND 4 to 14, the adrenal gland and pituitary have reduced response to a stressor (Schapiro et al., 1962; Schoenfeld et al., 1980). However, current studies question whether a true SHRP exists, since the HPA axis appears to

have stimuli specific activation in the neonatal rat pup (Kent et al., 1996). For example, in the rat pup a stressor may activate a portion of the HPA axis to initiate CORT synthesis and secretion; however, the response is generally muted and also depends on compounding environmental factors such as maternal behavior (Levin et al., 2000).

The predominant maternal behaviors that modulate the HPA axis during development are licking-grooming and arched back nursing. Rat pups that are reared under normal circumstances and receive ample maternal care do not respond to mild stressors such as brief periods of dam isolation, introduction to novel situations, placebo injections and restraint. However, rat pups maternally separated for a minimum of 8 hours respond to mild stressors that does not normally elicit an HPA response (Levin et al., 2000). In example, rat pups separated from the dam for 24 hours respond to saline injection by secreting ACTH. Conversely, rat pups, that are not separated, do not respond to similar injections with elevating ACTH levels (Suchecki et al., 1993b; Levine, 2002).

The type of maternal behavior experienced by rat pups is also responsible for regulating individual HPA axis components. Rat pups that are separated for 24 hours but are subjected to one of three behaviors (stroked, fed and stroked, or left undisturbed) by a handler respond differently to a stressor. Stroked animals respond to a saline injection with an increase in CORT levels; however, a change in ACTH is not detectable. Animals left undisturbed have the greatest stress response by elevating levels of both ACTH and CORT following a saline injection. Conversely, fed and

stroked animals do not respond with an increase in either ACTH or CORT (Suchecki et al., 1993a).

Depending on the stimulus, a stress response may be elicited regardless of the appropriate maternal behavior. Kainic acid injections cause an increase in ACTH levels in SHRP rats. However, even during the SHRP, rat pups of different ages respond to kainic acid injections with a differing amounts of ACTH secretion; the greatest response is observed at PND 6 (Kent et al., 1996). Stress responsive changes in CRH are also regulated in a stimulus and age specific manner. On PND 8, hypoglycemia upregulates CRH mRNA in some CRH neurons. Likewise, extreme cold upregulates CRH on PND 8, but not PND 6. Even though a stressor can elicit either an ACTH or CORT response, it is dampened compared to rats of other ages (Levine et al., 2000).

Excess glucocorticoids and the neonatal rat

Excess glucocorticoids act as neuroendangering compounds in the neonatal rat. Studies indicate that either perinatal or postnatal exposure to glucocorticoids leads to a variety of neuropathologies that persist throughout development and into adulthood (Flagel et al., 2002; Kamphuis et al., 2004; Neal et al., 2004; Burlet et al., 2005). Perinatal DEX administered to a pregnant dam results in decreased offspring weight at birth and delays in pup weight gain that persist for at least three weeks. Furthermore, neurological development was altered in the DEX-treated rat pups with deficits in righting reflex and grasping reflex detected at PND 3 and 10, respectively. HPA axis development was also changed in the DEX-treated pups with decreased basal ACTH and CORT levels at PND 1. Moreover, decreases in CRH mRNA and

protein in the paraventricular nucleus and median eminence was also reported (Burlet et al., 2005). Restraint stress of a pregnant dam and perinatal stress of rat pups has also been correlated with an increase in seizure vulnerability following kindling in both PND 14 male and female rat pups. However, a predisposition to kindled seizures in adulthood was only found in male rats (Edwards et al., 2002).

Postnatal DEX treatment also alters normal development with reduced somatic growth (Flagel et al., 2002; Neal et al., 2004) and abnormal neurological development (Kamphuis et al., 2004; Neal et al., 2004). Following postnatal DEX treatment rat pups have deficits in brain growth and learning and memory, altered social behavior and changes in HPA axis function (Kamphuis et al., 2004; Neal et al., 2004). In particular, DEX administered from PND 3 to 6 decreased somatic and brain growth (Flagel et al., 2002; Neal et al., 2004). DEX-treated rat pups are also less active in light and dark environments and have a decreased CORT response to novel environments when tested at PND 33 (Flagel et al., 2002). Additional studies have found that DEX-treatment from either PND 1-3 or 8-10 in female rat pups, but not male rat pups, results in deficits in spatial learning and memory tasks later in life (PND 31-63). However, there appears to be an age-related component of this response since DEX treatment at PND 28-30 caused impaired learning and memory in both the adult male and female rats (Machhor et al., 2004).

Neonatal cortisol treatment is also capable of altering HPA axis function in both immature and adult rats. Rat pups treated with cortisol during the third week of life have decreased morning basal plasma CORT levels when tested at 30 days of age.

These animals also have a prolonged stress response to restraint in adulthood (Ordyan et al., 2001).

Although postnatal glucocorticoid treatment can alter normal brain development by inducing neuronal cell death, there are conflicting data regarding the mechanism by which DEX induces CNS cell death. In one study, postnatal DEX treatment induced dendrite degeneration in developing hippocampal neurons (Tan et al., 2002). However, these authors suggest that despite the dendritic degeneration, apoptotic cell death did not occur. The markers used in these studies to measure the prevalence of hippocampal apoptosis in DEX-treated and control rats included immunohistochemical detection of Bcl-2, Bax and caspase3 and RT-PCR for changes in Bcl-2 and Bax mRNA expression. These data suggest that experimental and control treated rats have similar levels of both protein and mRNA expression for the chosen markers (Tan et al., 2002). However, Haynes et al., (2001) found that following a single DEX injection, immature male rats have an increased incidence of apoptosis within the hippocampus and striatum, but not the septum (Haynes et al., 2001). Consistent with this, studies show that DEX administered to the juvenile rat increases apoptosis in the dentate gyrus hilus and granule cell layers (Hassen et al., 1996), and when administered to the postnatal rat DEX can decrease the number and size of cortical neurons in the adult (Kreider et al., 2006).

7. Clinical Implications

In the prior sections of this chapter, the previous literature presented the concept that chronic GR activation in the developing and mature rat brain is neuroendangering. Clinical observations also support the hypothesis that GR agonists

may be harmful to the developing human brain (Fitzhardinge et al., 1974; Gibson et al., 1993; Bos et al., 1998; Shrivastava et al., 2000). Neonates exposed to excess glucocorticoid levels may be predisposed to developing neurological abnormalities and cognitive disorders such as schizophrenia (Lou et al., 1994; Koenig et al., 2002). Despite the putative neuroendangering aspects of glucocorticoids, DEX is commonly prescribed to treat premature infants for bronchopulmonary dysplasia. Such treatment paradigms have been reported to predispose them to various neuropathologies later in life, including cerebral palsy and changes in stress reactivity (Alkalay et al., 1996; Murphy et al., 2001; Murphy, 2001). Additionally, glucocorticoid and hypoxia exposure may increase the incidence of various neuropathologies in humans with underlying genetic predispositions for the disorders. However, due to the natural limitations associated with investigating human neuropathologies the postnatal rat has been utilized as an animal model for human brain development (Dobbing, 1974, , 1981; Hagberg et al., 1997). Therefore, changes in the postnatal rat brain following hypoxia and glucocorticoid treatment could potentially be extrapolated to the developing human CNS.

Glucocorticoid treatment of chronic lung disease

The average gestation for a human fetus is approximately 40 weeks. However, in the United States 10% of infants are born prematurely with gestational periods ranging from 22 to 37 weeks. Premature infants born prior to complete lung development are at a risk for developing respiratory distress syndrome. Respiratory distress syndrome is associated with a decrease in endogenous production of lung surfactant and is treated with an oral surfactant, exogenous glucocorticoids and

oxygen therapy administered by intubation (Doyle et al., 2006). The ventilator treatment is a subsequent predisposition for the chronic lung disease bronchopulmonary dysplasia. However, 70% to 75% of infants born prior to 26 weeks of gestation are immediately diagnosed with bronchopulmonary dysplasia independent of respiratory distress syndrome or oxygen therapy (Ehrenkranz et al., 2005).

The most common glucocorticoids administered for bronchopulmonary dysplasia prevention are DEX and betamethasone; both are synthetic glucocorticoids that have a higher affinity for the GR than the MR (Bar-Lev et al., 2004). Glucocorticoids are included in the chronic lung disease treatment regiment because they serve as both an anti-inflammatory agent and bronchodilator. The glucocorticoid anti-inflammatory properties are conferred by leukocyte inhibition; while the bronchodilator properties are mediated by increased β-andrenergic receptor numbers and subsequent β-andrenergic activity. Currently the treatment guidelines for glucocorticoid intervention of chronic lung disease vary in regards to the drug concentration and treatment duration (Grier and Halliday, 2005). Glucocorticoid treatment for chronic lung disease is beneficial for reducing supplementary oxygen needs and increasing the number of successful extubations (Doyle et al., 2006). However, controversy exists as to the possibility that glucocorticoids decrease neonatal morbidity, such as long term hospitalization or home oxygen therapy, and neonatal mortality (Yeh et al., 2004; Rademaker et al., 2006).

Hypoxia, glucocorticoids and human neuropathologies

Hypoxic insults and glucocorticoid exposure in the developing infant may cause various neuropathologies. Furthermore, the possibility exists that glucocorticoid treatment of premature hypoxic infants may exacerbate developmental abnormalities and increase the incidence of other pathologies, such as cerebral palsy (Shinwell et al., 2000; Yeh et al., 2004).

Various neuropathologies and developmental disorders have been associated with neonatal hypoxia. Severely premature infants have an increased incidence of cerebellar atrophy and mild periventricular abnormalities that have been identified by magnetic resonance imaging as early as 5.5 to 7 years of age (Krageloh-Mann et al., 1999). Prematurity and hypoxic-ischemia have also been correlated to the development of behavioral disorders, such as attention deficit disorder (Krageloh-Mann et al., 1999) and schizophrenia (Jones and Cannon, 1998; Dalman et al., 1999).

Several lines of research also indicate that either pre- or postnatal exposure to elevated glucocorticoid levels alters normal brain development. During gestation excess endogenous glucocorticoid exposure may play a role in the onset of prenatal stress syndrome and result in abnormal brain development. Women that experienced mid-gestational stress compared to non-stressed pregnant women give birth to children with a small head circumference and suboptimal neurological scores (Lou et al., 1994). Additionally, infants from women exposed to severe stressors, such as family death or influenza infection, during the second trimester of gestation have an increased incidence of schizophrenia (Koenig et al., 2002).

Exogenous postnatal glucocorticoid treatment for bronchopulmonary dysplasia also has the potential for negative side effects throughout the body

including hypertension, infection, ventricular hypertrophy and altered metabolic function (Ng et al., 1990; Brownlee et al., 1992; Bensky et al., 1996; Leitch et al., 1999). Neurological side effects are also a potential with exogenous glucocorticoid treatment. Infants that receive glucocorticoid treatment present with neurological abnormalities within the first year of life (Fitzhardinge et al., 1974) and an increase in the incidence of neuromotor dysfunction by the age of two (Bos et al., 1998; Yeh et al., 2004). Furthermore, these infants also have decreased head circumference (Gibson et al., 1996; Shrivastava et al., 2000; Murphy, 2001). Clinical studies have also correlated DEX use in premature infants to an increased incidence of cerebral palsy (Shinwell et al., 2000; Murphy et al., 2001). Preterm infants that received DEX had a significantly higher incidence of cerebral palsy (49%) compared to children treated with saline (15%) (Shinwell et al., 2000). These data indicate that glucocorticoid treatment of hypoxic infants exacerbates abnormal brain development with subsequent onset of pathologies.

The rat as a model for human brain development

Data generated from studies utilizing animal experimentation can sometimes be extrapolated to the human condition. Neonatal rat pup brain development has been correlated to human brain development based on brain growth rate, neurochemical expression, electroencephalographic patterns and patterns of synapse formation (Dobbing, 1974, , 1981; Hagberg et al., 1997). Based on these criteria, the PND 3 rat brain corresponds to approximately a 28 to 29 week gestation human brain, while a PND 7 rat brain corresponds to a full gestation human brain of 38 to 40 weeks old (Flagel et al., 2002). Therefore, glucocorticoid treatment of PND 3rat pups

corresponds to the human developmental period when preterm infants may be treated with DEX to augment lung development. Thus, information gained from animal studies could be potentially relevant in determining the effects of DEX on the developing human brain.

Parallels in the human and rat cingulate cortex

Deficits or injury to the human cingulate cortex are similar to neuropathologies associated with hypoxia and glucocorticoids. The cingulate cortex is one of four brain regions that make up the limbic system. In addition to the cingulate cortex, the amygdala, hippocampus and parahippocampal gyri comprise the limbic system (Nolte and Sundsten, 2002). Retrograde and anterograde axon tracers have been utilized in both primates and rats to examine the afferent and efferent cingulate cortex connections. These connections include sensory and hypothalamic inputs, extensive intrinsic corticocortical connections, reciprocal limbic system connections and projections to the striatum, thalamus and hypothalamus (Ongur and Price, 2000; Nolte and Sundsten, 2002; Ongur et al., 2003). The cingulate cortex is further subdivided into two distinct brain regions the anterior and posterior cingulate cortex.

The anterior cingulate cortex is a region of the medial prefrontal cortex in both the human (Ongur and Price, 2000) and the rat (Shah and Treit, 2003). The anterior cingulate cortex afferent and efferent connections are similar between different species. Numerous lines of research have begun to establish the anterior cingulate cortex function. The human anterior cingulate cortex is extensively activated when challenged with executive processing or cognitively demanding reasoning. The role the anterior cingulate cortex plays in executive processing is to

inhibit a response to non-essential stimuli. Additional activities that recruit the anterior cingulate cortex are responses to manual, vocal or oculomoter stimuli (Awh and Gehring, 1999). The anterior cingulate cortex also mediates the brain's response to emotion associated with complex behaviors, such as pain (Eisenberger et al., 2003). Deficits in human anterior cingulate cortex function present as a variety of psychological disorders including attention deficit disorder, obsessive compulsive disorder and schizophrenia (Benes et al., 1987; Bush et al., 1999; Heidbreder and Groenewegen, 2003). Furthermore, post mortem cytoarchitecture analysis of brains from schizophrenic patients revealed a lower density of neurons in the anterior cingulate cortex laminar layers II (Benes and Bird, 1987) and IV (Benes et al., 1986).

The rat anterior cingulate cortex function has similarities with the human. A lesion to the rat anterior cingulate cortex causes a deficit in fear responses. Furthermore, an anterior cingulate cortex lesion decreases the pre-pulse inhibition response in the rat, which is considered by psychobiologists to be a characteristic of a schizophrenic pathology (Yee, 2000; Kung et al., 2003; Shah and Treit, 2003).

The posterior cingulate cortex is involved in processing complex emotions. fMRI studies reveal that the posterior cingulate cortex has extensive activation when a human is presented with either pleasant or unpleasant words (Maddock et al., 2003). The posterior cingulate cortex also has extensive activation during episodic memory retrieval, such as those which constitutes a person's autobiographical memories (Maddock et al., 2001; Piefke et al., 2003). Furthermore, deficits in the human posterior cingulate cortex are associated with developmental amnesia, a disorder which impairs episodic memory retrieval. Developmental amnesia is a

neuropathology associated with hypoxic-ischemic insults during the first year of life (Vargha-Khadem et al., 2003).

Lesion models in the rat are also utilized to investigate the role of the posterior cingulate cortex in learning and memory by testing both allothetic and idiothetic navigation. Allothetic navigation requires the use of external cues, while idiothetic requires internal cues. Rats with posterior cingulate cortex lesions have deficits in learning both forms of navigation, while anterior cingulate cortex ablations result in moderate deficits in allothetic navigation (Whishaw et al., 2001).

Considering human cingulate cortex function, hypoxia and glucocorticoids may directly injure this region, which may cause the various observed hypoxic and glucocorticoid-induced neuropathologies such as schizophrenia and attention deficit disorder. Further, since overlap exists in rat and human cingulate cortex function hypoxia and glucocorticoid injury to the developing rat cingulate cortex may provide insight to the human neuropathologies.

8. Summary and Specific Aims

CNS development is characterized by the genesis of excess neurons, which are selected to die if inappropriate synaptic connections are made (Blaschke et al., 1996; Thomaidou et al., 1997). A transient period of NOCD has been identified during the first postnatal week in the rat cortex and hippocampus (Gould et al., 1991; Ferrer et al., 1994b; Spreafico et al., 1995). The putative mechanism that regulates NOCD is apoptosis. Apoptosis initiates death through a cellular cascade of events culminating with caspase activation, nuclear DNA fragmentation and cell disassembly (Kerr et al., 1972; Schweichel and Merker, 1973). The Bcl-2 gene

family, which consists of both pro- and anti-apoptotic members, mediates apoptosis; however, the characterization of these genes expression patterns and regulation during development is incomplete in the literature.

Bnip3 is a pro-apoptotic BH3-only member of the Bcl-2 gene family. However, Bnip3 is unique in that the BH3 domain does not confer Bnip3 dimerization with the anti-apoptotic members or mediates the protein's killing activity. Conversely, studies reveal that the TM domain is essential to Bnip3 protein function (Chen et al., 1997a; Ray et al., 2000; Vande Velde et al., 2000). Bnip3 regulation has predominantly focused on hypoxic-induction of Bnip3 gene transcription. In peripheral cell culture lines, studies have established that hypoxic-regulation of Bnip3 is mediated by HIF1 via a HRE within the promoter (Bruick, 2000). Bnip3 protein expression also increases in both non-neuronal and neuronal cells following hypoxia (Bruick, 2000). In the CNS, Bnip3 is regulated by hypoxia in the adult rat cortex and hippocampus and in differentiated oligodendrocytes (Schmidt-Kastner et al., 2004; Althaus et al., 2006; Burton et al., 2006).

DEX is a synthetic glucocorticoid that is neuroendangering to the developing brain. A single DEX injection in the juvenile rat increases levels of apoptosis in the hippocampus, cortex and striatum (Hassan et al., 1996; Haynes et al., 2001). A component of DEX neuroendangerment is conferred by GR regulation of the Bcl-2 gene family. However, glucocorticoid regulation of the Bcl-2 family is not conserved throughout development. In the juvenile rat, DEX selects for apoptosis by downregulating expression of the anti-apoptotic genes Bcl-2 and Bcl-X_L and upregulating expression of the pro-apoptotic gene Bax (Almeida et al., 2000).

Conversely in the postnatal rat, DEX does not regulate expression of Bcl-2, Bcl-X_L or Bax (Tan et al, 2002), despite reports of DEX-induced cell death (Kreider et al., 2006) Thus, the mechanisms of glucocorticoid mediated cell death during development have yet to be elucidated.

DEX is also commonly prescribed to treat human preterm infants with bronchopulmonary dysplasia (Halliday, 1999). However, clinical observations have correlated DEX use in premature infants to an increased predisposition of neurological abnormalities including: cerebral palsy, neuromotor dysfunction and schizophrenia (Shinwell et al., 2000; Shrivastava et al., 2000; Koenig et al., 2002).

Based on these reports from the current literature I hypothesized that glucocorticoids exacerbate hypoxia induced cortical neuron death, which alters normal CNS development. Further, the glucocorticoid enhancement of hypoxia cell death is mediated by GR modulation of hypoxia induced Bnip3 expression. The specific aims that address this hypothesis and conducted for this dissertation are described as follows:

1) To identify brain regions that endogenously express Bnip3 mRNA in the postnatal male and female rat and to subsequently determine the developmental ontogeny for Bnip3 mRNA in the male and female anterior and posterior cingulate cortices and hippocampus.

2) To determine if glucocorticoids exacerbate hypoxia induced cell death in primary cortical neurons through regulation of Bnip3 gene transcription and to elucidate GR and HIF1 regulation of the Bnip3 promoter.

Since DEX is used clinically to hasten lung development in premature infants, often with little regard to its neurodevelopmental consequences, these studies could identify a mechanism by which glucocorticoids modulate hypoxia sensitive targets in development to potential impact later neuropathology.

Chapter 3

Localization and developmental ontogeny of the pro-apoptotic Bnip3 mRNA in the postnatal rat cortex and hippocampus

Abstract

Naturally occurring cell death occurs during the first two postnatal weeks in the rat cortex and hippocampus. During this process, apoptosis is initiated by activating or altering expression of pro-apoptotic members of the Bcl-2 family. Bnip3 is a pro-apoptotic member of the Bcl-2 family that induces cell death by opening the mitochondrial permeability transition pore. To date, Bnip3 expression in the central nervous system has only been examined during hypoxia-mediated apoptosis in the adult rat brain. In this study, we investigated the localization and ontogeny of Bnip3 mRNA expression in the postnatal male and female rat brain. Bnip3 mRNA was localized by in situ hybridization in the neonatal cortex, hippocampus, habenula and thalamus. Using quantitative real time RT-PCR, Bnip3 mRNA levels were found to be greatest at postnatal day 6.5 in the female anterior and posterior cingulate cortices and hippocampus. Bnip3 mRNA expression also increased in the male anterior cingulate cortex at postnatal day 6.5. However, a developmental change in Bnip3 levels did not occur in the male posterior cingulate cortex and hippocampus. In the anterior cingulate cortex on postnatal day 6.0 and adulthood, female rats had significantly greater levels of Bnip3 mRNA compared to that of males. Altering levels of testosterone in the neonatal rat did not alter the sex differences in Bnip3

mRNA levels. The transient increase in Bnip3 mRNA expression correlates with naturally occurring cell death in the neonatal rat cortex and hippocampus. Thus, Bnip3 may be a mediator of developmental apoptosis in the postnatal rat brain.

Introduction

Development of the central nervous system (CNS) is marked by the genesis of greater numbers of neurons than is needed. Apoptotic removal of these excess neurons in response to growth factor withdrawal is a normal developmental process of the mammalian brain (Oppenheim, 1991). In the hippocampus and cortex of the developing rat brain, this process of naturally occurring cell death (NOCD) extends into postnatal life. In the neonatal rat hippocampus, an increase in postnatal apoptosis occurs during the first week. Subsequently, as the rat matures the number of apoptotic cells decreases (Gould et al., 1991; Ferrer et al., 1994b). Similarly, the rat cortex has a progressive increase in apoptosis during the first postnatal week, with a surge between postnatal day (PND) 5 and 8, which subsequently tapers (Ferrer et al., 1994b; Spreafico et al., 1995).

NOCD is mediated by the Bcl-2 gene family. The members of the Bcl-2 family are divided into two subfamilies, pro- and anti-apoptotic, which all share homology with at least one of the four conserved Bcl-2 homology (BH) domains of the anti-apoptotic Bcl-2 gene. The pro-apoptotic members of the Bcl-2 family are further divided into two groups based on which of the BH domains they possess. One group possesses multiple BH domains, while the second group possesses only Bcl-2 homology domain 3 (BH3) (Adams and Cory, 2001). The BH3 domain typically mediates an interaction between a BH3-only member and either a pro-apoptotic

multi-domain member or anti-apoptotic member such as Bcl-2 and Bcl-X_L, which results in disruption of mitochondrial function and onset of apoptosis (Yin et al., 1994b; Adams and Cory, 2001; Cheng et al., 2001; Letai et al., 2002).

The pro- and anti-apoptotic members of the Bcl-2 family respond to apoptotic stimuli to either initiate or prevent programmed cell death. However, the apoptotic mechanism governing NOCD is not conserved between brain regions (Lindsten et al., 2005). For example, apoptosis induced by survival factor withdrawal in sympathetic neurons is mediated by the pro-apoptotic members Bax and Bim (Deckwerth et al., 1996; Putcha et al., 2001); while in cerebellar granule neurons Bax but not Bim induces cell death (Miller et al., 1997; Putcha et al., 2002). Since, multiple mechanisms are implicated in controlling apoptosis in different brain regions multiple pro-apoptotic members of the Bcl-2 family may mediate the increase in cell death in the postnatal rat cortex and hippocampus. Thus a transient increase in expression of a pro-apoptotic member would theoretically coincide with the increase in postnatal NOCD. Such a developmental ontogeny has been established for only a few of the pro-apoptotic Bcl-2 family members in the rat brain (Ferrer et al., 1994a; Hamner et al., 1999; Rickman et al., 1999; Mooney and Miller, 2000). Therefore, additional characterization of the developmental ontogeny for the pro-apoptotic members is necessary to identify putative mediators of NOCD.

Bnip3 is a unique BH3-only member of the Bcl-2 family (Chen et al., 1997a; Zhang et al., 2003) that does not require BH3 mediated interaction with an anti-apoptotic member to induce cell death (Ray et al., 2000) The cell-killing effects of Bnip3 are mediated by the transmembrane (TM) domain which localizes inactive

protein to the mitochondrial membrane and mediates Bnip3 homodimerization and heterodimerization with Bcl-X_L. However, over-expression of Bcl-X_L delays but does not prevent Bnip3 mediated cell death (Ray et al., 2000). An apoptotic stimulus that increases Bnip3 expression and activates the protein can induce cell death in a mitochondrial dependent manner. The morphological features associated with Bnip3 mediated apoptosis include the formation of cytoplasmic vacuoles (Vande Velde et al., 2000) similar to vacuoles formed in neuronal cells following withdrawal of growth factors (Xue et al., 1999). Furthermore, apoptosis induced by Bnip3 has been shown to be attenuated by epithelial- and insulin-like growth factors (Kothari et al., 2003).

The regulation of Bnip3 in brain has been restricted to studies whose results implicate the protein in mediating hypoxia-induced cell death in the adult rat hippocampus (Schmidt-Kastner et al., 2004) or show an upregulation of Bnip3 mRNA during oligodendroglial differentiation (Itoh et al., 2003). In the following studies, we investigated the localization and ontogeny of Bnip3 mRNA expression in the developing male and female cortex and hippocampus. Levels of Bnip3 mRNA were measured to determine if a transient increase in Bnip3 expression correlates to elevated levels of apoptosis during the first postnatal week, potentially implicating Bnip3 as an important player in normal postnatal brain development.

Materials and Methods

Animals

Timed pregnant female Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed at Colorado State

University's laboratory animal research facility. The rat offspring were utilized in experiments to determine both the developmental ontogeny and hormonal regulation of Bnip3. Adult male and female rats were also purchased from Charles River Laboratories and utilized as controls for comparison with neonates.

The pregnant dams and adult rats were housed under a 12:12 light:dark cycle (lights on at 0700h) for one week prior to being killed. Beginning on gestational day 21, pregnant dams were examined every two hours in order to record the litter's birth date and time. The day of birth was considered as PND 0. Upon birth all litters were culled into groups of 10 pups, 5 males and 5 females, and returned to the dam. Male and female neonates that were utilized to determine the anatomical expression and developmental ontogeny of Bnip3 mRNA expression were halothane anesthetized and sacrificed on PND 4.0, 6.0, 6.5, 7.0, 7.5, 8.0, 10.0, and 21.0. To examine gonadal hormone regulation of Bnip3 mRNA expression in the male rat pup, animals were cryoanesthetized and testis were either removed (gonadectomized, GDX) or left intact following a sham surgery on PND 0. These animals were subsequently sacrificed on PND 6.5. Gonadal hormone regulation of Bnip3 mRNA in the female rat pup was investigated by giving animals a daily subcutaneous injection of testosterone propionate (TP; 50μg/day) in 10μl of safflower oil on PND 3.5 to PND 5.5. Controls received vehicle alone at the same injection regimen. Twenty four hours after receiving the third injection animals were halothane anesthetized and killed. All animal protocols were previously approved by the Colorado State University Animal Care and Use Committee and carried out in accordance with the National Institutes of Health and Institutional Animal Care and Use Guidelines.

Bnip3 cDNA Plasmid Synthesis

A Bnip3 cDNA was generated using RT-PCR on rat cortical mRNA with a forward (5' TTT AAA CAC CCG AAG CGC ACA G-3') and a reverse (5' GTT GTC AGA CGC CTT CCA ATG TAG A-3') primer pair specific for the rat Bnip3 coding sequence. The primer design was based on an existing GenBank sequence (GenBank accession nos. AF243515, forward primer position 464 and reverse primer position 606) and analyzed using Oligo software, version 6.51 (Molecular Biology Insights, Cascade, CO, USA). Specificity of the designed Bnip3 primers was confirmed using a nucleotide Blast search (NCBI Entrez pubmed). PCR was conducted in the Hybaid Omnigene PCR Thermocycler (Thermo Electron Corp. Waltham, MA, USA) and amplified for 40 cycles. The initial melting step was 94°C for 2 minutes followed by 40 cycles of 94°C melting (10.0 second), 64°C annealing (30.0 seconds), and 72°C elongation (1 minute). The size of the amplified Bnip3 cDNA was confirmed by 1% agarose gel electrophoresis. Bnip3 cDNA was cloned into the pCRII-TOPO plasmid containing both an SP6 and T7 promoter site using the HTP TOPO TA Cloning Kit Dual Promoter system (Invitrogen Life Technologies, Carlsbad, CA, USA).

In Situ Hybridization

To examine the localization and developmental regulation of Bnip3 mRNA in brain, we performed in situ hybridization (ISH) on paraformaldehyde fixed rat brain tissue using a radiolabeled cRNA probe. The Bnip3 cDNA containing plasmid was linearized with either Not1 (antisense) or Kpn1 (sense). S^{35}-UTP (1000 ci/mmole) radiolabeled sense and antisense Bnip3 cRNA probes were transcribed *in vitro* using

either T7 or SP6 RNA polymerase, respectively. The sense and antisense probes were used for ISH.

Brain tissue was collected from rat pups that were halothane anesthetized and subsequently perfused via the left ventricle initially with 0.9% saline plus heparin followed by 4% neutral buffered paraformaldehyde. Brains were harvested and placed in 30% sucrose phosphate buffered saline (PBS) until the brains were saturated. 25μm coronal sections were taken with a Leitz 1720 Digital Kryostat. Brain sections were post fixed with 10% formaldehyde in PBS for 8 minutes. Subsequently tissue was acetylated with a 0.1M Triethanolamne-HCL/0.9% saline (TEA) with 0.25 % acetic anhydride then dehydrated in increasing concentrations of ethanol. Tissue was incubated overnight at 64°C in hybridization solution (50% formamide, 0.06 M NaCl, 0.02 M Tris, 0.01 M EDTA, 10% Dextran sulfate, 2 x Denhart's solution, 50 mM dithiothreitol, 0.2% SDS, 100 mg/ml salmon sperm DNA, 50 mg/ml total yeast RNA and 50 mg/ml yeast transfer RNA) containing the S^{35} Bnip3 cRNA (2 x 10^7 cpm/ml) probe. Tissue was subsequently washed with a 2x standard saline citrate (SSC) solution followed by digestion of nonhybridized probe with 30 mg/ml ribonuclease A for 30 minutes at 37°C. The final wash stringency was 0.1 x SSC at 64°C. Radiolabeled tissues were exposed to Kodak Biomax MR Autoradiographic Film (Eastman Kodak Company, Rochester, NY, USA) for 3 days.

Image analysis

The density of hybridization signal per fixed area was determined using a CCD camera (Sony XC-77, Tokyo, Japan) with a Nikon (Melville, NY, USA) lens and the Scion Image (Frederick, MD, USA) software package. The anterior cingulate,

posterior cingulate, primary motor and sensory cortices, in addition to the septum, striatum, thalamus and hippocampus (CA1, CA3 and dentate gyrus) were independently analyzed. The brain regions were identified using a rat brain atlas (Swanson, 1992). For each brain region two measurements were taken bilaterally from five different sections of each rat brain. The measurements obtained from the five sections were averaged to obtain a single value for each rat.

Generation of standard curves for quantitative real time RT-PCR

To determine the absolute concentration of Bnip3 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in experimental tissue, cDNAs for Bnip3 and GAPDH were used. The Bnip3 cDNAs used to generate a standard curve were obtained from the previously described Bnip3 plasmid. The GAPDH cDNAs were generated by RT-PCR on cortical rat brain tissue with forward (5' ACG GCA AAT TCA ACG GCA CAG-3') and reverse (5' TCC AGG CGG CAC GTC AGA-3') primer pairs specific for the rat GAPDH coding sequence. Amplification consisted of 40 cycles with the initial melting step of 94°C for 2 minutes followed by 40 cycles of 94°C melting (10.0 second), 64°C annealing (30.0 seconds), and 72°C elongation (1 minute). Size of the amplified GAPDH PCR product was confirmed by 1% agarose gel electrophoresis. Bnip3 and GAPDH cDNA concentrations were calculated from an absorbance reading at wavelength 260 (OD260) using a Beckman D6530 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). The cDNAs were diluted to a stock concentration of 1ng/ml then subsequently serially diluted from 1ng/ml to 10fg/ml in PCR grade sterile water (Roche Inst. Indianapolis, IN) to generate six working standards for each primer pair.

Brain Dissection for Quantitative Real Time RT-PCR

Quantification of Bnip3 mRNA expression and Bnip3 mRNA expression relative to GAPDH was determined using real time RT-PCR with the LightCycler 1.25 system (Roche Inst. Indianapolis, IN, USA). Brain tissue utilized for real time RT-PCR was collected from rat pups that were halothane anesthetized and subsequently sacrificed by decapitation. Brains were harvested, immediately frozen in -20°C 3-methylbutane. The anterior and posterior cingulate cortices and hippocampus were dissected from frozen 150μm coronal sections. Total RNA was isolated using the protocol of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Concentration of total RNA was determined using spectrophotometry (OD 260/280).

Quantitative Real Time RT-PCR

0.5 μg of total RNA was reverse transcribed in duplicate to account for variance associated with the reverse transcription procedure. Reverse transcription was accomplished with MMLV reverse transcriptase and oligoDT primers (Invitrogen, Carlsbad, CA, USA). Quantitative real time RT-PCR reactions were performed on the duplicate cDNA samples with the Bnip3 and GAPDH primer pairs. The individual PCR reactions for each of the primer pairs included 0.5 U Platinum Taq antibody (Invitrogen, Carlsbad, CA, USA), 100 mM Tris-Cl, 0.5 U Taq polymerase, 2 μl of 10x stock of SYBR green I (Roche Inst. Indianapolis, IN, USA), 0.5μM of forward primer and reverse Bnip3 primer and 0.125μM of forward and reverse GAPDH primer. In addition to the above reagents 3mM and 5mM MgCl2 was added to the Bnip3 and GAPDH reactions, respectively. The Bnip3 amplification protocol was 92^{oC} for 2 minutes, followed by 45 cycles of 95°C (melting) for 1

second, 62°C (annealing) for 5 seconds and 72°C (elongation) for 15 seconds. The GAPDH amplification protocol matched the Bnip3 protocol with the exception of 66°C annealing temperature. Samples were assayed in duplicate alongside the Bnip3 or GAPDH cDNA standard curve for determination of the absolute concentration of the experimental Bnip3 or GAPDH mRNAs. In all experiments, samples which included water in place of template were used as negative controls. The absolute concentration of unknown Bnip3 and GAPDH mRNA was determined by the LightCycler Data Analysis Software (Roche Inst. Indianapolis, IN, USA). The relative levels of Bnip3 mRNA expression for the developmental ontogeny experiment were generated by dividing the absolute Bnip3 concentration by the absolute GAPDH concentration for each experimental mRNA. Levels of Bnip3 mRNA expression in studies measuring hormonal regulation of Bnip3 mRNA were reported as the absolute concentration of Bnip3 (pg/ng of cDNA).

Statistical analysis

Data were analyzed by a two way ANOVA using the Statview data analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). Analysis of variance was performed for each experiment. Student-Newman-Keul's and t-test procedures were used posthoc for individual comparisons. Differences were considered significant when $p<0.05$. Data was expressed as group means $\pm$ SEM.

Results

Distribution of Bnip3 mRNA expression in the developing and mature rat brain

Using ISH we first identified regions of the developing and adult rat brain that express Bnip3 mRNA. In the neonate, Bnip3 mRNA is found throughout the cortex

with intense expression in the anterior cingulate cortex, posterior cingulate cortex, and piriform cortex (Fig. 9A-B). Additionally, Bnip3 mRNA is expressed in the hippocampus (regions CA1-CA3 and dentate gyrus), habenula and medial thalamus (Fig. 9A-B). In contrast, Bnip3 mRNA expression is restricted to only a few brain regions in the adult rat brain. Bnip3 mRNA was found in the adult anterior and posterior cingulate cortices and hippocampal regions CA1-CA3 (Fig. 9C).

Endogenous Bnip3 mRNA expression changes during the development of the neonatal rat brain

Using ISH, we qualitatively investigated the developmental ontogeny of Bnip3 expression in the male and female anterior and posterior cingulate cortex and hippocampus. On PND 7.0 male rat pups have significantly ($F_{1,29}$=16.96; $p<0.05$) greater Bnip3 mRNA expression in the anterior cingulate cortex compared to levels found on PND 4.0 (Fig. 10A). In contrast, Bnip3 expression in the anterior cingulate cortex of the PND 7.0 female was not different from that on PND 4.0 (Fig. 10A). In the posterior cingulate cortex of both males and females, there was significantly ($F_{1,30}$=15.04; $p<0.05$) more Bnip3 mRNA on PND 7.0 then on PND 4.0 (Fig. 10B). In the hippocampus Bnip3 mRNA expression was significantly ($F_{1,18}$=10.06; $p<0.05$) elevated on PND 7.0 in the female neonate compared to Bnip3 levels on PND 4.0 (Fig 10C). Conversely, the male hippocampus has similar levels of Bnip3 mRNA expression on PND 4.0 and PND 7.0 (Fig. 10C).

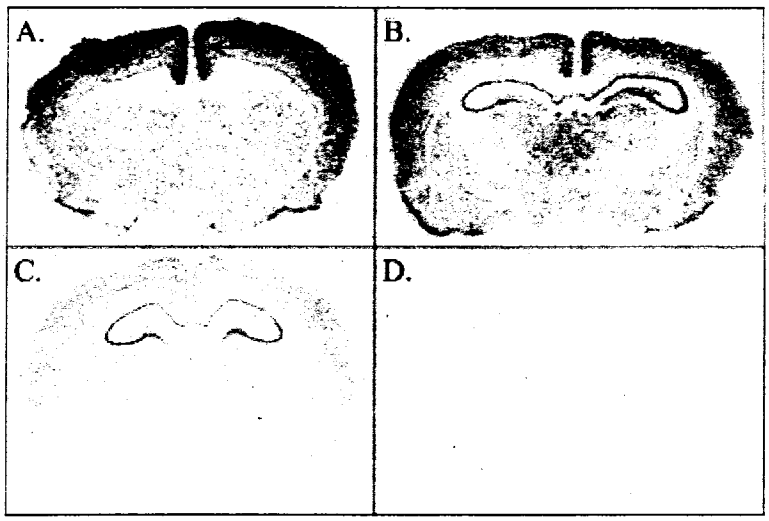


Figure 9. In situ hybridization analysis of Bnip3 mRNA expression in developing male and female rat brain. Bnip3 mRNA expression in the (A) rostral and (B) caudal rat brain at PND 7 and (C) adult rat brain. (D) Sense directed probe in adult rat brain shows no hybridization. The arrow identifies the anterior cingulate cortex in panel A. The asterisk identifies the posterior cingulate cortex in panel B.

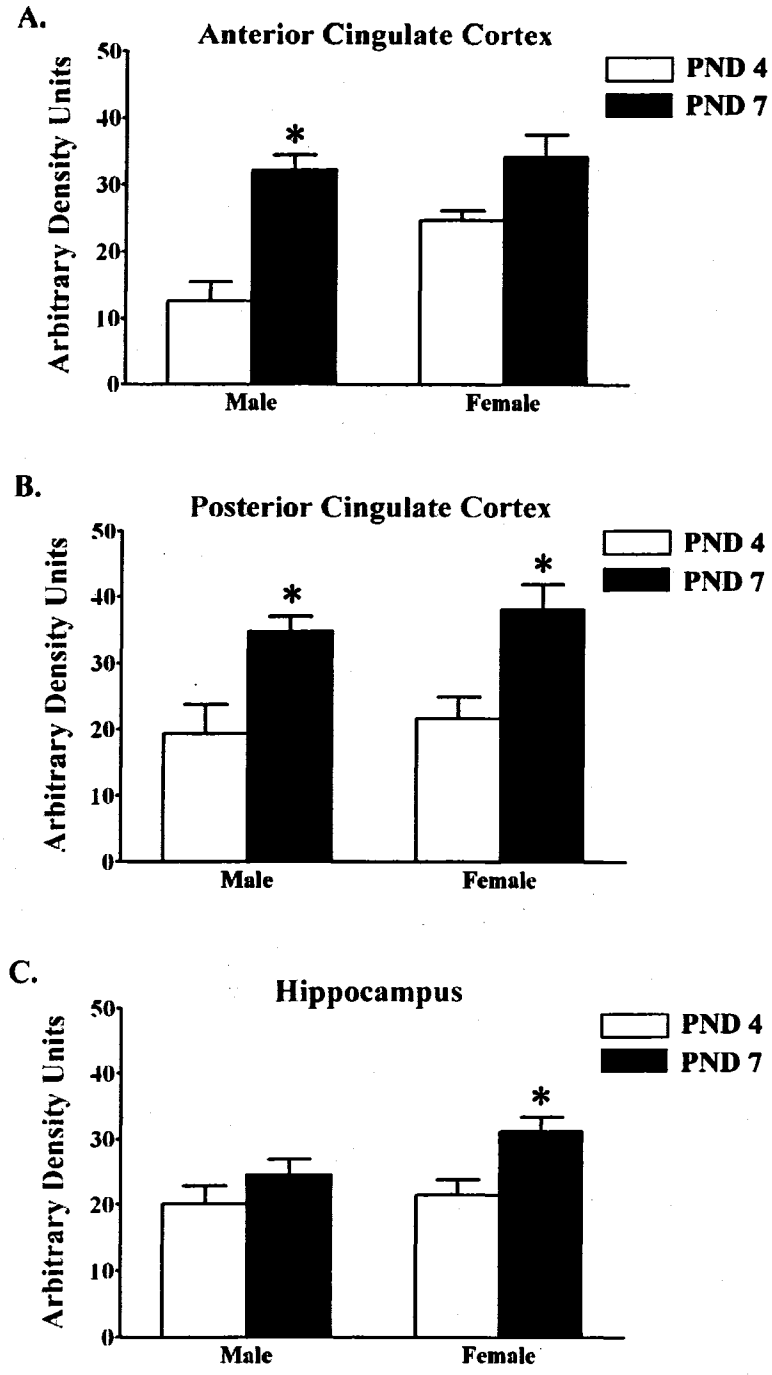


Figure 10. Comparison of Bnip3 mRNA expression in the (A) anterior cingulate cortex, (B) posterior cingulate cortex and (C) hippocampus of male and female rats on PND 4 (white bars) and PND 7 (black bars). Each bar represents the mean ± S.E.M. of 5-13 determinations. Two-way ANOVA (sex by age) followed posthoc by Student's t-test revealed significant differences (*$p<0.05$) between ages.

Developmental ontogeny of Bnip3 mRNA expression in the anterior and posterior cingulate cortices and hippocampus

In light of the changes observed in Bnip3 mRNA expression between PND 4.0 and 7.0 animals, we further investigated the developmental ontogeny of Bnip3 mRNA at additional postnatal ages using real time RT-PCR. In the anterior cingulate cortex Bnip3 mRNA levels were significantly elevated on PND 6.0 and 6.5 ($F_{8,76}$=7.61; $p<0.05$) in female rat pups and on PND 6.5 ($F_{8,75}$=4.43; $p<0.05$) in male rat pups compared to all other ages (Fig. 11A). Furthermore, in the male anterior cingulate cortex Bnip3 levels on PND 6.5 were eight times greater than in the adult; while Bnip3 levels on PND 6.5 in the females were five times greater than the adult. The elevated levels of Bnip3 mRNA on PND 6.5 decreased to adult levels by PND 7.0 in both the male and female anterior cingulate cortex. Additionally, on PND 6.0 and in the adult the female anterior cingulate cortex had significantly ($F_{1,151}$=4.43; $p<0.05$) greater levels of Bnip3 than the male anterior cingulate cortex (Fig. 11A). Similar to the anterior cingulate cortex, Bnip3 levels were significantly greater on PND 6.5 in the female posterior cingulate cortex ($F_{8,77}$=4.27; $p<0.05$) compared to all other ages (Fig. 11B). However, the increase in Bnip3 levels on PND 6.5 in the female posterior cingulate cortex was only two times greater than the adult. In the male posterior cingulate cortex no significant change in Bnip3 levels were measured throughout postnatal development (Fig. 11B). In the female hippocampus Bnip3 levels were significantly ($F_{8,60}$=2.70; $p<0.05$) elevated on PND 6.5 compared to all other ages (Fig. 11C). The levels of Bnip3 on PND 6.5 in the female

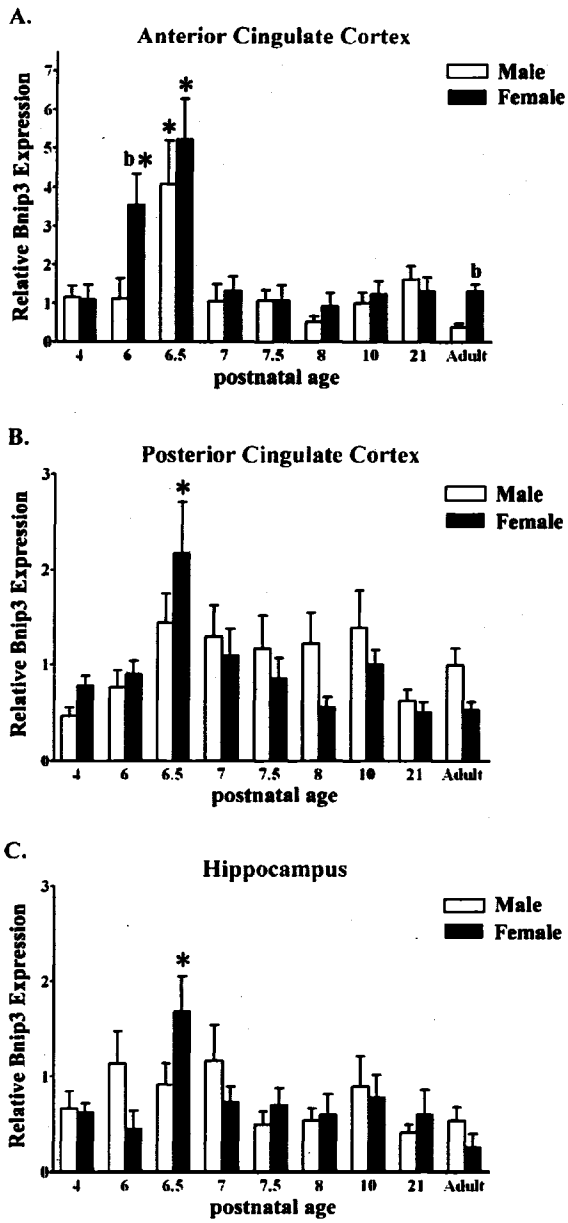


Figure 11. Developmental ontogeny of Bnip3 mRNA expression in the rat anterior and posterior cingulate cortices and hippocampus. Bnip3 mRNA expression was determined using quantitative real time RT-PCR on brain tissues taken from PND 4.0, 6.0, 6.5, 7.0, 7.5, 8.0, 10.0, 21.0 and adult male (white bars) and female (black bars) rats. Bnip3 mRNA levels were calculated as a ratio with GAPDH mRNA levels measured in the same samples of anterior cingulate cortex (A) posterior cingulate cortex (B) and hippocampus (C). Each bar represents the mean $\pm$ S.E.M. of 6-13 determinations. Data were analyzed by two-way ANOVA (sex by age) followed by Student-Newman Keul's posthoc test. Significant differences (*$p<0.05$) in Bnip3 mRNA expression across age. Multiple t-test comparisons indicate significant differences between the sexes at a given age ($^{b}p<0.05$).

hippocampus were approximately two times greater than Bnip3 levels in the adult female hippocampus. Bnip3 mRNA levels did not change across postnatal age in the male hippocampus (Fig. 3C).

Testosterone does not alter Bnip3 mRNA expression in the postnatal rat brain

Gonadal hormones have been shown to regulate members of the Bcl-2 gene family (Belcredito et al., 2001; Hur et al., 2004), Additionally, a transient increase in postnatal estrogen receptor expression has been reported in the cortex and hippocampus at ages that correspond to our measured increase in Bnip3 mRNA (MacLusky et al., 1979; O'Keefe and Handa, 1990; O'Keefe et al., 1993; O'Keefe et al., 1995). Therefore, we investigated the hormonal regulation of Bnip3 mRNA in the postnatal male and female rat pup to determine if the previously observed sex differences were a result of organizational actions of testosterone. At PND 6.5 GDX male rat pups had Bnip3 mRNA levels that were similar to the intact males in all brain regions examined (Table 2). Bnip3 mRNA was also measured in postnatal female rats after a daily subcutaneous injection of either TP or vehicle from PND 3.5-5.5. At PND 6.5 there was no significant affect of TP on Bnip3 mRNA expression in the anterior cingulate cortex, posterior cingulate cortex and hippocampus compared to the vehicle treated controls (Table 2).

Discussion

The experiments outlined in this study describe the localization and developmental ontogeny of Bnip3 mRNA in the postnatal and adult male and female rat brain. Bnip3 mRNA is expressed in multiple brain regions including the cingulate

Table 2

Bnip3 mRNA (pg/ng cDNA) levels in the anterior cingulate cortex, posterior cingulate cortex and hippocampus of postnatal GDX and intact male rats and TP and vehicle treated female rats.

Subject	Anterior cingulate cortex	Posterior cingulate cortex	Hippocampus
Male GDX	5.2 ± 0.9	5.6 ± 1.1	3.7 ± 0.7
Male sham	6.2 ± 0.7	4.0 ± 0.6	4.5 ± 0.9
Female TP	6.1 ± 0.9	9.3 ± 1.8	3.8 ± 0.8
Female vehicle	5.7 ± 1.4	6.7 ± 1.4	3.4 ± 0.4

Quantitative real time RT-PCR with primers specific to Bnip3 were utilized to measure Bnip3 expression levels in intact and gonadectomized (GDX) PND 6.5 male rat pups and PND 6.5 female rat pups treated with either vehicle or testosterone propionate (TP). Bnip3 mRNA levels (pg/ng of cDNA) in the anterior cingulate cortex, posterior cingulate cortex and hippocampus were not changed following gonadectomy of male rat pups or testosterone treatment of female rat pups (values represent mean ± S.E.M. for 10-13 determinations).

cortex and hippocampus in the developing and adult rat brain. Additionally, Bnip3 mRNA is expressed at higher levels and in a greater number of regions in the neonatal rat brain compared to the adult rat brain. The developing rat brain also exhibits differences in the ontogeny of Bnip3 expression depending on the animal's sex and the brain region examined. In the neonatal female anterior cingulate cortex, posterior cingulate cortex and hippocampus, we detected a transient increase in Bnip3 expression with peak levels on PND 6.5. Similarly, a peak in Bnip3 mRNA expression was detected in the developing male anterior cingulate cortex on PND 6.5. However, no change in Bnip3 expression was measured in the neonatal male posterior cingulate cortex and hippocampus. An additional sex difference was detected in the anterior cingulate cortex with greater levels of Bnip3 expression on PND 6.0 and in adult females compared to males of comparable age. Despite these sex differences in Bnip3 mRNA expression, levels in the neonatal rat brain were not changed following testosterone treatment at birth.

Previous *in vivo* localization of Bnip3 protein in the rat brain has been limited to the adult forebrain, cortex, lateral thalamus, hypothalamus and hippocampus (Schmidt-Kastner et al., 2004). Utilizing ISH we also detected Bnip3 mRNA in the adult rat cortex, including the cingulate cortex, and hippocampal regions CA1-CA3. In contrast, we were unable to identify Bnip3 mRNA in the thalamus and hypothalamus. Such a discrepancy could be the result of a greater rate of Bnip3 mRNA versus protein degradation in those regions. However, this is unlikely as the Bnip3 protein contains an endogenous PEST domain the targets it for rapid proteasome degradation (Chen et al., 1999). Alternatively, this difference could be

due to our use of ISH for mapping mRNA expression whereas Schmidt-Kastner et al., (2004) utilized a Bnip3 polyclonal antibody derived against the hamster antigen for immunocytochemistry. Additionally, the Bnip3 protein in that study was detected in the nucleus of the neurons (Schmidt-Kastner et al., 2004), which is a novel location as endogenous Bnip3 has been previously localized to only the mitochondria (Vande Velde et al., 2000; Zhang et al., 2003).

In this study, we also examined expression in the neonatal brain, which has not been previously characterized. By ISH, Bnip3 mRNA was detected in a greater number of brain regions in the PND 7.0 brain compared to the adult, including the cortex, with intense staining in the cingulate cortex and piriform areas, hippocampus (regions CA1-CA3 and dentate gyrus), and habenula. Given that Bnip3 is a pro-apoptotic protein, it can be postulated that the brain regions which endogenously express Bnip3 mRNA may be undergoing apoptosis. Furthermore, these regions may be more susceptible to apoptosis induced by stimuli that increase expression or activate Bnip3 protein.

Our studies have also shown that there is a transient increase in expression of the pro-apoptotic gene Bnip3 in the postnatal rat cingulate cortex and hippocampus. The increase in Bnip3 mRNA expression correlates with the increase in NOCD in the neonatal rat brain (Gould et al., 1991; Ferrer et al., 1994b; Spreafico et al., 1995). Bnip3 expression peaked on PND 6.5 in the male and female anterior cingulate cortex and this timing correspond well with the transient increase in cell death from PND 5 to 8 in the rat cortex (Spreafico et al., 1995). Furthermore, the increase in Bnip3 expression measured on PND 6.5 in the female posterior cingulate cortex and

hippocampus correlates with increased levels of postnatal cell death during early postnatal development in the hippocampus (Gould et al., 1991; Ferrer et al., 1994b). We did not observe changes in Bnip3 mRNA expression in male posterior cingulate cortex or hippocampus. These results suggest that if Bnip3 is a mediator of NOCD then it is likely to regulate cell death in both sexes in the anterior cingulate cortex, but only regulate developmental cell death in the female posterior cingulate cortex and hippocampus. However, studies characterizing NOCD in the cortex and hippocampus have not investigated cell death in relation to the sex of animal in the anterior and posterior cingulate cortices or hippocampus (Gould et al., 1991; Spreafico et al., 1995). The possibility exists that sex differences in NOCD occur in the cingulate cortex and hippocampus, as has been reported for other brain regions including the primary visual cortex (Nunez et al., 2001). Therefore, additional studies investigating the sex differences in developmental cell death are necessary in order to clarify the discrepancy between the ontogeny of Bnip3 and levels of NOCD. Further studies to examine the nature of NOCD should also be considered as recent studies indicate that Bnip3 is capable of inducing autophagic cell death (Daido et al., 2004; Kanzawa et al., 2005).

Although the increase in Bnip3 mRNA expression in the neonatal rat cortex and hippocampus implicates Bnip3 as a putative regulator of NOCD, other pro-apoptotic proteins have also been identified as mediators for developmental apoptosis. For example, double knockout mice deficient in both Bax and Bak, proteins which possess multiple BH domains, prematurely die and have a greater number of cells in the CNS compared to wild type mice (Lindsten et al., 2000; Wei et

al., 2001; Lindsten et al., 2003) or mice deficient in only one of the proteins (Lindsten et al., 2000; Lindsten et al., 2005). Similarly, the pro-apoptotic BH3-only proteins have also been implicated in regulating normal CNS development. Sympathetic ganglion neurons deficient in the BH3-only protein, Bim, are resistant to growth factor withdrawal, while deficiency in the BH3-only proteins, Bid and Bad, does not protect neurons from growth factor withdrawal (Deckwerth et al., 1996; Putcha et al., 2002; Lindsten et al., 2005). The use of transgenic mice has implicated many pro-apoptotic members as mediators in CNS development. The developmental ontogeny of Bnip3 suggest it may be involved with a component of cortical and hippocampal development. However, our data are only correlative and additional studies that disrupt Bnip3 function during CNS development are necessary to determine the exact relationship between Bnip3 and NOCD.

BH3-only pro-apoptotic proteins, such as Bnip3, induce apoptosis by heterodimerizing with either Bcl-2 or Bcl-X_L, and subsequently disrupting mitochondrial function (Green and Kroemer, 2004). Consequently, the elevated levels of Bnip3 that were found on PND 6.5 may play a role in regulating NOCD via interactions with anti-apoptotic proteins such as Bcl-X_L. *In vitro* studies characterizing Bnip3 interactions with the anti-apoptotic Bcl-2 family members reveal that Bnip3 heterodimerizes with Bcl-X_L and induces cell death in a mitochondrial dependent manner (Ray et al., 2000). Furthermore, Bcl-X_L has been established as an essential mediator of mammalian development and cell death. Mice deficient in Bcl-X_L die at embryonic day 13 and have excessive apoptosis within the intermediate zone of the spinal cord and brainstem as well as in the dorsal root

ganglion (Motoyama et al., 1995; Shindler et al., 1997; Lindsten et al., 2005). In the rat cortex and hippocampus Bcl-X_L expression is decreased by PND 6.0 and remains depressed through the second postnatal week (Hamner et al., 1999). This decrease in Bcl-X_L expression correlates with the surge in Bnip3 expression that we detected on PND 6.5 in the cingulate cortex and hippocampus. Thus, decreases in Bcl-X_L coupled with increases in Bnip3 expression could alter the ratio of pro- to anti-apoptotic genes in the cingulate cortex and hippocampus, which subsequently leads to NOCD.

In addition to the transient increase in Bnip3 mRNA measured on PND 6.5 we also identified a sex difference in Bnip3 mRNA expression in the cingulate cortex and hippocampus. On PND 6.0, Bnip3 mRNA is greater in anterior cingulate cortex of females compared to males. Furthermore, Bnip3 mRNA expression is significantly upregulated in the posterior cingulate cortex and hippocampus of PND 6.5 female rats, but not males. In order to ascertain the cause of the observed sex differences, we treated neonatal male and female rats with testosterone. Gonadal hormones, such as estrogen regulate transcription of the pro-apoptotic Bcl-2 family members such as Bik and Bnip2, closely homologous relatives to Bnip3 (Belcredito et al., 2001; Hur et al., 2004). Furthermore, estrogen mediated gene transcription is conferred by the estrogen receptor, which is transiently increased during the first postnatal week in the rat cortex and hippocampus (MacLusky et al., 1979; O'Keefe and Handa, 1990; O'Keefe et al., 1993; O'Keefe et al., 1995). Correspondingly, the postnatal male rat brain is exposed to estrogen as a result of the intracellular conversion of testosterone to 17β-estradiol by the aromatase enzyme and this is

thought to be responsible for the defeminization of the male brain (MacLusky et al., 1979; MacLusky and Naftolin, 1981; MacLusky et al., 1986). In females, circulating estradiol is sequestered by alpha-fetoprotein in the developing brain, thus rendering the estradiol inactive and preventing its actions in neurons (MacLusky et al., 1979; MacLusky and Naftolin, 1981; MacLusky et al., 1986). Therefore, we addressed the hypothesis that elevated estrogen in the postnatal male rat cortex and hippocampus attenuated the rise in Bnip3 mRNA expression on PND 6.5. However, removal of circulating testosterone from neonatal males or administration of testosterone to neonatal females did not alter baseline levels of Bnip3 in the PND 6.5 male and female rat anterior and posterior cingulate cortices and hippocampus. Therefore, we conclude that the sex differences we detected in Bnip3 expression are not due to early postnatal hormone exposure.

In summary, the experiments conducted in this study established the localization and ontogeny of the pro-apoptotic gene, Bnip3, in the developing male and female rat brain. The transient increase in Bnip3 mRNA expression, which closely correlates with the increase in postnatal NOCD, implicates Bnip3 as a putative participant in the regulation of developmental apoptosis. Furthermore, regions of the developing brain which endogenously express Bnip3 may subsequently be susceptible to insults that either induce expression or activate Bnip3 protein. Since Bnip3 has been previously shown to be upregulated in the adult rat hippocampus following a hypoxic-ischemic insult, (Schmidt-Kastner et al., 2004), Bnip3 could act as a mediator of hypoxia induced cell death in the neonatal brain.

Chapter 4

Glucocorticoid exacerbation of hypoxia induced neuronal death and expression of the pro-apoptotic Bnip3 in the developing cortex

Abstract

Neonatal administration of the synthetic glucocorticoid, dexamethasone (DEX) retards brain growth, alters adult behaviors and induces cell death in the rat brain, thereby implicating glucocorticoids as developmentally neuroendangering compounds. Glucocorticoids also increase expression of pro-apoptotic Bcl-2 family members and exacerbate expression of hypoxic responsive genes. Bnip3 is a pro-apoptotic Bcl-2 family member that is up regulated in response to hypoxia. In these studies, we investigated the interactions of glucocorticoid receptor and hypoxia in the regulation of Bnip3 mRNA in cortical neurons. Using quantitative real time RT-PCR, we found that DEX treatment of neonatal rat pups from postnatal day 4-6 caused a significant increase in Bnip3 mRNA expression compared to vehicle controls. A significant increase in Bnip3 mRNA was also measured in primary cortical neurons 72 hours after treatment with RU28362, a glucocorticoid receptor selective agonist. In primary cortical neurons, hypoxia increased Bnip3 mRNA expression and this was exacerbated with RU28362 treatment. These changes corresponded to changes in cell viability as measured using LDH and MTT assays. Glucocorticoid receptor and hypoxia mediated regulation of a transfected Bnip3 promoter - luciferase reporter

construct was further examined in primary cortical neurons. Regulation of the Bnip3 promoter was mediated by a glucocorticoid response element and hypoxic response element. Finally, over-expression of Bnip3 in primary cortical neurons increased cell death. This effect was eliminated when the transmembrane domain was removed from Bnip3. These studies identify a novel pathway in the developing cortex through which glucocorticoids may exacerbate a metabolic insult, such as hypoxia.

Introduction

Corticosterone, the endogenous glucocorticoid secreted by the rat adrenal gland, binds with high affinity to two different receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Reagan and McEwen, 1997). MR activation is associated with a neuroprotective phenotype (Almeida et al., 2000), whereas GR activation is implicated in the induction of an endangered neural phenotype (Sapolsky et al., 1986; Reagan and McEwen, 1997). For example, in the adult, GR activation by the synthetic glucocorticoid dexamethasone (DEX) reduces anterior cingulate cortex volume (Cerqueira et al., 2005).

Elevated glucocorticoid levels also endanger cells in the neonatal brain as evidenced by decreased hippocampal volume (Coe et al., 2003) and retarded overall brain growth and neurological development (Flagel et al., 2002). Postnatal DEX treatment decreases both the number and size of cortical neurons (Kreider et al., 2006), which persists into adulthood as these rats show altered social behavior and learning and memory deficits (Kamphuis et al., 2004; Neal et al., 2004).

In juvenile rats, GR activation increases the incidence of apoptosis in the hippocampus and striatum (Hassan et al., 1996; Haynes et al., 2001). This is

consistent with their ability to regulate Bcl-2 family member expression. In the juvenile rat hippocampus, DEX downregulates the anti-apoptotic genes Bcl-2 and Bcl-X_L; while upregulating the pro-apoptotic gene Bax (Almeida et al., 2000). In contrast, in the neonatal rat hippocampus, DEX does not alter Bcl-2 or Bax (Tan et al., 2002). These data suggest that glucocorticoid regulation of apoptosis is mediated through a novel mechanism in the neonatal rat brain.

Pro and anti-apoptotic members of the Bcl-2 family initiate apoptotic events. Bnip3 is a unique pro-apoptotic member of the Bcl-2 homology domain 3 (BH3) subfamily (Boyd et al., 1994; Yasuda et al., 1998) in that Bnip3 induced cell death is independent of the BH3 domain (Ray et al., 2000), and is not prevented by Bcl-2 and Bcl-X_L over-expression (Ray et al., 2000). Bnip3 gene regulation has been predominantly investigated following hypoxia. During hypoxic events, hypoxia inducible factor-1 (HIF-1) mediates the transcription of Bnip3 via interaction with a hypoxic response element (HRE) in the promoter region (Bruick, 2000; Sowter et al., 2001). Furthermore, hypoxia induces Bnip3 expression in adult rat hippocampus, cortex and striatum which correlates with increases in cell death (Schmidt-Kastner et al., 2004; Althaus et al., 2006). Notwithstanding, Bnip3 expression in response to hypoxia, its regulation by mechanisms independent of hypoxia, or interactions between the two have not been investigated in the developing brain.

Consequently, the following studies examined glucocorticoid regulation of Bnip3 in the neonatal rat cingulate cortex and in primary cortical neurons. We found that glucocorticoids increased Bnip3 mRNA expression both *in vivo* and *in vitro*. We also determined that hypoxia-upregulation of Bnip3 mRNA expression is exacerbated

by glucocorticoids. Consistent with this, glucocorticoid and hypoxic exposure decreased cortical neuron viability. Finally, our studies show that the GR and hypoxic regulation of Bnip3 is mediated through interactions between a glucocorticoid response element (GRE) and HRE within the Bnip3 promoter.

Materials and Methods

Animals

Timed pregnant female Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed at Colorado State University's laboratory animal research facility. For *in vivo* experiments that used postnatal offspring, pregnant dams were monitored on a daily basis in order to record the litter's birth date and time. The day of birth was considered as postnatal day (PND) 0. Upon birth all litters were culled into groups of 10 pups, 5 males and 5 females, and returned to the dam. Male and female neonates were subcutaneously injected with a daily dose of DEX (0.2mg/kg in 100μl safflower oil) from PND 4 through 6. Controls received vehicle at the same injection regimen. Twenty four hours after receiving the third injection (PND7) animals were halothane anesthetized and killed. For *in vitro* studies using primary cortical neurons, pregnant dams were halothane anesthetized on either gestational day 17 or 18 and the rat fetuses were delivered by cesarean section. The dam was subsequently sacrificed by decapitation. All animal protocols were previously approved by the Colorado State University Animal Care and Use Committee and carried out in accordance with the National Institutes of Health and Institutional Animal Care and Use Guidelines.

Primary Cortical Neurons

The primary cortical neurons were harvested from E17-18 rat fetuses using modified protocols (Banker and Cowan, 1977; Brewer et al., 1993). During the dissection, brain tissue was kept in a CMF Ringers-glucose solution (0.155M NaCl, 5.0mM KCl, 10.0mM Hepes, 11.0mM D-glucose) on ice. Dissected cortical tissue was incubated at room temperature in a solution consisting of 50% CMF Ringers-glucose plus 50% trypsin (62,000units/ml) for 15 minutes. Cortical tissue was washed once with Dulbecco's Modified Eagles Medium (Invitrogen, Carlsbad, CA, USA [DMEM]) containing 15% Fetal Bovine Serum then dissociate by titration in the same media. Cortical neurons were plated in Neurobasal medium without phenol red (Invitrogen, Carlsbad, CA, USA) supplemented with 1.0x B27 (Invitrogen, Carlsbad, CA, USA), 100μg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), 0.5mM L-glutamine (Mediatech, Inc. Herndon, VA, USA) and 0.025mM L-glutamic acid (Sigma-Aldrich Co. St. Louis, MO, USA). Primary cortical neurons were plated at a density of $5x10^5$ cells/9.4cm^2 for RNA isolation studies, $3x10^5$ cells/1.9cm^2 for MTT, lactate dehydrogenase (LDH) and Bnip3 promoter studies, and $.3x10^5$ cells/0.3cm^2 for Bnip3 expression plasmid studies. All cultures were maintained for 3 days in vitro (DIV) in plating media. All experiments were begun after 4-5 DIV and concluded by 8 DIV so that all results were from primary cortical neurons of an immature phenotype.

Experiments that involve RNA isolation and cell death as a result of glucocorticoid and hypoxia treatment were conducted in cells grown in Neurobasal media without phenol red, and supplemented with 0.1x B27, 100μg/ml penicillin-streptomycin and 0.5mM L-glutamine. Transfection studies using the Bnip3 promoter

constructs were conducted in cells grown in the above media minus penicillin-streptomycin. The B27 was diluted in order to obtain a final concentration of 2.8nM corticosterone in the treatment media. Primary cortical neurons treated with glucocorticoids received either vehicle (0.005% of 95%EtOH in the previously described media) or doses of the serially diluted GR agonist RU28362 (0.1, 1.0, 5.0 or 10.0nM; Roussel-UCLAF, France). Throughout the experiment cultures were maintained at 37°C in ambient air infused with 5% CO_2, or in Modulator Incubator Chambers (Billups-Rothenberg Inc. Del Mar, CA, USA) filled with 20% O_2, 5% CO_2, N_2 balanced (normoxic) or 1% O_2, 5% CO_2, N_2 balanced (hypoxic) for either 48 or 72 hours. Transfection of primary cortical neurons with the Bnip3 and Bnip3ΔTM expression plasmids were conducted in Neurobasal medium minus phenol red, supplemented with 1x B27, and 0.5mM L-glutamine.

Bnip3 cDNA plasmid synthesis

A Bnip3 cDNA plasmid was designed for use in situ hybridization (ISH) studies and for generating the Bnip3 cDNA standards for real time RT-PCR. The Bnip3 cDNA was generated using RT-PCR on rat cortical RNA with a forward (5' TTT AAA CAC CCG AAG CGC ACA G-3') and a reverse (5' GTT GTC AGA CGC CTT CCA ATG TAG A-3') primer pair specific for the rat Bnip3 coding sequence. The Bnip3 primers were also used for quantitative real time RT-PCR experiments. The primer design was based on an existing GenBank sequence (GenBank accession nos. AF243515, forward primer position 464 and reverse primer position 606) and analyzed using Oligo software, version 6.51 (Molecular Biology Insights, Cascade, CO, USA). Specificity of the Bnip3 primers was confirmed using a

nucleotide Blast search (NCBI Entrez pubmed). PCR was conducting in a Hybaid Omnigene PCR Thermocycler (Thermo Electron Corp. Waltham, MA, USA) and amplified for 40 cycles. The initial melting step was 94°C for 2 minutes followed by 40 cycles of 94°C melting (10 seconds), 64°C annealing (30 seconds), and 72°C elongation (1 minute). The size of the amplified Bnip3 cDNA was confirmed by 1% agarose gel electrophoresis. Purified Bnip3 cDNA was cloned into the pCRII-TOPO plasmid containing both an SP6 and T7 promoter site using the HTP TOPO TA Cloning Kit Dual Promoter system (Invitrogen Life Technologies, Carlsbad, CA, USA).

Expression Plasmids

The Bnip3 and Bnip3ΔTM expression plasmids were kindly provided by Dr. Don Dubik (Manitoba Institute of Cell Research, Manitoba, CA). The expression plasmids were synthesized as previously described (Chen et al., 1997a). The pcDNA 3.0 (Invitrogen, Carlsbad, CA) expression plasmid was transfected as a negative control.

Promoter Reporter Plasmids

The Bnip3 and mutHRE promoter luciferase reporter plasmids were kindly provided by Dr. Richard Bruick (University of Texas Southwestern Medical Center, Dallas, TX, USA). The promoter/reporter gene plasmids were generated as previously described (Bruick, 2000). The Bnip3 and mutHRE promoters were inserted into the pGL3-Basic plasmid (Promega Corp. Madison, WI, USA). The Bnip3 promoter luciferase reporter plasmid was used to generate the GRE1 (GRE1 luc), GRE2 (GRE2 luc), GRE3 (GRE3 luc) and Bnip3ΔGRE2 promoter luciferase reporter plasmids. The

locations of the three putative GREs within the Bnip3 promoter (GenBank accession nos. AF283504) are -528 to -514 (GRE1), -367 to -353 (GRE2) and -211 to -197 (GRE3). To generate the GRE1 luc and GRE 2 luc plasmids, forward and reverse primer pairs were designed to individually isolate the Bnip3 promoter regions containing the putative GRE1 and GRE2 sites. The forward (5' CAC AGG TAC CGC AGG AGG AGG TCC CCA ACC C 3') and reverse (5' CAC AGC TCA GCG AGC AAC ACT GAG GCG CTA GG 3') primer pairs for amplifying GRE1 are at positions -500 and -455. The forward (5' CAC AGG TAC CGC TCA GTG TTG CTC GCA CCT CG 3') and reverse (3' CAC AGC TCA GCG GGG TGG AGC CTG GTT GG 3') primer pairs for amplifying GRE2 are at position -499 to -263. The forward primers include a 5' KpnI restriction site; while the reverse primers have a 5' BlpI restriction site. PCR amplification of GRE1 and GRE2 was conducted using the Bnip3 promoter as template. Amplified PCR product was digested with the restriction enzymes KpnI and BlpI. Double digest with KpnI and BlpI was also performed on the full length Bnip3 promoter to excise the three putative GREs. The size of the GRE1 and GRE2 cDNAs and digested plasmid was confirmed by 1% agarose gel electrophoresis. GRE1 and GRE2 were subsequently directional cloned into the digested Bnip3 plasmid using the Rapid DNA Ligation Kit (Roche Inst. Indianapolis, IN, USA). The GRE3 luc plasmid was generated by double digest of the full length Bnip3 promoter luciferase reporter plasmid with BstXI and KpnI to excise the promoter region containing the putative GRE1 and GRE2 sites. The digested plasmid was blunt end ligated with the Rapid DNA Ligation Kit. The Bnip3ΔGRE2 luc plasmid was generated by site directed mutagenesis using the QuikChangeII XL Site-

Directed Mutagenesis Kit (Stratagene,La Jolla, CA, USA) per the manufacturer's specifications. Forward (5' CGT GCA GGT CCC GGC TAG CCT CAA GG 3') and reverse (5' CCT GAG GCT AGC CGG GAC CTG CAC G 3') primers were designed to eliminate GRE2 within the Bnip3 promoter without introducing nucleotide mutations. PCR amplification of the Bnip3ΔGRE2 plasmid was conducted using the Bnip3 promoter luciferase reporter plasmid as template. Sequencing reactions were performed with the GRE1 luc, GRE2 luc, GRE3 luc and Bnip3ΔTM luc plasmids to confirm the cDNA sequence for each plasmid.

In Situ Hybridization

To examine the localization and developmental regulation of Bnip3 mRNA in brain, we performed ISH on paraformaldehyde fixed neonatal rat brain tissue using a ^{35}S-labeled cRNA probe. The Bnip3 cDNA containing plasmid was linearized with either Not1 (antisense) or Kpn1 (sense). ^{35}S-UTP (1000 ci/mmole) radiolabeled sense and antisense Bnip3 cRNA probes were transcribed in vitro using either T7 or SP6 RNA polymerase, respectively. The sense and antisense probes were used for ISH.

Brain tissue was collected from rat pups that were halothane anesthetized and subsequently perfused via the left ventricle initially with 0.9% saline plus heparin, followed by 4% neutral buffered paraformaldehyde. Brains were harvested and placed in 30% sucrose phosphate buffered saline (PBS) until the brains were saturated. 25μm coronal sections were taken with a Leitz 1720 Digital Kryostat (VWR Scientific, West Chester, PA, USA). Brain sections were post fixed with 10% formaldehyde in PBS for 8 minutes. Subsequently tissue was acetylated with a 0.1M Triethanolamne-HCL/0.9% saline (TEA) with 0.25 % acetic anhydride then

dehydrated in increasing concentrations of ethanol. Tissue was incubated overnight at 64°C in hybridization solution (50% formamide, 0.06M NaCl, 0.02M Tris, 0.01M EDTA, 10% Dextran sulfate, 2 x Denhart's solution, 50mM dithiothreitol, 0.2% SDS, 100mg/ml salmon sperm DNA, 50mg/ml total yeast RNA and 50mg/ml yeast transfer RNA) containing 2 x 10^7 cpm/ml of the radiolabeled probe. Tissue was subsequently washed with a 2x standard saline citrate (SSC) solution followed by digestion of nonhybridized probe with 30 mg/ml ribonuclease A for 30 minutes at 37°C. The final wash stringency was 0.1 x SSC at 64°C. Radiolabeled tissues were exposed to Kodak Biomax MR Autoradiographic Film (Eastman Kodak Company, Rochester, NY, USA) for 3 days.

Image analysis

The density of hybridization signal per fixed area was determined using a CCD camera (Sony XC-77, Tokyo, Japan) with a Nikon (Melville, NY, USA) lens and the Scion Image (Frederick, MD, USA) software package. The anterior cingulate cortex and posterior cingulate cortex were independently analyzed. The brain regions were identified using a rat brain atlas (Swanson, 1992). For each brain region two measurements were taken bilaterally from five different sections of each rat brain. The measurements obtained from the five sections were averaged to obtain a single value for each rat.

RNA Isolation for Quantitative Real Time RT-PCR

Brain tissue utilized for real time RT-PCR was collected from rat pups that were halothane anesthetized and subsequently sacrificed by decapitation. Brains were harvested and immediately frozen in -20°C 3-methylbutane. The anterior and

posterior cingulate cortices and hippocampus were dissected from frozen 150μm coronal sections. Total RNA was also isolated from primary cortical neurons for real time RT-PCR. Prior to RNA isolation treatment media was removed and the cortical neurons were washed once with PBS. Cortical neurons were collected into PBS by manually removing the cells from the culture dish with a rubber policeman. PBS was removed by centrifugation at 2000rpm for 2 minutes. The neurons were resuspended then homogenized in GIT extraction buffer (4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% N-laurel sarcosine, 0.1M β-mercaptoethanol). Total RNA was isolated from the rat brain tissue and primary cortical neurons using the Chomczynski and Sacchi protocol (Chomczynski and Sacchi, 1987). Concentration of total RNA was determined using spectrophotometry (O.D. 260/280).

Quantitative Real Time RT-PCR

0.5μg of total RNA was reverse transcribed with MMLV reverse transcriptase and oligoDT primers (Invitrogen, Carlsbad, CA, USA). The concentration of reverse transcribed cDNA was measured in the primary cortical neuron studies using Oligreen ssDNA Quantitation Reagent and Kit (Molecular Probes, Inc. Eugene, OR, USA). Quantification of Bnip3 mRNA expression was determined using real time RT-PCR with the LightCycler 2.0 system (Roche Inst. Indianapolis, IN, USA). Real time RT-PCR reactions were performed on the cDNA samples with the Bnip3 primers previously described. PCR reactions included 3mM $MgCl_2$, 100mM Tris-Cl, 0.5U Taq polymerase, 2μl of 10X stock of Faststart SYBR green I (Roche Inst. Indianapolis, IN, USA) and 0.5μM of forward primer and reverse Bnip3 primer. The initial melting step for the reaction was 92°C for 10 minutes followed by 45 cycles of

95°C (melting) for 1 second, 62°C (annealing) for 5 seconds and 72°C (elongation) for 15 seconds. Samples were assayed alongside a Bnip3 cDNA standard curve for determination of the absolute concentration of the experimental Bnip3 mRNAs. Bnip3 cDNA was used to generate the standard curve. The Bnip3 cDNA used was obtained by digesting the previously described Bnip3 cDNA plasmid with BamHI. The concentration of the digested Bnip3 cDNA was calculated from an absorbance reading at wavelength 260 (OD260) using a Beckman D6530 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). The cDNAs were diluted to a stock concentration of 1ng/ml then subsequently serially diluted from 1ng/ml to 10fg/ml in PCR grade sterile water (Roche Inst. Indianapolis, IN) to generate six working standards. The absolute concentration of unknown Bnip3 mRNA was determined by the LightCycler Data Analysis Software 4.0 (Roche Inst. Indianapolis, IN, USA). Studies measuring Bnip3 mRNA in brain tissue were reported as an absolute concentration (pg/μl of cDNA); while Bnip3 mRNA levels in primary cortical neurons were reported as pg of Bnip3/ng of cDNA. In all experiments, samples that included water in place of template were used as negative controls.

Transfection of Primary Cortical Neurons

Primary cortical neurons were dual transfected with either the Bnip3, Bnip3ΔTM, or empty pcDNA expression plasmids in conjunction with a β-galactosidase reporter. The β-galactosidase reporter is a β-actin LacZ reporter construct that is regulated by the human actin promoter and expresses the β-galactosidase gene. Prior to transfection, media was changed to a Neurobasal medium minus phenol red (1x B27, and 0.5mM L-glutamine). Expression plasmids were

transfected using the FuGENE 6 Transfection reagent (Roche Applied Science, Indianapolis, IN, USA as per the manufacturer's recommendations). The transfection reagent:DNA concentration ratio for each of the expression plasmids was 3:1 (0.18μl FuGENE 6:0.05μg expression plasmid and 0.01μg β-galactosidase). Cortical neurons were incubated with the transfection reagent for 2 hours at 37°C. Culture media was changed to Neurobasal medium minus phenol red (1x B27, and 0.5mM L-glutamine) and neurons were incubated at 37°C for an additional 70 hours. After 72 hours, cell viability of the transfected neurons was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corp. Madison, WI, USA). β-galactosidase activity was measured in the transfected neurons using the Tropix GalactoLight kit assay system (Applied Biosystems, Foster City, CA, USA) according to manufacturer's recommendations. Luciferase and β-galactosidase activity were measured with the 20/20 TD luminometer (Turner Designs, Sunnyvale, CA, USA). Neuron viability following transfection was normalized to β-galactosidase activity for each transfection paradigm to account for variations in transfection efficiency. The normalized data was reported as percent change in viability from $0.3x10^5$ cortical neurons that were not transfected. The viability of the non-transfected cortical neurons was measured using the CellTiter-Glo Luminescent Cell Viability Assay.

Primary cortical neurons were dual transfected with either the empty pGL3-basic, Bnip3, mutHRE, GRE1, GRE2, GRE3 or Bnip3ΔGRE promoter luciferase reporter plasmids and the β-galactosidase reporter. Prior to transfection, culture media was changed to Neurobasal minus phenol red containing 0.1x B27 and 0.5mM L-

glutamate. Transfection was performed using the FuGENE 6 Transfection Reagent. The transfection reagent:DNA concentration ratio for each of the plasmids was 3:1 (1.5μl FuGENE 6:0.4μg promoter reporter plus 0.1μg β-galactosidase). Cultures were incubated in transfection reagent for 2 hours at 37°C. After 2 hours, transfected cultures were treated with either vehicle (0.005% 95% EtOH) or doses of RU28362 (0.1, 1.0, 5.0 or 10.0nM) in Neurobasal minus phenol red (0.1x B27 and 0.5mM L-glutamate) for 40 hours. Cultures were maintained in normoxic, hypoxic or ambient air conditions depending on the experimental design. Forty two hours after transfection the cells were lysed. Luciferase activity was measured by adding 20μl of lysate into 100μl of luciferan substrate (Promega Corp. Madison, WI, USA). β-galactosidase activity was measured by using 40μl of lysate with the Tropix-GalactoLight kit. Luciferase and β-galactosidase activity were measured with the 20/20 TD luminometer and reported as relative light units (RLU); luciferase/β-galactosidase activity.

Lactate Dehydrogenase and MTT Assays

The percent of cell death was measured in primary cortical neurons treated with 1 of 4 doses of RU28362 (0.1, 1.0, 5.0 or 10.0nM) or vehicle (0.005% 95%EtOH) and incubated at 37°C for 48 and 72 hours in ambient air conditions. Cell death was determined by measuring LDH release into the culture media after RU28362 treatment using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corp. Madison, WI, USA). Data was reported as the percent cell death, experimental LDH release as a result of treatment relative to maximal LDH release.

The percent change in viability was measured in primary cortical neurons treated with 1 of 3 doses of RU28362 (0.1, 1.0 and 10.0nM) or vehicle and maintained in either normoxic (20% O_2) or hypoxic (1% O_2) for 48 and 72 hours. Cell viability was measured using an MTT assay. After treatment primary cortical neurons were washed once with sterile PBS and media was replaced with 250µl of a 0.25mg/ml solution of MTT (Thiazolyl Blue Tetrazolium Bromide; Sigma-Aldrich, St. Louis, MO, USA) in Neurobasal media minus phenol red, containing 0.1x B27, 100µg/ml penicillin-streptomycin and 0.5mM L-glutamine. Cortical neurons were incubated at 37°C for 1.5 hours, the MTT solution was removed and 200µl of 0.04M HCL in absolute isopropanol was added to each experimental sample. 100ml of each sample was transferred to a 96 well plate and the absorbance of the converted dye was read at 570nm on a Bio-Rad Model 680 micro plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The percent change in cell viability in hypoxic versus normoxic conditions, for the vehicle treated cultures, was calculated relative to the normoxic controls for each time. For each time point the percent change in viability for the RU28362 treated cortical neurons under hypoxic conditions was calculated relative to the vehicle hypoxic controls and the viability for RU28362 treated neurons under normoxic conditions was calculated relative to the vehicle treated-normoxic controls.

Statistical analysis

Data were analyzed by a two way ANOVA using the Statview data analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). Student-Newman-Keul's and

t-test procedures were used posthoc for individual comparisons. Differences were considered significant when $p<0.05$. Data were expressed as group means $\pm$ SEM.

Results

Bnip3 mRNA expression is upregulated by dexamethasone in the neonatal rat brain

Using ISH we localized Bnip3 mRNA expression in the brain of vehicle (Fig. 12A-B) or DEX- treated PND 7 rats (Fig. 12C-D). Bnip3 mRNA was expressed in the same brain regions of animals from both treatment groups with greatest hybridization density throughout the cortex, including the anterior and posterior cingulate cortices and piriform cortex. Additionally, Bnip3 mRNA was expressed in the CA1 through CA3 regions of the hippocampus, as well as in the dentate gyrus, medial thalamus and habenula (Fig. 12A-D). Quantitation of film autoradiograms showed that DEX significantly ($F_{1,10}=5.23$, $p<0.05$) increased Bnip3 mRNA levels in the female, but not male anterior cingulate cortex compared to vehicle treated males (Fig. 12E). Conversely, in the posterior cingulate cortex DEX-treatment significantly ($F_{1,19}=8.07$, $p<0.05$) increased Bnip3 mRNA levels in males, but not females (Fig. 12F).

To confirm our finding that DEX upregulates Bnip3 mRNA expression in the developing rat brain, we quantified Bnip3 mRNA levels using real time RT-PCR. On PND 7, female rat pups treated with DEX had significantly elevated levels of Bnip3 mRNA in the anterior ($F_{1,40}=7.72$, $p<0.01$) and posterior ($F_{1,40}=8.29$, $p<0.01$) cingulate cortices compared to vehicle treated controls (Fig. 13A-B). There was no effect of DEX in male rats (Fig. 13A-B). Additionally, we detected a significant sex by treatment effect in the anterior cingulate cortex ($F_{1,40}=7.27$, $p<0.05$). In the PND

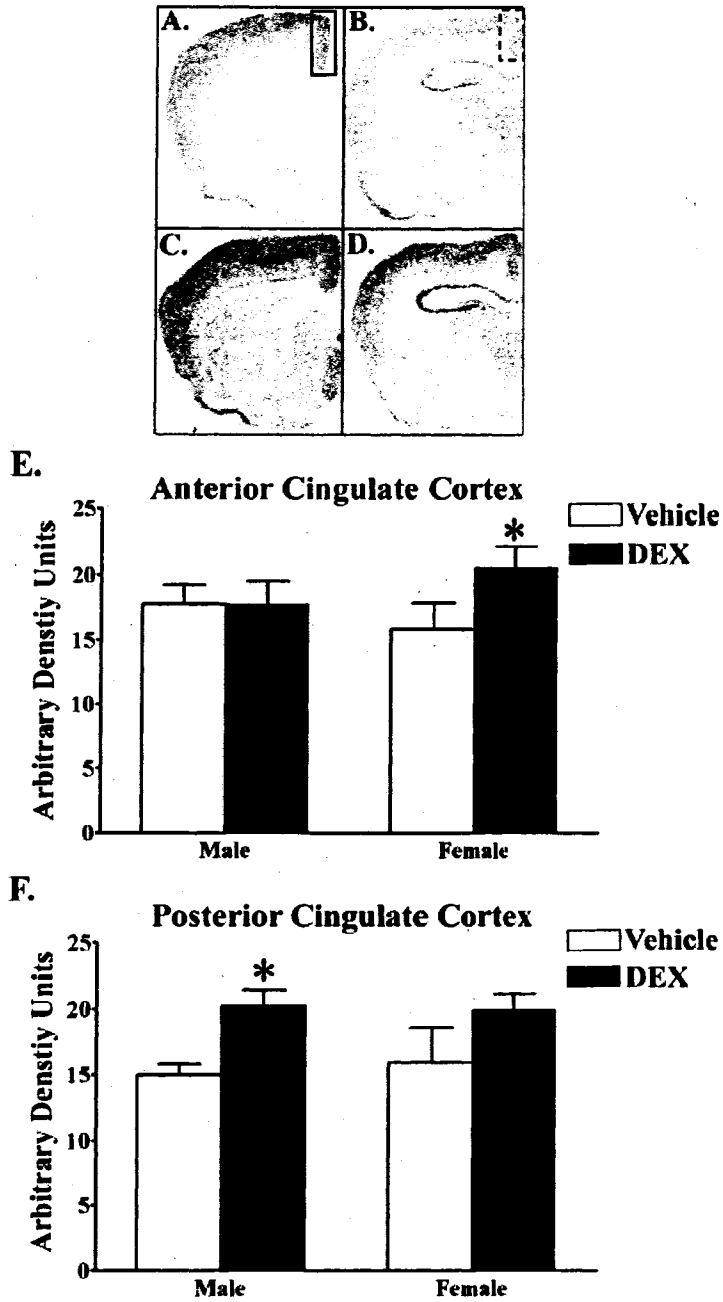


Figure 12. Photomicrographs showing in situ hybridization analysis of Bnip3 mRNA expression in DEX treated rats. Bnip3 mRNA expression in the PND 7 (A-B) vehicle and (C-D) DEX treated rat brain. The anterior and posterior cingulate cortices are demarcated by solid (A) and dashed (B) boxes, respectively. Comparison of Bnip3 mRNA expression in the (E) anterior cingulate cortex and (F) posterior cingulate cortex of PND 7 male and female rats after either vehicle (white bars) or DEX (black bars) treatment. Each bar represents the mean $\pm$ S.E.M. of 5-7 determinations. Data were analyzed by two-way ANOVA (sex by treatment). Multiple t-test comparisons were used to indicate groups that have significant differences (*$p<0.05$) in Bnip3 mRNA expression versus vehicle treated controls.

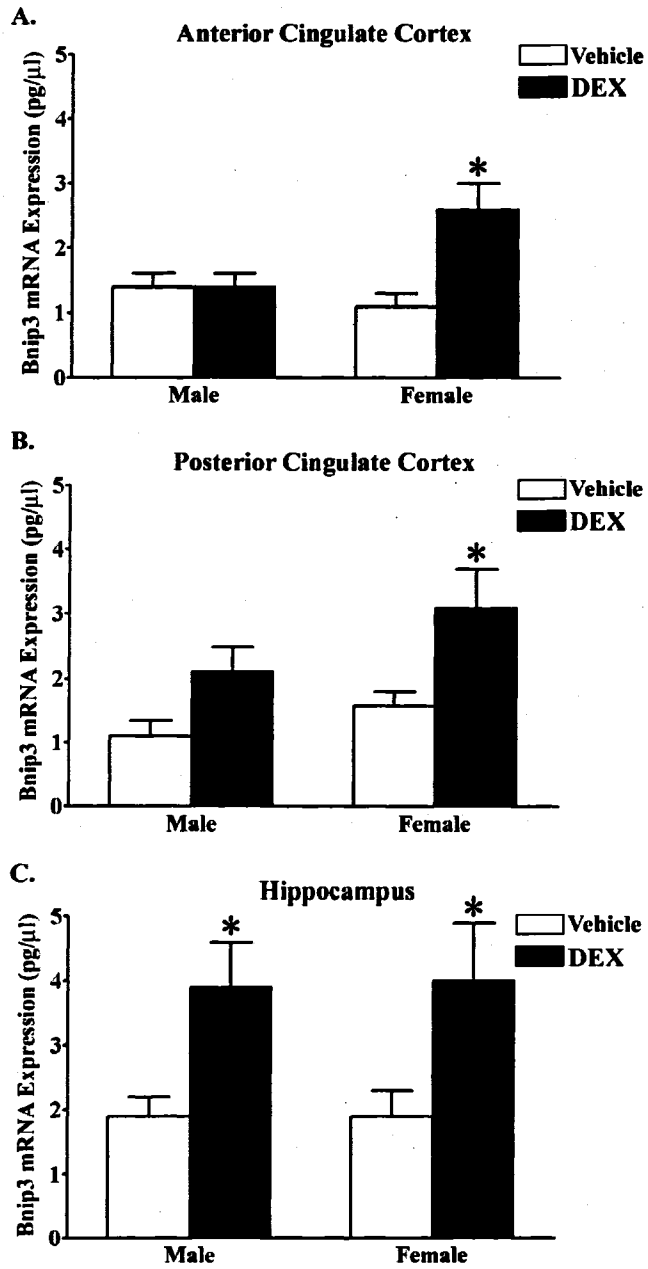


Figure 13. DEX upregulates Bnip3 mRNA expression in the neonatal anterior and posterior cingulate cortices and hippocampus. Quantitative real time RT-PCR was utilized to measure Bnip3 expression levels (pg/µl cDNA) in the (A) anterior cingulate cortex, (B) posterior cingulate cortex and (C) hippocampus of PND 7.0 male and female rat pups treated with either vehicle (white bars) or DEX (black bars). Each bar represents the mean ± S.E.M. of 10-14 determinations. Data were analyzed by two-way ANOVA (sex by treatment). Multiple t-test comparisons was used to indicate groups that were significantly different (*$p<0.05$) in Bnip3 mRNA expression as a result of DEX treatment.

7 hippocampus DEX treatment significantly ($F_{1,40}$=9.57, $p<0.005$) upregulated Bnip3 mRNA expression in both the male and female rat compared to vehicle treated controls (Fig. 13C).

Glucocorticoids increase Bnip3 mRNA expression in primary cortical neurons

To determine if GR regulates Bnip3 gene expression in developing neurons, we treated primary cortical neurons with the selective GR agonist, RU28362. Bnip3 mRNA expression was quantified using real time RT-PCR. Bnip3 mRNA levels were not changed after treatment with RU28362 for 48 hours compared to vehicle treated controls (Fig. 14A). However, after 72 hours there was a significant ($F_{3,32}$=5.26, $p<0.05$) increase in Bnip3 mRNA levels in neurons treated with 10.0nM RU28362 compared to neurons treated with vehicle and 0.1nM RU28362 (Fig. 14A). Additionally, there was a significant ($F_{3,63}$=4.53, $p<0.05$) interaction between RU28362 treatment and cultures treated for either 48 or 72 hours.

RU28362 treatment of primary cortical neurons induces cell death

We next examined the putative neuroendangering affects that low doses of GR agonist have on cortical neuron survival. Levels of LDH were used as a measure of cell death. After 72 hours, a significant ($F_{1,149}$=4.41, $p<0.05$) increase in the percent of cell death was found after treatment of cortical neurons with 5.0 and 10.0nM RU28362 compared to neurons treated with 5.0 and 10.0nM RU28362 for 48 hours, (Fig. 14B). Approximately a 6% and 10% increase in cell death was measured following 5.0nM and 10.0nM RU28362 treatment for 72 hours compared to 48 hours (Fig. 14B). However, RU28362 treatment for either 48 or 72 hours, at any of the

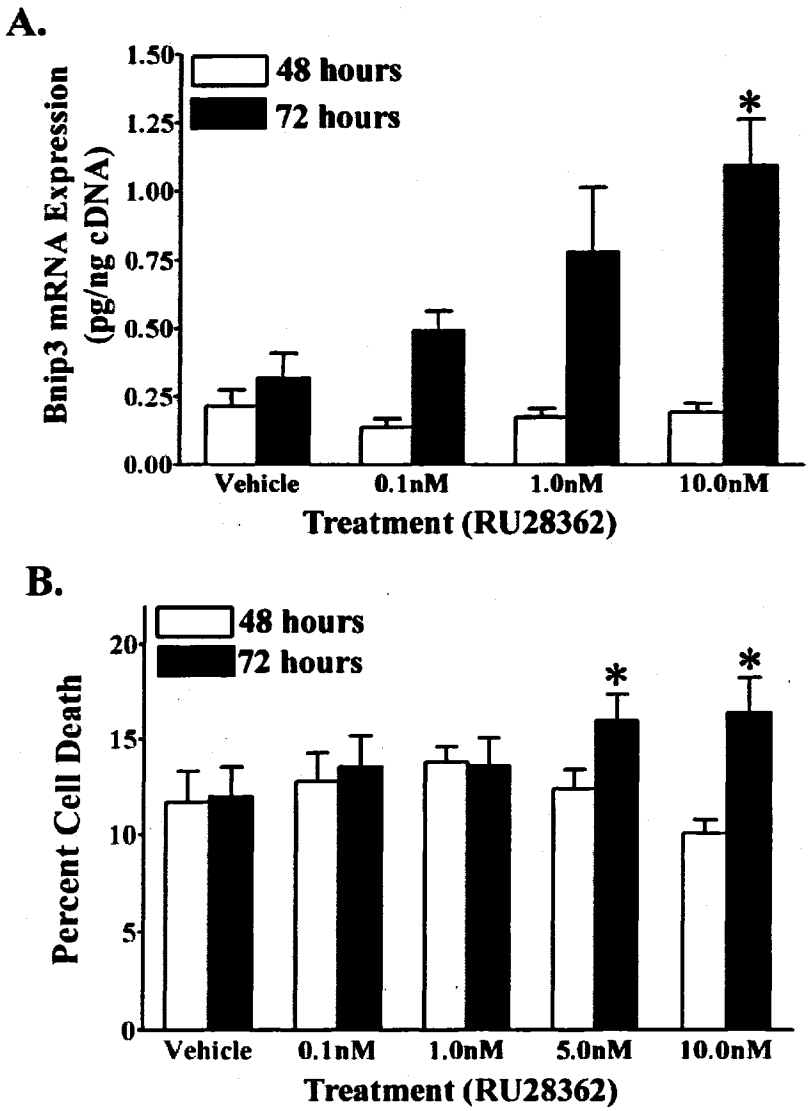


Figure 14. A GR selective agonist increases Bnip3 mRNA expression and induces cell death in primary cortical neurons. (A) Quantitative real time RT-PCR of Bnip3 mRNA levels (pg/ng cDNA) in primary cortical neurons treated with either RU28362 or vehicle and maintained in normoxic (20% O_2) conditions for 48 (white bars) or 72 (black bars) hours. (B) Lactate dehydrogenase assay determination of percent cell death in primary cortical neurons treated with either RU28362 or vehicle and maintained in ambient air for 48 (white bars) and 72 (black bars) hours. Each bar represents the mean $\pm$ S.E.M. of 8-21 determinations. Data were analyzed by two-way ANOVA (treatment by time). (A) Student-Newman Keul's posthoc test indicates significant differences (*$p<0.05$) in Bnip3 mRNA levels relative to vehicle treated controls. (B) Multiple t-test comparisons were used to indicate groups with

doses tested, did not significantly increase levels of cell death compared to vehicle treated controls (Fig. 14B).

Hypoxia increases Bnip3 transcription through a hypoxic response element

To determine if hypoxia regulates Bnip3 transcription in cortical neurons, we maintained primary cortical neurons in either normoxic (20% O_2) or hypoxic (1% O_2) conditions for 48 and 72 hours and subsequently quantified Bnip3 mRNA levels. Bnip3 mRNA was significantly ($F_{1,33}$=10.42, $p<0.05$) elevated, to levels approximately 2 fold of that of normoxic controls when primary cortical neurons were maintained in hypoxic conditions for 72 hours (Fig. 15A). Bnip3 mRNA was not changed in cortical neurons after 48 hours of hypoxia compared to normoxic controls (Fig. 15A). In order to establish a mechanism for hypoxia-mediated Bnip3 gene transcription, we next transfected primary cortical neurons with a luciferase reporter construct that contained either the full length Bnip3 promoter or the Bnip3 promoter with a mutation in the HRE site (mutHRE luc). Promoter activity was assessed in transfected cortical neurons maintained in normoxic or hypoxic conditions. We first measured a significant ($F_{1,76}$=25.15; $p<0.0001$) effect in regards to plasmid transfected, either Bnip3 luc or mutHRE luc, by air condition (Fig. 15B). Additionally, a significant ($F_{1,76}$=18.96; $p<0.0001$) increase in promoter activity was measured in cortical neurons transfected with Bnip3 luc and maintained in hypoxic conditions, compared to Bnip3 luc transfected neurons maintained in normoxic conditions (Fig. 15B). This effect of hypoxia was not seen when the mutHRE was transfected into cortical neurons and exposed to hypoxic conditions (Fig. 15B).

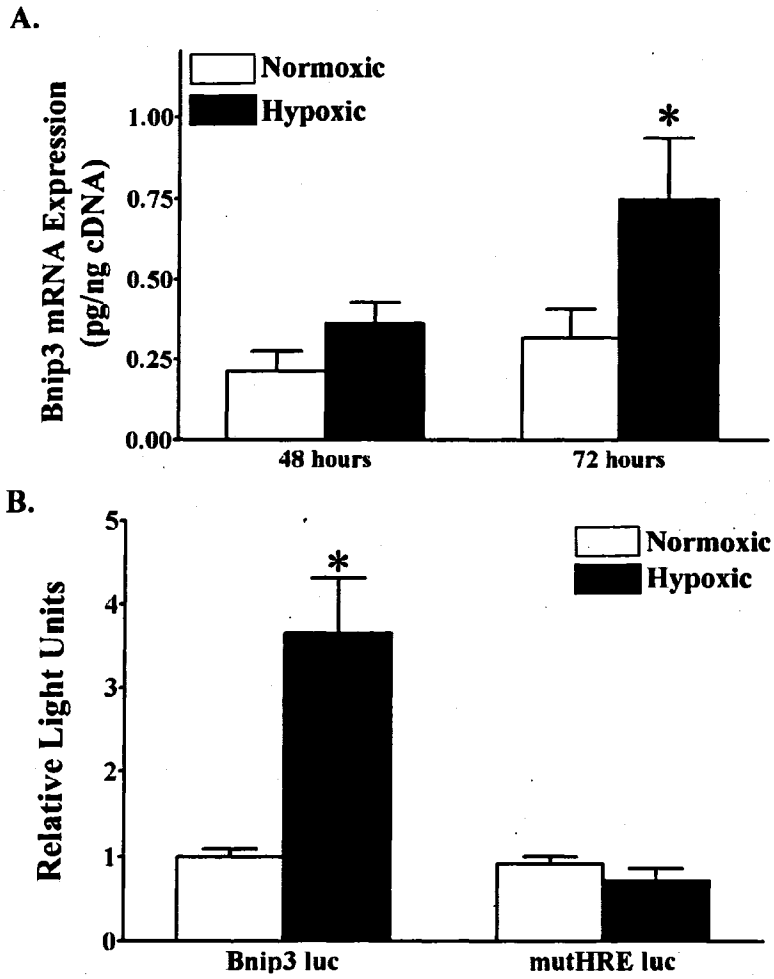


Figure 15. Hypoxia increases Bnip3 mRNA levels in primary cortical neurons through the hypoxic response element. (A) Quantitative real time RT-PCR was used to measure Bnip3 mRNA levels (pg/ng cDNA) in primary cortical neurons maintained in either normoxic (20% O_2) or hypoxic (1% O_2) conditions for 48 (white bars) or 72 (black bars) hours. Each bar represents the mean $\pm$ S.E.M. of 9-11 determinations. Data were analyzed by two-way ANOVA (treatment by time). (B) Luciferase reporter gene assay was used to measure promoter activity in primary cortical neurons dual transfected with either a Bnip3 luc or mutHRE luc reporter plasmid and a β-galactosidase reporter and maintained in either normoxic (white bars) or hypoxic (black bars) conditions. Relative light units (RLU) were calculated as a ratio of luciferase:β-galactosidase activity for each transfected culture and normalized to the promoter activity of the normoxic cultures. Each bar represents the mean $\pm$ S.E.M. of 11-15 determinations generated in 5 separate transfection assays. Data were analyzed by two-way ANOVA (plasmid type by oxygen condition). Multiple t-test comparisons were used to indicate significant differences (*$p<0.05$) from control groups.

Glucocorticoids exacerbate hypoxic induced Bnip3 mRNA expression in primary cortical neurons

The previous experiments established that Bnip3 mRNA expression and cell death are increased following administration of the GR selective agonist. Furthermore, we also established that hypoxia induces Bnip3 mRNA levels in cortical neurons through the HRE in the Bnip3 promoter. We subsequently investigated whether GR activation exacerbated the effects of hypoxia on Bnip3 mRNA expression. Treatment with RU28362 increased Bnip3 mRNA above that of cultures exposed to hypoxia alone. There was as significant ($F_{3,35}$=3.51, $p<0.05$) 2 fold increase in Bnip3 mRNA levels compared to hypoxic, vehicle treated controls when cortical neurons were treated with either 0.1, 1.0 or 10 nM RU28362 for 72 hours (Fig. 16). Such a synergistic affect of glucocorticoids and hypoxia on Bnip3 expression was not present after 48 hours of RU28362 treatment (Fig.16), which was consistent with the timing of the effects of RU28362 on cortical neuron cell death. A significant ($F_{3,70}$=3.12; $p<0.05$) interaction between RU28362 treatment and treatment time was also measured.

Glucocorticoids enhance hypoxia induced primary cortical neuron death

We next determined if glucocorticoid treatment in conjunction with hypoxia decreased primary cortical neuron viability, as measured with the MTT assay. We first established the percent change in cortical neuron viability after 48 and 72 hours of hypoxic exposure in the absence of glucocorticoids. A significant ($F_{1,32}$=65.57, $p<0.05$) decrease in cell viability (25%) was measured in neuronal cultures maintained in hypoxic conditions for 48 when compared to cultures maintained in

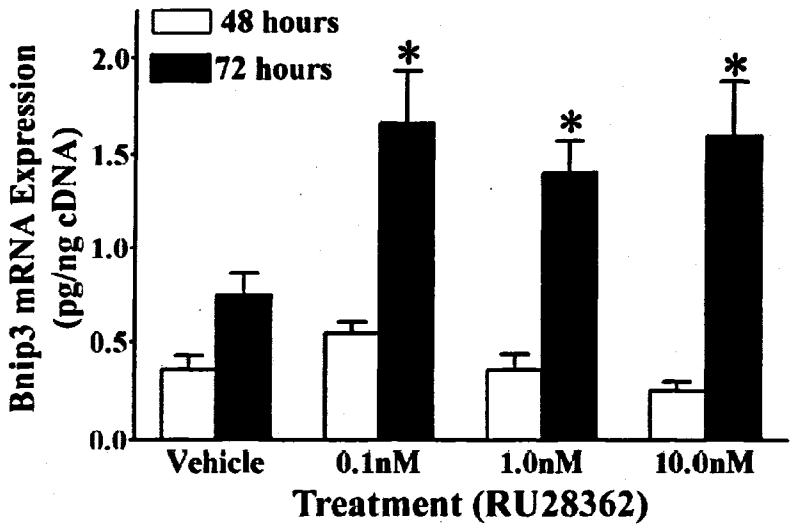


Figure 16. Glucocorticoids enhance hypoxia induced Bnip3 mRNA expression in primary cortical neurons. Bnip3 mRNA expression (pg/ng cDNA) in primary cortical neurons treated with either RU28362 or vehicle and maintained in hypoxic (1% O_2) conditions for 48 (white bars) or 72 (black bars) hours. Each bar represents the mean $\pm$ S.E.M. of 9-11 determinations. Data were analyzed by two-way ANOVA (treatment by time). Student-Newman Keul's posthoc test was used to indicate groups with significant differences (*$p<0.05$) in Bnip3 mRNA levels relative to vehicle treated controls.

normoxic conditions (Fig. 17A-B). Cortical neurons maintained in hypoxic conditions for 72 hours also had a significant ($F_{1,33}$=195.79, $p<0.05$) decrease in cell viability (50%) compared to vehicle treated normoxic controls.

We subsequently investigated the synergistic affects of glucocorticoids and hypoxia on primary cortical neuron viability. In order to focus on the exacerbation of cortical neuron death as a result of glucocorticoid treatment, the data for hypoxic RU28362 treated cultures was normalized to hypoxic vehicle treated controls. Primary cortical neurons treated with 10.0nM RU28362 for 48 hours and maintained in hypoxic conditions had a significant ($F_{3,67}$=4.30, $p<0.05$) decrease in cell viability compared to 1.0nM RU28362 treated hypoxic cultures, but not to vehicle treated hypoxic cultures (Fig. 17C). However, cortical cultures treated with 1.0 or 10.0nM RU28362 and maintained in hypoxic conditions for 72 hours had a 12% decrease in viability ($F_{3,61}$=5.14, $p<0.05$) relative to hypoxic vehicle treated controls (Fig. 17D). Furthermore, in cultures treated for 72 hours there was a significant ($F_{3,123}$=2.89; $p<0.05$) interaction between RU28362 treatment and air conditions (Fig. 17D).

Bnip3 protein decreases primary cortical neuron viability

Primary cortical neurons were transfected with a Bnip3 expression plasmid and cell viability was subsequently determined after 72 hours. Over-expression of Bnip3 protein significantly ($F_{3,55}$=16.41, $p<0.0001$) decreased cortical neuron viability by 38% relative to the non-transfected controls and controls transfected with the empty pcDNA plasmid (Fig. 18). Because the transmembrane domain of the Bnip3 protein is thought to be as essential for the induction of Bnip3 mediated cell death (Ray et al., 2000), we also used a plasmid that encodes for a Bnip3 protein with

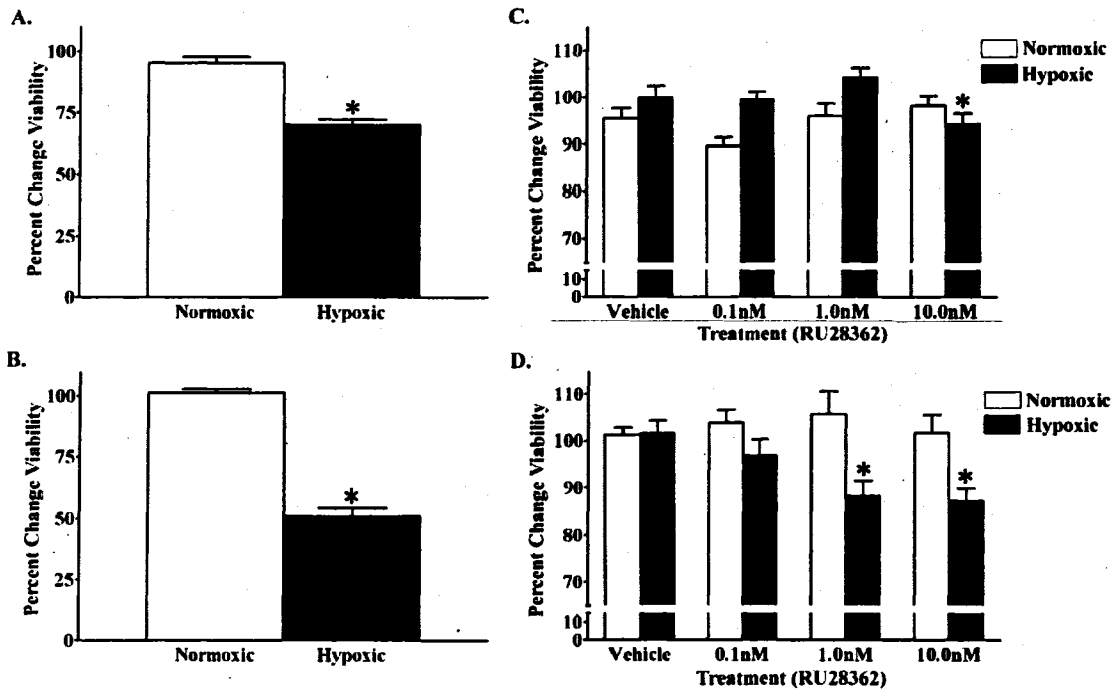


Figure 17. The GR selective agonist RU28382 enhances hypoxia-induced death of primary cortical neurons. (A-B) MTT assay to measure percent change in viability of vehicle treated primary cortical maintained in either normoxic or hypoxic conditions for (A) 48 and (B) 72 hours. Percent change in viability is relative to normoxic vehicle treated controls for each time point. (C-D) MTT assay to measure percent change in viability of RU28362 or vehicle treated primary cortical neurons maintained in either normoxic (white bars) or hypoxic (black bars) for (C) 48 and (D) 72 hours. Percent change in viability for normoxic cultures is relative to vehicle treated normoxic controls. Percent change in viability for hypoxic cultures is relative to vehicle treated hypoxic controls. Each bar represents the mean $\pm$ S.E.M. of 14-18 determinations. Data were analyzed by two-way ANOVA (A-B air conditions by time; C-D RU28362 by air conditions). (A-B) Multiple t-test comparisons indicate significant differences (*$p<0.05$) in percent change viability as a result of hypoxia at a given time. Student-Newman Keul's posthoc test was used to indicated groups with significant differences (*$p<0.05$) in percent change viability relative to hypoxic 1.0nM (C) or vehicle- treated (D) cultures.

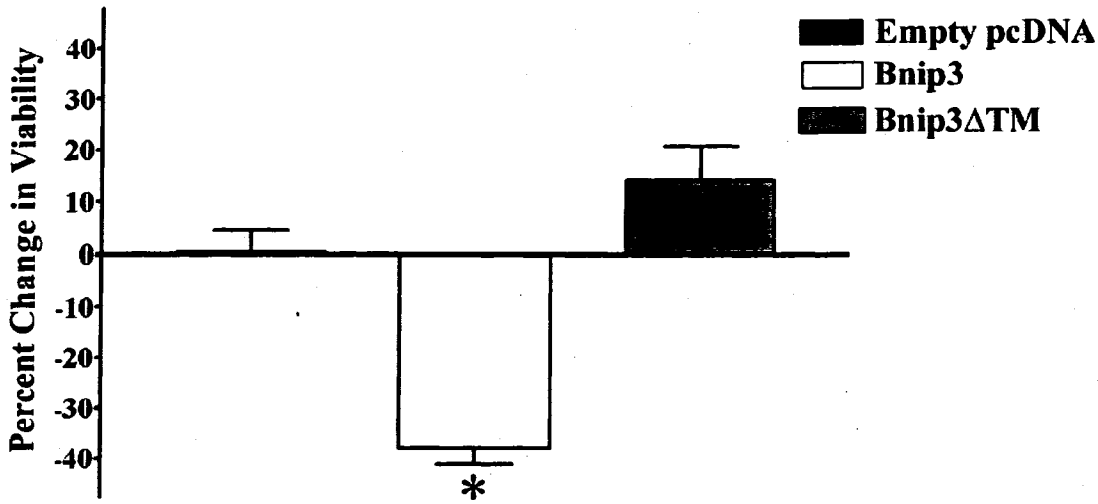


Figure 18. Over-expression of Bnip3 in primary cortical neurons induces cell death. Percent change in viability was measured in primary cortical neurons dual transfected with Bnip3, Bnip3ΔTM or empty PCDNA expression plasmid and a β-galactosidase reporter for 72 hours. Viability of transfected neurons was normalized to β-galactosidase activity and reported as percent change in viability relative to the viability of non-transfected controls. Each bar represents the mean ± S.E.M. of 18-24 determinations generated in 3 transfections. Data were analyzed by one-way ANOVA followed by Student-Newman Keul's posthoc test. * indicates groups that were significantly different ($p<0.05$) in percent change in viability relative to non-transfected control.

a deletion of the transmembrane domain (Bnip3ΔTM). Deletion of the transmembrane domain prevented the decrease in cell viability relative to the full length Bnip3 expression plasmid (Fig. 18). Transfection of primary cortical neurons with an empty plasmid was not different from cell viability in non-transfected control neurons. These data confirm that our transfection protocol does not by itself, alter baseline cell numbers (Fig. 18).

GR regulation of Bnip3 promoter activity in primary cortical neurons is dependent on a GRE

Since the previous experiments indicated that GR increased levels of Bnip3 mRNA, we sought to determine whether GR acts to increase Bnip3 mRNA levels by inducing gene transcription. Primary cortical neurons were transiently transfected with a Bnip3 promoter - luciferase reporter containing plasmid and then treated with RU28362 or vehicle for 42 hours. Bnip3 promoter activity was significantly ($F_{4,95}$=4.55, $p<0.05$) elevated in transfected neurons treated with 1.0, 5.0 and 10.0nM RU28362 compared to vehicle-treated, transfected neurons (Fig. 19).

We further examined the Bnip3 promoter *in silico*, and identified 3 consensus GRE sites. Figure 20A illustrates the Bnip3 promoter with the locations of the 3 putative GRE sites relative to the transcription start site. In order to determine which of the three putative GREs mediates GR regulation of Bnip3 gene transcription, three mutant Bnip3 promoter - luciferase reporter constructs were generated to isolate the individual putative GREs (Fig. 20A, bottom 3 illustrations). Primary cortical neurons were transfected with either GRE1-luc, GRE2-luc or GRE3-luc and treated with varying doses of RU28362 or vehicle for 42 hours. Basal activity for GRE1-luc,

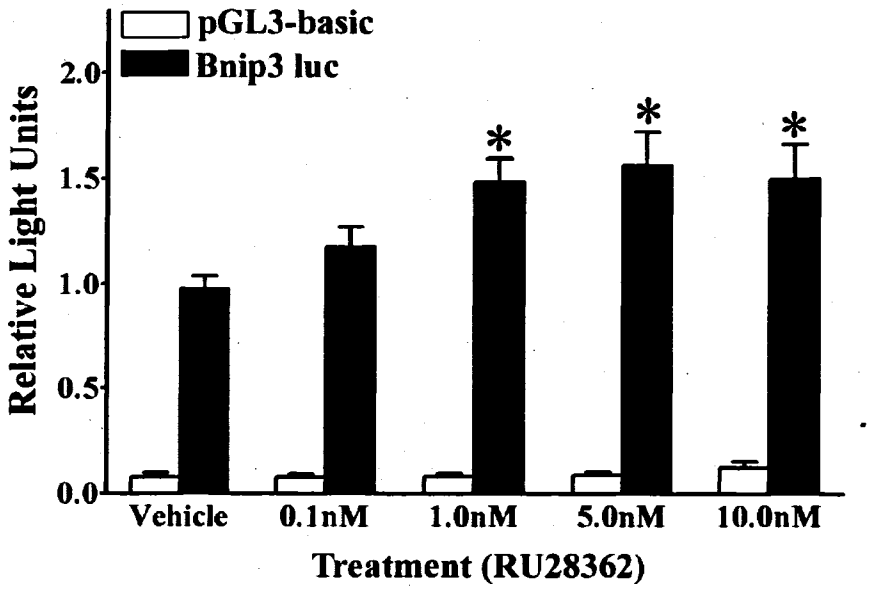


Figure 19. GR induces Bnip3 promoter activity in primary cortical neurons. A luciferase based reporter gene assay was used to measure promoter activity in primary cortical neurons dual transected with either the empty pGL3-basic luciferase reporter (white bars) or Bnip3 promoter luciferase reporter (black bars) containing plasmids and a β-galactosidase reporter. Transfected neurons were treated with either RU28362 or vehicle. Relative light units (RLU) were calculated as a ratio of luciferase:β-galactosidase activity for each transfected culture and normalized to the mean RLU of vehicle treated neurons transfected with the Bnip3 promoter. Each bar represents the mean ± S.E.M. of 18-24 determinations generated in 8 separate transfection assays. Data were analyzed by two-way ANOVA (treatment by plasmid) followed by Student-Newman Keul's posthoc test. * indicates those groups with significant differences (*$p<0.05$) in promoter activity relative to vehicle treated controls.

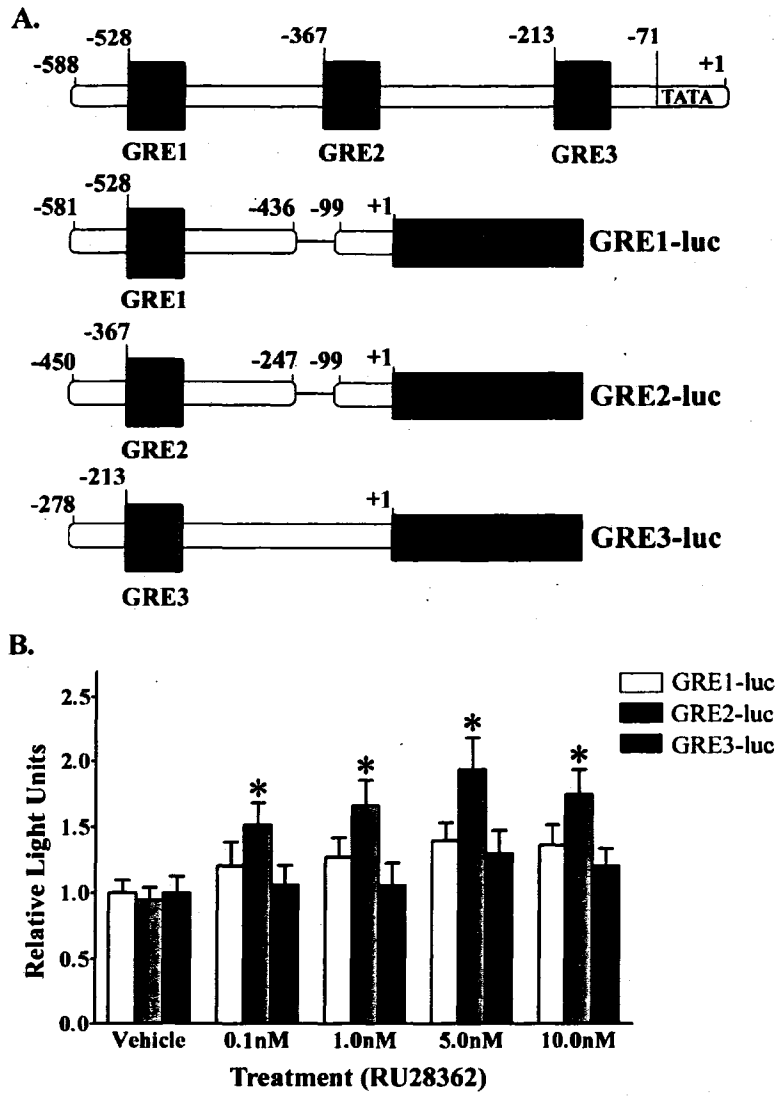


Figure 20. The Bnip3 promoter region containing the putative GRE2 is responsive to activated GR. (A) Illustration depicting the endogenous Bnip3 promoter with the relative locations of the three putative GREs and a schematic of the three mutant Bnip3 promoter luciferase reporters (GRE1 luc, GRE2 luc and GRE3 luc) generated to isolate each individual GRE. (B) Luciferase based reporter gene assay was used to measure promoter activity in primary cortical neurons dual transfected with either GRE1 luc (white bars), GRE2 luc (grey bars) or GRE3 luc (black bars) containing plasmids and a β-galactosidase reporter and subsequently treated with vehicle or RU28362. Relative Light Units (RLU) were calculated as a ratio of luciferase:β-galactosidase activity for each transfected culture and normalized to the mean vehicle treated RLUs for each promoter reporter tested. Each bar represents the mean ± S.E.M. of 11-15 determinations generated in 5 separate transfection assays. Data were analyzed by two-way ANOVA (treatment by plasmid type) followed by Student-Newman Keul's posthoc test. * indicates those groups that were significantly different (*$p<0.05$) in promoter activity relative to vehicle treated controls.

GRE2-luc and GRE-3-luc were not significantly different from that of the full length Bnip3 promoter - luciferase reporter (data not shown). Primary cortical neurons transfected with GRE1-luc and GRE3-luc did not respond to RU28362 with a significant change in promoter activity relative to vehicle treated controls (Fig. 20B). However, cortical neurons transfected with GRE2-luc had a significant ($F_{4,62}$=4.24, $p<0.05$) increase in promoter activity in response to RU28362 treatment relative to vehicle treated transfected controls (Fig 20B). The magnitude of this increase was approximately 2- fold greater after RU28362 treatment (Fig. 20B), and achieved levels equivalent to the promoter activity of the full length Bnip3 promoter after RU28362 treatment (Fig. 19). Additionally, we identified a significant increase in the GRE2-luc activity following 0.1nM RU28362 treatment relative to vehicle treated controls (Fig. 20), which did not occur in cultures transfected with Bnip3 luc and treated with 0.1nM RU28362 (Fig. 19). The increase in GRE2-luc promoter activity following 0.1nM RU28362 treatment may result from removal of the other putative GRE 1 or 3 sites. These GRE sites may serve as regions that suppress basal Bnip3 expression levels possible through MR interactions with the promoter. However, additional experiments are necessary to elucidate the cause of these results.

Finally, to confirm that the putative GRE2 is the region that mediates GR regulation of Bnip3 gene transcription we transfected primary cortical neurons with a Bnip3 promoter - luciferase reporter plasmid, with a disrupted GRE2 site (Bnip3ΔGRE2-luc; Fig. 21A). The Bnip3ΔGRE2-luc transfected cortical neurons had basal promoter activity that was not different from the basal promoter activity in Bnip3 luc transfected cortical neurons (data not shown). Cortical neurons transfected

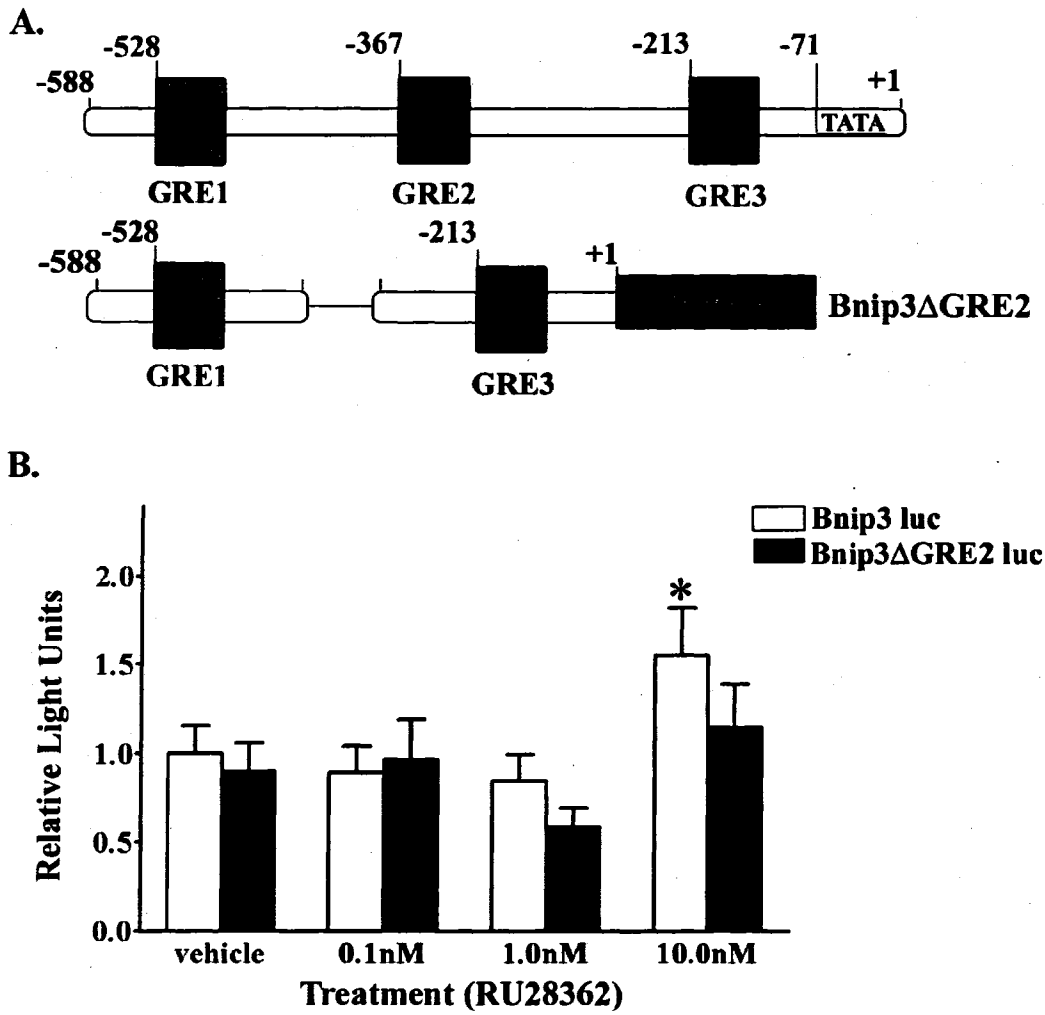


Figure 21. GR regulation of Bnip3 gene transcription is mediated through a GRE. (A) Upper illustration depicting the endogenous Bnip3 promoter with the relative locations of the three putative GREs. The lower illustration is a schematic of the mutant Bnip3ΔGRE2 promoter luciferase / reporter (Bnip3ΔGRE2 luc) generated to eliminate the 2nd GRE site while leaving the remainder of the promoter is intact (B) Luciferase based reporter gene assay was used to measure promoter activity in primary cortical neurons dual transfected with either Bnip3 luc (white bars) or Bnip3ΔGRE2 luc (black bars) containing plasmids and a β-galactosidase reporter and subsequently vehicle or RU28362 treated. Relative light units (RLU) were calculated as a ratio of luciferase:β-galactosidase activity for each transfected culture and normalized to the vehicle treated mean RLUs for each promoter reporter. Each bar represents the mean ± S.E.M. of 10-15 determinations generated in 5 transfections. Data were analyzed by two-way ANOVA (treatment by plasmid) followed by Student-Newman Keul's posthoc test. * indicates those groups with significant differences (*$p<0.05$) in Bnip3 luc promoter activity relative to vehicle and 0.1nM RU28362 treated cultures.

with Bnip3 luc and treated with 10.0nM RU28362 had significantly ($F_{2,149}$=3.96; $p<0.05$) increased promoter activity compared to vehicle and 0.1nM RU28362 treated Bnip3 luc transfected cultures (Fig. 21B). Conversely, RU28362 did not induce luciferase activity in cortical neurons transfected with Bnip3ΔGRE2-luc when compared to vehicle-treated Bnip3ΔGRE2 luc transfected cultures (Fig 21B).

Discussion

The results of the experiments in this study identified the pro-apoptotic gene Bnip3 as a mediator of cortical neuron death and also as a glucocorticoid and hypoxia responsive gene in the rat cortex. DEX treatment of postnatal female rat pups increased Bnip3 mRNA expression in the anterior and posterior cingulate cortices and the hippocampus. However, in male rat pups, DEX increased Bnip3 mRNA levels in the hippocampus, but not the cingulate cortex. Glucocorticoid induction of Bnip3 transcription was mediated by a GRE site within the 5' untranslated region of the Bnip3 gene. Despite the upregulation of Bnip3 mRNA expression in response to glucocorticoid treatment, this was insufficient to cause neuronal death. However, glucocorticoids exacerbated the secondary metabolic insult of hypoxia in cortical neurons as concomitant treatment with glucocorticoids and hypoxia increased cell death relative to those maintained in hypoxic conditions alone. Moreover, the enhanced increase in Bnip3 mRNA expression following glucocorticoid and hypoxia treatment parallels the observed increase in cortical neuron death.

Previous studies have established that glucocorticoids can endanger the CNS by increasing levels of the pro-apoptotic protein Bax and decreasing levels of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, in the juvenile and adult rat hippocampus

(Almeida et al., 2000) and in primary hippocampal neurons (Crochemore et al., 2005). Furthermore, DEX treatment results in an increase in apoptosis in the adult rat hippocampus and striatum (Hassan et al., 1996; Haynes et al., 2001). Consistent with these studies, our results indicate that DEX similarly induces expression of Bnip3 in the postnatal male and female rat hippocampus. This may be an additional component of the mechanism mediating DEX induced hippocampal cell death. However, Bnip3 interactions with the Bcl-2 family and subsequent implications for cell viability are only partially defined (Ray et al., 2000; Gao et al., 2005). It is known that Bnip3 protein heterodimerizes with the anti-apoptotic proteins Bcl-2 and Bcl-X_L, but this does not seem to prevent Bnip3 mediated cell death. Furthermore, Bnip3 interactions with other proteins are mediated by the TM domain and not the BH3 domain (Chen et al., 1997a; Ray et al., 2000), the site within BH3-only proteins that are implicated as essential to Bax activation (Wei et al., 2001). Regardless, the putative interaction of Bnip3 and Bax has not been reported in the current literature.

Despite the observations showing glucocorticoid neuroendangerment in the hippocampus, and DEX dependent decreases in cortical neuron numbers (Crochemore et al., 2005; Kreider et al., 2006), to the best of our knowledge, glucocorticoid endangerment of cortical neuron responses to a secondary metabolic insult, have not yet been described. The results of our studies have established that activated GR increases Bnip3 transcription in the anterior and posterior cingulate cortices of the postnatal female rat, but not in the male cingulate cortex. This sex difference suggests the possibility that the female cingulate cortex may be more

susceptible to the neuroendangering aspects of glucocorticoids than the male cingulate cortex.

In a previous study, we investigated a potential mechanism governing sex differences in Bnip3 expression in the developing rat brain. It was determined that sex differences in postnatal gonadal steroid hormone levels are not responsible for the differing basal levels of Bnip3 mRNA expression in the male and female anterior and posterior cingulate cortices (Sandau and Handa, in press). It is possible that this sex difference may be the result of genetic differences; however, additional experiments are necessary to understand the underlying mechanism mediating sex difference Bnip3 gene expression.

Our *in vitro* studies also indicate that glucocorticoids act directly on cortical neurons to upregulate Bnip3 mRNA expression. Furthermore, activated GR directly regulates Bnip3 gene transcription via a GRE site within the Bnip3 promoter. Notwithstanding, earlier studies show that glucocorticoids can act indirectly to regulate expression of the Bcl-2 gene family by modulating NFκB (Ramdas and Harmon, 1998; Dhandapani et al., 2005) and p53 transcriptional activity (Miyashita et al., 1994; Miyashita and Reed, 1995; Maiyar et al., 1997; Crochemore et al., 2002; Iyer et al., 2003). Bnip3 expression has similarly been shown to be attenuated following inhibition of NFκB (Baetz et al., 2005), a gene that is glucocorticoid regulated (Webster and Cidlowski, 1999). Therefore, indirect regulation of Bnip3 gene transcription through glucocorticoid modulation of NF-κB remains a possibility. Conversely, Bnip3 gene transcription appears to be independent of p53 activity (Guo

et al., 2001; Fei et al., 2004), despite reports of glucocorticoid regulation of p53 (Crochemore et al., 2002).

Bnip3 regulation has been shown to be hypoxia responsive, both in peripheral cell types as well as in the CNS (Bruick, 2000; Althaus et al., 2006; Burton et al., 2006). Increases in hypoxia-induced cell death are associated with increases in Bnip3 expression, both *in vivo* and *in vitro* (Schmidt-Kastner et al., 2004; Althaus et al., 2006; Burton et al., 2006). Using *in vivo* approaches, Bnip3 protein levels were found to be increased following a hypoxic-ischemic insult in the adult rat hippocampus, cortex and striatum (Schmidt-Kastner et al., 2004; Althaus et al., 2006). Similarly, in a differentiated oligodendroglial cell line, Bnip3 protein levels are increased in response to hypoxia (Burton et al., 2006). Consistent with these studies, our data indicate that Bnip3 mRNA is upregulated by hypoxia in primary cortical neurons.

We have also determined that hypoxia regulates Bnip3 in cortical neurons via the HRE site within the promoter; a site that was previously only characterized in peripheral cell types (Bruick, 2000; Sowter et al., 2001). Accordingly, Bnip3 expression also decreased cortical neuron viability after hypoxia. We have also determined that removal of the TM domain of Bnip3 prevented the effects of Bnip3 and allowed partial recovery of primary cortical neuron viability following hypoxia. The Bnip3ΔTM has been previously reported to act as a dominant negative to the endogenous Bnip3 protein (Kanzawa et al., 2005; Burton et al., 2006) and negates hypoxia induced cell death in differentiated oligodendroglial cells (Burton et al., 2006). Taken together, these data suggest that Bnip3 mediates a component of

hypoxia induced cell death in cortical neurons, but additional factors may also contribute to the hypoxic response.

Elevated glucocorticoids causes an altered metabolic state that is partially conferred by decreased glucose transporter expression levels and inhibition of glucose uptake in neurons (Sapolsky et al., 1986; Horner et al., 1990; Virgin et al., 1991; Booth et al., 1998). These changes subsequently endanger hippocampal neurons when challenged with a second metabolic insult such as glutamate excitotoxicity or hypoxia-ischemia (Sapolsky and Pulsinelli, 1985; Roy and Sapolsky, 2003). Our studies demonstrate that low doses of GR agonist also exacerbate cell death associated with a hypoxic insult in primary cortical neurons. However, this affect was time dependent as a change in cortical neuron viability was detected after 72 hours of treatment, but not after 48 hours. In agreement with the increase in cell death we also showed that hypoxia-induced Bnip3 expression was exacerbated following 72 hours of glucocorticoid treatment. Activated GR can also stimulate or repress expression of additional hypoxic responsive genes including, glucose transporter 3 and vascular endothelial growth factor (Kodama et al., 2003; Leonard et al., 2005). Although some studies suggest that GR modulation of hypoxic responsive genes is conferred by transactivation of HIF-1, there are conflicting data in regards to whether the event is mediated through an HRE or GRE (Kodama et al., 2003; Leonard et al., 2005).

Our studies have also determined that increasing levels of Bnip3 protein, through the over-expression of Bnip3 in cortical neurons works independent of additional insults and is sufficient to induce cortical neuron death. Cell death directly attributed to increases in Bnip3 protein has been demonstrated in multiple cell types

including oligodendrocytes (Vande Velde et al., 2000; Burton et al., 2006). In each of the cell types tested, the TM domain of the Bnip3 protein is implicated as the region which confers its killing activity (Vande Velde et al., 2000; Yook et al., 2004; Burton et al., 2006). This aspect of Bnip3 protein function is conserved in cortical neurons as our data indicates that the TM domain, and not the BH3 domain, is essential to Bnip3 mediated cell death.

In summary, the experiments conducted in these studies have identified a cellular mechanism by which glucocorticoids can endanger the cerebral cortex and exacerbate hypoxia-induced cell death. These findings are in agreement with reports that identify glucocorticoids as compounds that enhance pathologies associated various secondary metabolic insults. Furthermore, our data have identified a candidate gene, Bnip3, as a mediator of these observed affects. However, additional studies are necessary to determine if Bnip3 acts in conjunction with the other Bcl-2 family members to mediate cell death. The results of these studies have potential clinical ramifications since synthetic glucocorticoids are commonly prescribed to prevent chronic lung disease in premature infants (Bar-Lev et al., 2004; Doyle et al., 2006). Furthermore, information gained in regards to GR and HIF-1 regulation of Bnip3 may serve as a therapeutic target for hypoxic insults in the developing and mature CNS.

Chapter 5

Discussion

The studies conducted in this dissertation focus on glucocorticoid modulation of Bnip3 transcription in the presence and absence of a secondary insult, such as hypoxia, in the developing rat cortex. The experiments described in this dissertation were designed to address or shed light on a more profound clinical observation; that glucocorticoids, acting in concert with hypoxia, are potentially harmful to the developing human brain. Glucocorticoid treatment of premature infants, which are hypoxic by nature, results in a higher incidence of cerebral palsy than premature infants not treated with steroids (Shinwell et al., 2000). Additionally, a theory has recently been proposed that infants exposed to either excess glucocorticoids or hypoxia are predisposed to developing schizophrenia (Koenig et al., 2002). These clinical studies indicate that an overlap exists between the neuropathologies associated with these two particulate insults. Furthermore, these reports suggest that glucocorticoids potentiate secondary metabolic insult in the developing human brain, similar to what has been described in the rodent.

One gene, Bnip3, was singled out in my studies as the putative effector protein that mediates glucocorticoid exacerbation of hypoxia induced cell death. The rational for this stems from studies in the literature which indicate that hypoxia increases Bnip3 mRNA and protein in every cell type tested (Bruick, 2000; Althaus et

al., 2006; Burton et al., 2006). In support of these studies, Bnip3 was found to be regulated by hypoxia in my treatment paradigms. Furthermore, glucocorticoids acting either independently or together with hypoxia can enhance Bnip3 gene transcription.

My data show that Bnip3 has the capability to act independently to regulate cell death in the developing brain associated with hypoxia and glucocorticoids. However, review of the current literature raises additional questions: Is Bnip3 a valid candidate for cell death associated with normal and abnormal brain development? Does Bnip3 act independently or in conjunction with additional effector proteins to regulate cell death associated with NOCD and injury? Are there alternate mechanisms that regulate Bnip3 expression in the brain? Despite the obvious differences in these questions, one underlying theme links each of these in my mind: Is my data relevant to neuropathologies associated with the developing human brain? Each question will be addressed in the following discussion in regards to normal development and injury associated with hypoxia and glucocorticoid exposure. However, due to the limitations associated with human studies, inferences drawn from experiments conducted with human tissues or in animal models are used to address the putative implications.

Is Bnip3 a valid candidate for mediating cell death associated with normal and abnormal brain development?

In consideration of the data from both this dissertation and the current literature, I believe that Bnip3 is an important mediator of both normal and abnormal brain development. However, I do not believe that Bnip3 is the sole effector protein of NOCD in the developing brain. Furthermore, our data and the available literature indicate that Bnip3 acts in conjunction with additional proteins to mediate cell death

resulting from hypoxia and / or glucocorticoids. A more complex model for these cell death events is plausible as this would confer greater control over a process that once complete, is irreversible. Additionally, a complex model allows for a greater number of interaction sites that factors such as hypoxia, glucocorticoids and growth factors can converge to modulate Bnip3 expression or activity.

A major indicator that Bnip3 is associated with either NOCD or death attributed to injury are reports of either a caspase-independent cell death mechanism or autophagy that correlate with the time of NOCD or injury. Cell death attributed to Bnip3 protein function is quite unique because it is dependent on mitochondrial function, but independent of caspase activity (Vande Velde et al., 2000). Furthermore, Bnip3 over-expression results in cell death induction characterized by mitochondrial permeability pore opening, electrochemical gradient suppression, increased reactive oxygen species, extensive cytoplasmic vacuoles and late chromatin condensation. Yet, Bnip3 over-expression is not associated with the typical apoptosis markers of cytochrome c release and increased caspase-3 and -9 and APAF-1 activity (Vande Velde et al., 2000).

This original characterization describes a cell death process that is neither apoptotic or necrotic. Instead the cell death more closely resembles autophagy due to the presence of cytoplasmic vacuoles and chromatin condensation (Schweichel and Merker, 1973). However, these studies did not confirm that Bnip3 mediated cell death is autophagic. More recent studies conducted in a malignant glioma cell line have determined that Bnip3 induces autophagic cell death that is caspase-independent. Furthermore, cell death resulting from arsenic trioxide or ceramide treatment, which

exclusively induce autophagy, is inhibited if Bnip3 protein function is rendered inactive (Daido et al., 2004; Kanzawa et al., 2005). cDNA microarray, RT-PCR and western blot data also identified an increase in Bnip3 expression following arsenic trioxide treatment. The other Bcl-2 family members typically induce a caspase-dependent mechanism of apoptosis. Furthermore, expression levels of the other pro- and anti-apoptotic Bcl-2 family member, with the exception of Bnip3L, are not altered in response to arsenic trioxide. Even though Bnip3L is upregulated in response to arsenic trioxide the protein does not mediate autophagy (Kanzawa et al., 2005). Cumulatively, these studies suggest that autophagic cell death is unique to Bnip3 protein function.

Autophagy is also associated with normal CNS development and injury resulting from hypoxia (Xue et al., 1999; Yu et al., 2003; Florez-McClure et al., 2004; Gu et al., 2004). Growth factor withdrawal in cerebellar granule neurons and sympathetic ganglion neurons induces autophagy, not apoptosis (Xue et al., 1999; Lum et al., 2005). Evidence of autophagy arises from studies performed in cells derived from Bax/Bak deficient mice. Despite the lack of Bax and Bak, which studies demonstrate are essential but redundant for apoptosis induction, cells subjected to growth factor withdrawal induce a delayed autophagic cell death if growth factors are not restored (Lum et al., 2005). Furthermore, cell death associated with growth factor withdrawal in wild type sympathetic ganglion neurons is inhibited by 3-methyladenine, a compound that prevents autophagy (Xue et al., 1999).

Studies also postulate that autophagy can mediate injury associated with hypoxia-ischemia in the neonatal rat hippocampus. Similar to growth factor

withdrawal, postnatal rats subjected to hypoxia-ischemia have delayed cell death that is not morphologically similar to either apoptosis or necrosis. The cell death is characterized by the presence of condensed nuclei with clumped chromatin and extensive cytoplasmic vacuoles. However, these studies did not determine if the vacuoles are lysosomal in nature (Fukuda et al., 1999; Sheldon et al., 2001). These reports implicate autophagy as a component of cell death associated with either growth factor withdrawal or hypoxic injury. However, studies have yet to identify the underlying mechanism that mediates these observed responses. Based on data identifying Bnip3 as the Bcl-2 family member that mediates autophagy, Bnip3 is a likely candidate to orchestrate both normal and abnormal cell death associated with autophagy.

Additional evidence, independent of autophagy, lends support that Bnip3 is a putative effector protein for NOCD. Our studies identified an increase in Bnip3 mRNA expression in the female anterior and posterior cingulate cortices and hippocampus that strongly correlates to previously measured increases in cortical and hippocampal NOCD (Gould et al., 1991; Ferrer et al., 1994b; Spreafico et al., 1995). However, in the male an increase in Bnip3 mRNA expression was only measured in the anterior cingulate cortex. These studies can be interpreted in multiple ways. One possible interpretation is that the mechanism of NOCD is different pending the brain region and the species sex. If this interpretation is correct then Bnip3 regulates NOCD in all brain regions tested of the female, but only regulates NOCD in the anterior cingulate cortex of the male. Thus the increased levels of NOCD in the postnatal male cortex and hippocampus may likely be attributed to a different cell-death protein. An

alternative interpretation is that in the posterior cingulate cortex and hippocampus, a sex difference exists in NOCD levels and Bnip3 mediates these sex differences. Sex differences in NOCD have been documented in select brain regions including the primary visual cortex (Nunez et al., 2001). However, the studies that identified the peak in postnatal NOCD in the rat cortex and hippocampus did not measure cell death in males and females separately (Gould et al., 1991; Ferrer et al., 1994b; Spreafico et al., 1995). Thus it is feasible that sex differences in NOCD also occur in the hippocampus and additional regions of the cortex. A final interpretation is that Bnip3 is not a primary mediator for postnatal NOCD, but instead the peaks in Bnip3 expression point to development periods that predispose a particular brain region or sex to an insult.

In order to more fully understand the significance of increased Bnip3 levels during postnatal development I have recently measured NOCD levels in the male and female anterior and posterior cingulate cortices and hippocampus. NOCD was measured by counting the number of pyknotic cells in cresyl violet stained brain sections from PND 5, 6.5, 8 and 10 rats. The pyknotic neurons were considered dying based on the presence of morphological features, such as apoptotic bodies and condensed chromatin. In the anterior and posterior cingulate cortices cell death was significantly elevated on PND 5 compared to all other age groups in the male. Conversely, the female rat did not have a transient increase in NOCD at any age tested in the anterior and posterior cingulate cortices. In both the male and female CA1 and CA3 hippocampal regions elevated NOCD levels were measured on PND 5 compared to all other ages.

These data indicate that the number of dying cells identified by morphological characterization is elevated in the cingulate cortex and hippocampus prior to the transient surge in Bnip3 expression. This suggests that Bnip3 may not be a mediator of NOCD. However, limitations exist in these studies as they did not specifically investigate the number of cells dying from autophagy. In order to more definitively determine if Bnip3 is a mediator of NOCD, studies counting autophagic cell numbers throughout development are necessary.

Additional evidence that indicates that Bnip3 is involved in CNS development stems from studies that investigated oligodendroglial differentiation and Bnip3 regulation by growth factors. The BH3-only member expression levels were measured during oligodendroglial cell differentiation. A majority of the BH3-only members including Bim, Biklk, DP5/Hrk, Bad, Bid, Noxa, Puma and Bnip3L do not change expression patterns during this period; however, the levels of Bnip3 and Bmf are increased (Itoh et al., 2003). This suggests that either Bnip3 or Bmf may mediate the removal of extra oligodendrocytes during development. Studies conducted in peripheral cell culture types also report that Bnip3 acts in the capacity to regulate cell death in response to growth factor withdrawal; as the presence of either EGF or IGF attenuates Bnip3 killing activity (Kothari et al., 2003).

Direct evidence links Bnip3 to hypoxia-induced cell death. Burton et al., (2006) have reported that oligodendroglial cells maintained in hypoxia have increased cell death levels in comparison to cells maintained in normoxic conditions. Furthermore, oligodendroglial cells transfected with Bnip3ΔTM prior to hypoxic exposure have decreased cell death levels compared to controls (Burton et al., 2006).

Bnip3ΔTM has been characterized as a dominant negative Bnip3 variant that heterodimerizes with endogenous Bnip3 protein and prevents cell death induction (Burton et al., 2006). In this dissertation, I report similar findings showing an increase in cortical neuron cell death following hypoxia that is partially attenuated by Bnip3ΔTM over-expression. In both studies, transfection with Bnip3ΔTM only partially attenuated the hypoxia induced cell death. In the oligodendroglial cells a 20% reduction in cell death levels was reported (Burton et al., 2006); while we measured a 10% decrease in our primary cortical neurons. The partial attenuation indicates that Bnip3 does mediate a component of hypoxia-induced cell death in the CNS. However, what remains unknown is whether (1) the partial attenuation is representative of the transfection efficiency for Bnip3ΔTM or (2) an additional protein, yet to be identified, also mediates a component of the cell death associated with a hypoxic insult.

Cumulatively, the results of my studies and those conducted by others, suggest that Bnip3 is a potential contributor to both normal and abnormal cell death. However, Bnip3's association with normal brain development is more causal as a direct link between Bnip3 function and NOCD has yet to be tested. In order to definitively address the role of Bnip3 during CNS development studies utilizing a Bnip3 knockout mouse, or using other methodology to specifically reduce Bnip3 expression, followed by CNS development characterization are essential to either confirm or negate the importance of Bnip3.

Potential regulation and actions of Bnip3 during CNS development

CNS development is demarcated by periods of NOCD in the rat cortex and hippocampus, which peaks during the first postnatal week (Gould et al., 1991; Ferrer et al., 1994b; Spreafico et al., 1995). A number of studies have investigated the Bcl-2 family members as mediators for developmental cell death induction. Many of these studies have utilized transgenic knockout mice to examine the putative roles for the Bcl-2 family members during CNS development and in response to growth factor withdrawal (Greenlund et al., 1995; Putcha et al., 2001; Putcha et al., 2002). Additionally, the developmental ontogeny for a few of the Bcl-2 members has been established (Ferrer et al., 1994a; Vekrellis et al., 1997; Vinet et al., 2002).

In order to contribute to this line of research, I investigated the developmental ontogeny of Bnip3 in the rat cortex and hippocampus. My studies identified a transient increase in Bnip3 mRNA expression that corresponds with the postnatal NOCD peak. The parallel increase in Bnip3 expression and NOCD suggests that the BH3-only pro-apoptotic member may be involved in the developmental process. Additional evidence from the literature also lends support for this hypothesis. Based on our data and studies from the current literature I have developed a proposed model (Fig. 22) for Bnip3 gene expression and protein actions during normal CNS development.

Growth factors serve as cell survival signals by modulating pro- and anti-apoptotic Bcl-2 family member expression (Fig. 22A). For example, growth factor activation of the PIP3-Akt pathway induces anti-apoptotic Bcl-2 and Bcl-X_L protein expression and decreases pro-apoptotic Bax protein expression (Yamaguchi et al., 2001; Yamaguchi and Wang, 2001; Dhandapani et al., 2005). Further, the regulation

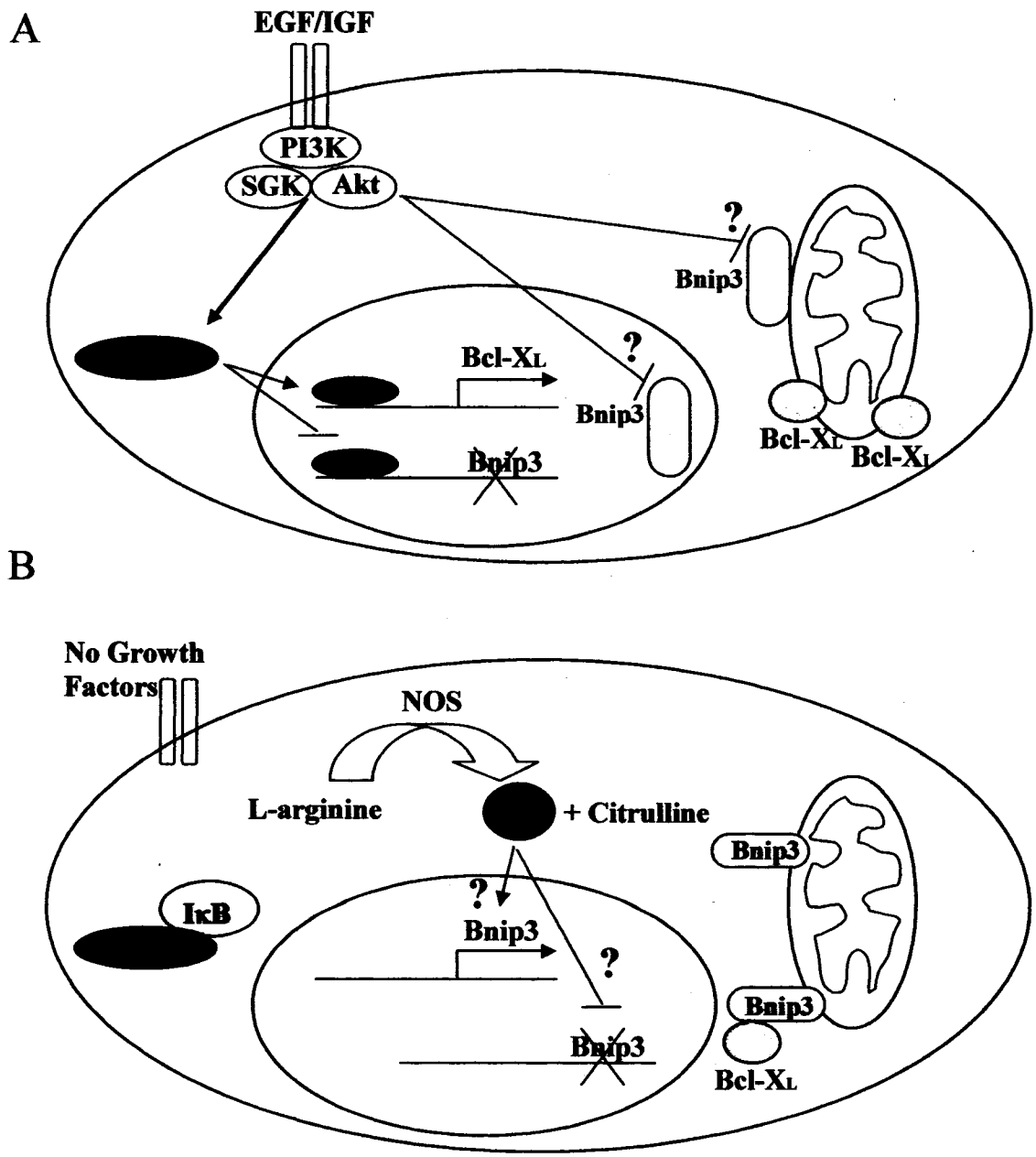


Figure 22. Schematic illustration depicting the putative regulation and actions of Bnip3 during NOCD. (A) Presence of growth factors prevents NOCD via NF-κB downregulation of Bnip3 and upregulation of Bcl-X_L. Growth factors also prevent basal Bnip3 protein activation. The question marks indicate areas of conflicting data in regards to inactive Bnip3 protein localization either in the nucleus or at the mitochondrial membrane. (B) Growth factor withdrawal induces Bnip3 expression and suppresses Bcl-X_L expression. Bnip3 protein is activated in absence of growth factor and disrupts mitochondrial function by either interaction with Bcl-X_L or directly opening the PT pore. The question marks indicate possibility of either NO induction or suppression of Bnip3 gene expression, which has not been investigated in neurons.

of the pro- and anti-apoptotic members is mediated by the intermediate protein NF-κB which is activated by growth factors via Akt (Romashkova and Makarov, 1999) (Fig. 22A). The increase in anti-apoptotic members selects for cell survival by stabilizing the mitochondrial membrane and preventing the apoptosis induction. A direct research line has not yet established that Bnip3 gene transcription is also modified in this manner, but studies indicate that growth factor regulation via PIP3-Akt and NF-κB is feasible.

Bnip3 expression levels in response to growth factors have yet to be measured in cortical and hippocampal cells. However, studies reveal that the basal and inducible expression of Bnip3 gene transcription is modulated by activated NF-κB. Furthermore, NF-κB directly suppresses Bnip3 expression within the Bnip3 promoter at an NF-κB element (Baetz et al., 2005). Despite these experiments being conducted in ventricular myocytes, the presence of NF-κB regulatory element site within the Bnip3 promoter indicates that NF-κB can directly modulate Bnip3 expression in any cell type, including neurons and glia. Therefore, I speculate that during cortical development the presence of growth factors not only induces Bcl-2 and Bcl-X_L expression, but also suppresses Bnip3 expression (Fig. 22A).

Conversely, growth factor removal during NOCD would negate the NF-κB suppression of Bnip3. Bnip3 expression levels would subsequently rise, like we measured on PND 6.5 in the cingulate cortex, and select for cell death (Fig. 22B). However, in order to confirm this hypothesis, measurement of Bnip3 expression in response to growth factors must be conducted. If it can be established that Bnip3 gene

transcription is responsive to growth factors, then studies investigating the NF-κB necessity would need to be performed.

Developmental changes in Bnip3 expression levels may also be due to fluctuations in postnatal nitric oxide (NO) levels. In the rat cerebral cortex, brain nitric oxide synthase levels are endogenously elevated during the first postnatal week with greatest levels achieved on PND 7 (Labuda et al., 2003). The increase in nitric oxide synthase levels correlates with both the transient increase in Bnip3 expression and NOCD. Nitric oxide synthase converts L-arginine to NO and citrulline. Studies show that NO acts as either a neuroendangering or neuroprotective compound (Ciani et al., 2002b; Ciani et al., 2002c; Ciani et al., 2002a; Feng et al., 2002). Furthermore, multiple studies indicate that NO is a component of a second messenger signaling system associated with NOCD (Farinelli et al., 1996). NO also regulates Bnip3 expression, by either suppressing or enhancing transcription depending on the cell type (Zamora et al., 2001; Yook et al., 2004). Therefore, the transient increase in Bnip3 expression that we observed on PND 6.5 in the cingulate cortex and hippocampus may result from changes in NO levels. One possibility is that the combination of increases in nitric oxide synthase and growth factor withdrawal could cause the increase in Bnip3 on PND 6.5. Alternatively, if NO suppresses Bnip3 expression in cortical neurons, the peak in nitric oxide synthase on PND 7 may cause the decrease in Bnip3 expression after PND 6.5 (Fig. 22B).

Typically, the BH3-only pro-apoptotic proteins induce apoptosis by heterodimerizing with either Bcl-2 or Bcl-X_L, and subsequently disrupting mitochondrial function (Green and Kroemer, 2004). Consequently, the elevated

Bnip3 levels found on PND 6.5 may play a role in regulating NOCD via interactions with an anti-apoptotic protein such as Bcl-X_L (Fig. 22B). *In vitro* studies characterizing Bnip3 interactions with the anti-apoptotic Bcl-2 family members reveal that Bnip3 heterodimerizes with Bcl-X_L and induces cell death in a mitochondrial dependent manner (Ray et al., 2000). Furthermore, Bcl-X_L has been established as an essential mediator of mammalian development and cell death. Mice deficient in Bcl-X_L die at E13 and have excessive apoptosis within the intermediate zone of the spinal cord and brainstem as well as in the dorsal root ganglion (Motoyama et al., 1995; Shindler et al., 1997; Lindsten et al., 2005). Bcl-X_L has also been shown to be an essential component of telencephalic neuron development (Roth et al., 1996; Shindler et al., 1997; Lindsten et al., 2005). The developmental ontogeny for Bcl-X_L has been established and in the rat cortex and hippocampus, Bcl-X_L expression is decreased by PND 6.0 and remains depressed through the second postnatal week (Hamner et al., 1999). This decrease in Bcl-X_L expression correlates with the rapid rise in Bnip3 expression that we detected on PND 6.5 in the cingulate cortex and hippocampus. Thus, decreases in Bcl-X_L coupled with increases in Bnip3 expression could alter the ratio of pro- to anti-apoptotic genes in the cingulate cortex. The elevated levels of Bnip3 protein may interact with and sequester the Bcl-X_L protein, subsequently disrupting mitochondrial membrane stabilization, opening the permeability transition pore and inducing NOCD (Fig. 22B).

In addition to either inducing or inhibiting cell death through Bnip3 transcriptional regulation, cell death may be modulated by altering the protein's activities. Kothari et al., (2003) report that either EGF or IGF prevents the onset of

Bnip3 mediated cell death. Furthermore, these studies indicate that second messenger signaling, mediated by activated growth factor receptor, modify the Bnip3 protein within the BH3 domain, but not the PEST or amino terminus domains, thereby rendering it inactive (Kothari et al., 2003). Consequently, elevated levels of either EGF or IGF may prevent Bnip3 protein interaction with either the mitochondrial membrane or Bcl-X_L (Fig. 22A).

Growth factor modification of the Bnip3 protein may also affect the protein's intracellular localization. However, the current literature is riddled with mixed reports in regards to Bnip3 intracellular localization during the non-death state. Initial characterization of the Bnip3 protein in peripheral cell types determined that the protein contains a TM domain that targets it to the mitochondrial membrane (Chen et al., 1997; Ray et al., 2000). Furthermore, studies reveal that the endogenous protein is loosely associated with the mitochondria in the absence of a death initiating signal; but upon cell death the carboxyl terminal becomes incorporated within the mitochondrial membrane (Vande Velde et al., 2000). Conversely, two studies performed *in vivo* found that in the cortex, hippocampus and striatum Bnip3 is localized to the cytoplasm in the inactive state, but upon cell death Bnip3 translocates to the nucleus. However, in both studies cell death was not directly attributed to Bnip3 activity (Schmidt-Kastner et al., 2004; Althaus et al., 2006). Finally, studies conducted in an oligodendroglial cell culture line report that in the absence of a death stimulus Bnip3 protein is localized to the nucleus, but must translocate to the mitochondria to initiate cell death (Burton et al., 2006). These reported differences in expression patterns make it difficult to draw conclusions concerning the mechanism

of Bnip3 mediated cell death. However, I hypothesize that during CNS development growth factor inactivation of the Bnip3 protein either sequesters the protein to the vicinity of mitochondrial membrane or nucleus (Fig. 22A). The removal of growth factors subsequently activates the protein where it translocates to the mitochondria, becomes integrated with the outer membrane and opens the permeability transition pore (Fig. 22B).

Hypoxia and glucocorticoid induction of cell death

In addition to the potential role Bnip3 may play during normal brain development, the pro-apoptotic protein is a cell death mediator of hypoxia (Bruick, 2000; Burton et al., 2006). Our data also indicated that Bnip3 is regulated by glucocorticoids. However, glucocorticoid treatment independent of an additional insult does not induce a significant amount of cell death in comparison to vehicle treated neurons. The lack of cell death in cortical neurons treated with only glucocorticoids is contradictory to reports that reveal a single injection of DEX in the postnatal animal results in a reduced cortical neuron numbers in the adult animal (Kreider et al., 2006). However, this could result from a change in the rate of neurogenesis, not cell death. Indeed, studies examining hippocampal neurogenesis in postnatal animal dentate gyrus has demonstrated that glucocorticoids can reduce this rate (Tanapat et al., 1998; Coe et al., 2003). An additional possibility is that the dose I used versus the dose used in the other studies was different and subsequently caused a different result. For example, studies indicate that steroid hormone dose response curves are biphasic in nature being U-shaped, either upright or inverted. Furthermore,

the U-shape response is postulated to be a mechanism employed by a cell to maintain homeostasis (Calabrese and Baldwin, 2001).

Regardless, our data indicates that glucocorticoids can act to endanger cortical neurons when they are challenged by a secondary metabolic such as hypoxia. Low doses of GR agonist (0.1 and 1.0nM RU28362), which should not maximally occupy GR are sufficient to exacerbate hypoxia-induced Bnip3 expression in cortical neurons. This affect also carries over to increased death levels in neurons treated with glucocorticoids and hypoxia. These findings are not too surprising as GR functions to decrease expression of glucose transporters (Xia et al., 1995; Booth et al., 1998; Royer et al., 2000; Vega et al., 2006) and subsequently glucose surplus in neurons (Sapolsky et al., 1986). Furthermore, Sapolsky and Pulsinelle (1986) reported that glucocorticoids exacerbate hypoxic-ischemic insults in the adult rat brain.

My studies are the first to identify a mechanism by which glucocorticoids might act to modulate a secondary insult. A potential concern is that a majority of these studies were conducted *in vitro*. However, I believe that our data has *in vivo* relevance in regards to the developing brain. Evidence in support of this stems from animal studies in the current literature and my own. In research chapter 2, I reported that Bnip3 mRNA is increased in response to glucocorticoid treatment in the cingulate cortex and hippocampus of the developing rat brain. Additional studies have established that Bnip3 is upregulated during hypoxia in the rat cortex and hippocampus (Schmidt-Kastner et al., 2004; Althaus et al., 2006). Cumulatively, these studies indicate that *in vivo* both hypoxia and glucocorticoids individually induce Bnip3 expression. Since the individual insults result in the same outcome both

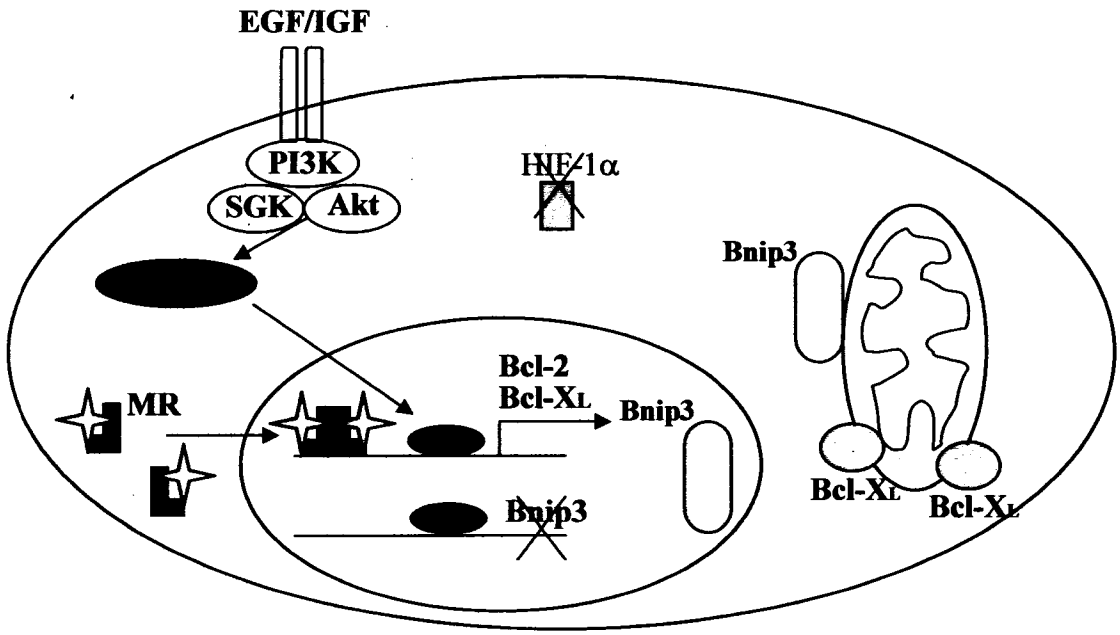
in vivo and *in vitro*; it is feasible that the exacerbation of Bnip3 expression in response to glucocorticoid and hypoxia treatment would also occur *in vivo*. However, *in vivo* glucocorticoid and hypoxia regulation of Bnip3 expression and cell death would be complicated by additional endogenous compounds such as CORT and growth factors. In light of these additional complications, I have developed schematic models (Fig. 23) for the putative *in vivo* regulation of Bnip3 in normoxic, hypoxic, and glucocorticoid plus hypoxic conditions in the developing brain.

Normoxia

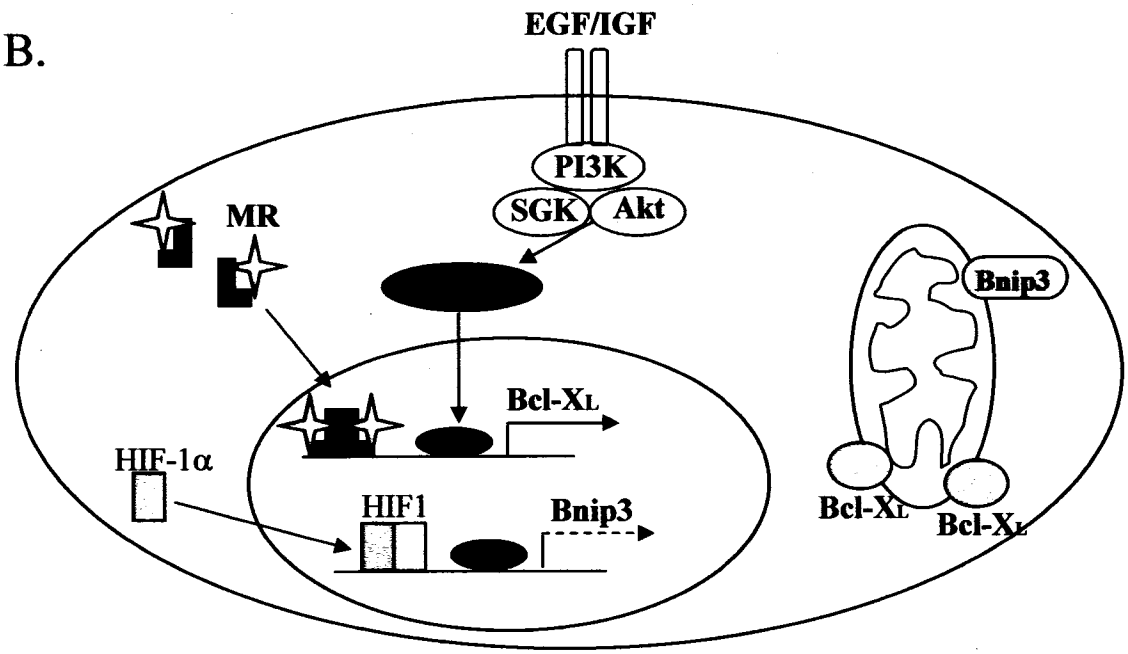
Bnip3 regulation in the normoxic brain is very similar to the previous description of Bnip3 regulation during CNS development in Figure 22. During normoxia neuroprotective factors including growth factors and endogenous CORT are present (Fig. 23A). Growth factors confer a protective affect through the PIP3-Akt pathway. Activated Akt phosphorylates IκB allowing the release and translocation of NF-κB to the nucleus (Ozes et al., 1999; Romashkova and Makarov, 1999). NF-κB acting as a transcription factor induces expression of Bcl-X_L (Bui et al., 2001) and presumably decreases expression of Bnip3 (Baetz et al., 2005).

In addition to growth factors, the developing brain would be exposed to low levels of circulating CORT. The plasma CORT levels in neonates are at a concentration that predominantly results in MR occupation and subsequent activation; while GR would be unoccupied and inactive (Dallman et al., 2000; Watts, 2000). Similar to growth factors, MR also confers a neuroprotective phenotype by altering Bcl-2 family member expression levels. In the juvenile rat hippocampus MR activation increases Bcl-2 and Bcl-X_L expression and decreases Bax expression.

A.



B.



C.

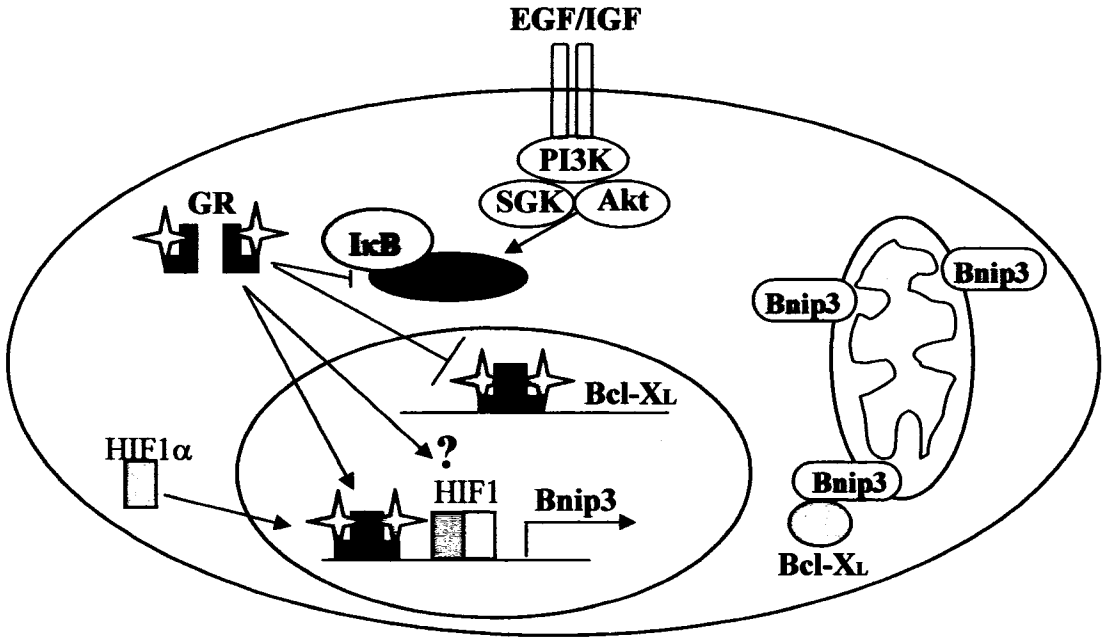


Figure 23. Schematic illustration depicting the putative regulation and actions of Bnip3 during either normoxic, hypoxic or glucocorticoid plus hypoxic conditions. (A) Normoxic conditions select for cell survival. During normoxia HIF-1α is degraded. The presence of growth factors downregulates Bnip3 and upregulates Bcl-X_L expression through NF-κB. Endogenous CORT levels protect neurons through MR activation and induction of Bcl-X_L expression. (B) Hypoxic conditions prevent HIF-1α degradation. HIF-1α translocates to the nucleus and heterodimerizes with ARNT to form HIF1. HIF1 induces Bnip3 transcription at an HRE. Elevated Bnip3 levels select for cell death; however, the presence of growth factors and CORT confers protection by increasing Bcl-X_L expression. Growth factors also prevent maximal Bnip3 expression (depicted by dashed line). (C) Glucocorticoids acting through the GR and in conjunction with hypoxia further induces Bnip3 expression. The hypoxia induces HIF1 formation which increases Bnip3 transcription. GR inhibits the protective effects of growth factors by suppressing NF-κB transcriptional activity; further increasing Bnip3 expression and decreasing Bcl-X_L expression.

However, these studies did not establish if MR regulates Bcl-2, Bcl-X_L and Bax expression through a hormone response element or second messenger system (Almeida et al., 2000). The elevated levels of Bcl-2 and Bcl-X_L subsequently act to stabilize the mitochondrial membrane (Fig. 23A).

Full oxygenation results in oxygen dependent degradation of HIF-1α, which prevent the expression of hypoxic responsive genes such as Bnip3 (Fig. 23A). HIF1α degradation is mediated by HIF-1α prolyl hydroxylase, which hydroxylates the proline residues of the HIF-1α protein (Bruick and McKnight, 2001; Yu et al., 2001). The hydroxyl groups subsequently targets the protein for ubiquitination by the Von Hippel-Lindau protein and rapid proteasome degradation (Kamura et al., 2000; Ohh et al., 2000; Jaakkola et al., 2001).

Hypoxia

During hypoxia, the affinity of HIF-1α prolyl hydroxylase for oxygen is below the partial pressure of oxygen in tissue. The lack of oxygen subsequently prevents the addition of hydroxyl groups to the HIF-1α protein. By default, this leads to HIF-1α protein stabilization. Stabilized HIF-1α translocates to the nucleus and heterodimerizes with ARNT to form the transcription factor HIF1 (Wang et al., 1995). HIF1 induces Bnip3 transcription via an HRE within the 5' untranslated region of the gene (Fig. 23B). Hypoxia dependent induction of Bnip3 transcription through the HRE was first reported in peripheral cell culture types (Bruick, 2000). Our studies indicate that hypoxic induction of Bnip3 in cortical neurons is also mediated by the same 5' HRE site. Hypoxia induced Bnip3 expression also occurs in the adult rat cortex, hippocampus and striatum as well as in cultured oligodendroglial cells

(Schmidt-Kastner et al., 2004; Althaus et al., 2006; Burton et al., 2006). However, these studies did not determine if Bnip3 is directly regulated by hypoxia through the HRE.

In addition to regulating Bnip3 expression during hypoxia, HIF1 mediates tumor suppressor protein p53 stabilization. Studies reveal that Bcl-2 gene family expression is modulated by p53. It has been shown that hypoxia induces Bnip3L expression which is conferred by activated p53 (Fei et al., 2004). Under hypoxic conditions, p53 also increases multi-domain member Bax expression and decreases expression of Bcl-X_L. However, Bcl-2 gene family regulation by p53 does not appear to be universal as regulation of Bnip3 transcription is independent of p53 activity (Lee et al., 2002; Aoyagi et al., 2003; Fei et al., 2004).

Based on the altered expression levels of Bnip3, hypoxia selects for cell death. However, hypoxia does not result in maximal Bnip3 expression and cell death (Fig. 23B). Our studies support this conclusion as exacerbations of both Bnip3 expression and cell death are seen with the dual insult of hypoxia and glucocorticoids. A possible mechanism for these results may be that the presence of growth factors activates NF-κB which suppresses Bnip3 expression levels during hypoxia (Fig. 23B). Therefore, periods of development when growth factor levels are depressed, such as during NOCD, neurons may be more susceptible to hypoxic insults.

Despite the partial induction of Bnip3 expression, hypoxia alone is sufficient to induce cell death. In primary cortical neurons we measured a 50% reduction in viable cells following 72 hours of hypoxic treatment. Hypoxic treatment has also been reported to decrease oligodendroglial cell viability (Burton et al., 2006).

Additionally, both studies utilized the Bnip3ΔTM expression plasmid as a dominant negative to confirm that a component of the cell death is mediated by Bnip3. Studies performed *in vivo* also correlate hypoxic-ischemic infarcts with an induction of Bnip3 expression in the cortex, hippocampus and striatum (Schmidt-Kastner et al., 2004; Althaus et al., 2006).

Hypoxia and glucocorticoids

Synthetic glucocorticoids such as DEX are commonly administered for bronchopulmonary dysplasia treatment in premature infants (Bar-Lev et al., 2004). DEX acts as an anti-inflammatory agent in the lung, and studies indicate that this prevents complications associated with intubation (Doyle et al., 2006). The anti-inflammatory properties of DEX are conferred by selective GR activation.

In the developing brain activated GR results in a neuroendangering phenotype at the cellular level with subsequent causes in long-term behavior abnormalities (Kamphuis et al., 2004; Machhor et al., 2004; Neal et al., 2004). DEX administered to the postnatal rat causes a decrease in the total number of cortical neurons in the adult animal (Kreider et al., 2006). Additionally, DEX treatment increases the incidence of apoptosis in the developing hippocampus (Almeida et al., 2000). Consequently, I also investigated cell death as a result of GR activation. My data indicate that glucocorticoids, when acting independently do not induce cortical neuron death. However, glucocorticoids function to endanger cortical neurons by increasing Bnip3 expression levels, a response that is mediated by a GRE within the Bnip3 promoter. Additional Bcl-2 family members have also been shown to be regulated by GR (Fig. 23C). In the hippocampus, DEX decreases Bcl-2 and Bcl-X_L expression and increases

Bax expression (Almeida et al., 2000). However, these studies did not determine if GR directly regulates expression of these Bcl-2 members via a GRE.

Several studies have found that glucocorticoids can exacerbate secondary metabolic insults, such as kainic acid and hypoxia-ischemia, in the rat brain (Sapolsky and Pulsinelli, 1985; Roy and Sapolsky, 2003). In accordance with these studies, I determined that glucocorticoids, administered in conjunction with hypoxia, potentiate cortical neuron death when compared to vehicle-treated hypoxic cells. Cell death exacerbation also correlates closely with the increases in Bnip3 expression (Fig. 23C). The enhanced levels of Bnip3 expression may result from concomitant Bnip3 promoter activation by both GR and HIF1 through the GRE and HRE, respectively. Alternatively, studies reveal that glucocorticoid modulation of hypoxic responsive genes is mediated through GR transactivation of HIF1 at either the GRE or HRE (Kodama et al., 2003; Leonard et al., 2005). Activated GR specifically interacts with the HIF-1α subunit of the HIF1 heterodimer protein. The protein:protein interactions of GR and HIF-1α are mediated by the LBD of activated GR (Kodama et al., 2003), presumably through the AF2 domain, and the carboxyl terminal of HIF-1α (Kodama et al, 2003). The HIF-1α carboxyl terminal is an oxygen dependent transactivation domain, which is inhibited during normoxia by asparaginyl hydroxylase (Jiang et al., 1997; Pugh et al., 1997; Dann et al., 2002).

GR may also indirectly regulate Bnip3 gene transcription by inhibiting growth factor actions (Fig. 23C). The results of some studies reveal that glucocorticoids prevent NF-κB transcriptional activity. Activated GR has been reported to directly interact with NF-κB and suppress its transcriptional activity (Auphan et al., 1995;

Scheinman et al., 1995; Ramdas and Harmon, 1998; De Bosscher et al., 2003). Additionally, GR induces IκB synthesis, which indirectly inhibits NF-κB by sequestering the transcription factor in the cytoplasm (Auphan et al., 1995; Scheinman et al., 1995; Ramdas and Harmon, 1998). Glucocorticoids, acting through GR, may subsequently prevent NF-κB induced expression of Bcl-X_L (Bui et al., 2001) and suppression of Bnip3 (Baetz et al., 2005). This process would further select for cell death (Fig. 23C.)

The elevated levels of Bnip3 protein induce cell death by disrupting the mitochondrial membrane. However, there are multiple potential mechanisms by which Bnip3 mediates this process. One possibility is that activated Bnip3 protein, which integrates into the outer mitochondrial membrane (Vande Velde et al., 2000), directly induces PT pore opening (Vande Velde et al., 2000; Kim et al., 2002; Baetz et al., 2005; Webster et al., 2005). A second possibility is that Bnip3 heterodimerizes with Bcl-X_L (Chen et al., 1999; Ray et al., 2000) sequesters the protein and subsequently disrupts mitochondrial function (Letai et al., 2002). The final mechanism involves Bnip3 interactions with a pro-apoptotic multi-domain member. Gao et al., (2005) suggest that interactions between Bnip3 and a multi-domain member occur in peripheral tissue types. In MCF-7 and HeLa cell culture lines the human homolog to the multi-domain member Bok (hBok) is upregulated during hypoxia. Furthermore, Bnip3 directly interacts with hBok and induces protein oligomerization (Gao et al., 2005). Bnip3 interaction with hBok is similar to previously described cell death mechanisms where a BH3-only member is the intermediate between the apoptotic stimulus and multi-domain member activation

(Wei et al., 2001). However, hBok, as well as rat and mouse homologs are not expressed in the brain (Hsu et al., 1997; Inohara et al., 1998; Zhang et al., 2000; Gao et al., 2005). Even though hBok is not expressed in the brain the possibility exists that Bnip3 is still capable of inducing hypoxia-stimulated cell death through a multi-domain member in the brain. A putative candidate for such a multi-domain member is Bax; because Bax deficient mice are partially protected from hypoxic-ischemic insults (Gibson et al., 2001). Nonetheless, additional studies investigating Bnip3 and Bax interactions and their relative contributions to cell death during hypoxia are necessary.

Is my data relevant to or have implications associated with neuropathologies in the developing human brain?

Premature infants diagnosed with bronchopulmonary dysplasia are treated with glucocorticoids to prevent additional complications and potentially accelerate recovery. However, the developing human brain is susceptible to pathology associated with hypoxic and glucocorticoid exposure in a similar fashion to the developing rodent brain. Hargitai et al., (2001) report that bronchopulmonary dysplasia increases the incidence of cortical apoptosis in humans. Our studies identify a potential mechanism by which cell viability is altered in the developing brain through upregulation of Bnip3 in response to glucocorticoid treatment and hypoxic exposure.

The evolutionarily conserved nature of Bnip3 protein suggests that it is a mediator of hypoxia-induced cell death in the human CNS. Throughout evolution, from the worm to the rodent to the human, Bnip3 protein function and structure has

been preserved. Numerous homologs to Bnip3 have been identified including homologs in c. elegans (ceBnip3) and the human (Bnip3h, Bnip3L and Nix) (Matsushima et al., 1998; Chen et al., 1999; Cizeau et al., 2000; Farooq et al., 2001). Similar to Bnip3, ceBnip3, Bnip3h, Nix and Bnip3L all have BH3, TM and PEST domains (Matsushima et al., 1998; Chen et al., 1999; Cizeau et al., 2000; Farooq et al., 2001). Additionally, homodimerization, heterodimerization and the killing activity of the ceBnip3 and Nix proteins is conferred by the TM, not the BH3 domain. ceBnip and Nix also interact with Bcl-X_L; however, Bcl-X_L does not prevent cell death (Chen et al., 1999; Cizeau et al., 2000).

The hypoxia-dependent regulation of Bnip3, Nix and Bnip3L has also been examined in numerous studies. Hypoxia induced cell death is directly attributed to Bnip3 protein function in a human-derived oligodendroglial cell culture line. Similar to Bnip3, Nix and Bnip3L are also upregulated during a hypoxic insult (Bruick, 2000; Fei et al., 2004; Birse-Archbold et al., 2005). The results of several studies reveal that hypoxia regulation of both Nix and Bnip3 is dependent on HIF-1α (Vengellur and LaPres, 2004). However, promoter analysis for the presence of a functional HRE has yet to be performed on Nix. Bnip3L is also upregulated during hypoxia; but unlike Bnip3, the hypoxia-induced expression of Bnip3L is dependent on p53 activity (Fei et al., 2004). Taken together, these studies suggest that proteins similar in structure and function to Bnip3 exist in the human. It can be argued that one of these proteins may integrate a response to glucocorticoids and hypoxia in the developing human brain similar to the observed results in my studies. However, studies examining the glucocorticoid regulation of these homologs have yet to be conducted.

Assuming that Bnip3 mediates hypoxia-induced cell death in the developing human brain, potential therapies protecting against this type of insult can be formulated. Peripheral administration of iodinated EGF shows that EGF rapidly passes the blood brain barrier to gain entry into the mouse CNS (Pan and Kastin, 1999). Therefore, treatment of premature infants that are hypoxic with EGF may reduce the levels of CNS cell death by attenuating Bnip3 activity. However, growth factor treatment may not afford much protection against the neuroendangering affects of glucocorticoids given that GR regulates NF-κB activity.

Cumulatively, both the studies of others and my own implicate Bnip3 as a putative mediator of hypoxia-induced cell death in the developing CNS. However, prior to developing treatments which attenuate Bnip3 activities in the hypoxic human brain; studies confirming Bnip3 as the mediator to hypoxic-cell death are necessary.

Conclusions

The experiments conducted for this dissertation identify a protein, Bnip3, which may mediate both normal and abnormal cortical development. Bnip3 may act in the developing cortex to regulate a component of growth factor withdrawal induced autophagy. Additionally, my data indicate that Bnip3 mediates cortical neuron death associated with the glucocorticoid exacerbation of a hypoxic insult. These studies have clinical significance since glucocorticoids are commonly prescribed for bronchopulmonary dysplasia treatment in hypoxic, premature infants. Correspondingly, glucocorticoid treatment of premature infants is associated with increased levels of cortical apoptosis and incidence of cerebral palsy. My studies are the first to identify a mechanism and potential effector protein that mediates glucocorticoid endangerment of secondary metabolic insults in the developing human brain. In light of these clinical observations coupled with my current findings, the use of glucocorticoids in the clinic, for treatment of hypoxic neonates should be reevaluated.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AF	activation function
AHR	aryl hydrocarbon receptor
ALDO	aldosterone
ANT	adenine nucleotide translocator
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
AVP	arginine vasopressin
Bnip3	bcl-2 and nineteen kDa interacting protein-3
Bnip3h	*Homo sapiens* Bnip3h
Bnip3L	bnip3-like
Bnip3ΔTM	bnip3 with excluded transmembrane domain
BH	bcl-2 homology domain
BH3	bcl-2 homology domain 3
bHLH-PAS	basic helix loop helix containing Per/ARNT/Sim
ceBnip3	*Caenorhabditis elegans* Bnip3
CRH	corticotrophin releasing hormone
CORT	corticosterone
CNS	central nervous system
DEX	dexamethasone

DNA	deoxyribonucleic acid
E	embryonic day
EGF	epidermal like growth factor
FOXO	forkhead box transcription factor, class O
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDX	gonadectomized
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA	hypothalamo-pituitary adrenal axis
HIF1	hypoxia inducible factor1
HIF-1α	hypoxia inducible factor1-α
HRE	hypoxic response element
HSP-90	heat shock protein-90
IAP-2	inhibitor of apoptosis-2
IGF-1	insulin like growth factor-1
ISH	in situ hybridization
KD	dissociation constant
LDH	lactate dehydrogenase
MMP	mitochondria membrane permeabilization
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-κB
NGF	nerve growth factor

NO	nitric oxide
NOCD	naturally occurring cell death
N-terminal	amino terminal domain
PCR	polymerase chain reaction
PI3K-Akt	phosphatidylinositol-3-OH kinase- protein kinaseA pathway
PND	postnatal day
PNS	peripheral nervous system
PT pore	permeability transition pore
RT-PCR	reverse transcription-polymerase chain reaction
RNA	ribonucleic acid
Sim	single-minded
TM	transmembrane domain
TP	testosterone propionate
VDAC	voltage dependent anion channel