ROLE OF AUTOPHAGY IN NEURONAL INJURY MODELS OF THE CENTRAL NERVOUS SYSTEM

By

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Om Sai Ram
To my beloved parents for their encouragement, vision and blessings during my academic career
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ROLE OF AUTOPHAGY IN NEURONAL INJURY MODELS OF THE CENTRAL NERVOUS SYSTEM

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Autophagy is an intracellular phenomenon which is induced under conditions of stress. It is characterized by the presence of double membrane vesicles in the cytoplasm, autophagosomes. Autophagosomes sequester cytoplasmic organelles and finally fuse with the lysosomes. The lysosomal hydrolases subsequently breakdown the sequestered organelles in the vesicles into its constituent amino acids. The amino acids are then recycled into the protein machinery of the cell to sustain cell survival. Autophagy has been reported to aid cell survival under conditions of stress such as nutrient starvation or cell injury. Prolonged autophagy has also been known to result in autophagic cell death. While the apoptotic and necrotic cell death pathways have been well studied, there lacks a comprehensive understanding of the molecular events involving autophagic cell death. We examined the potential roles of the apoptosis-linked caspase-3 and the necrosis/apoptosis-linked calpain-1 after autophagy induction under prolonged amino acid (AA) starvation conditions in PC-12 cells. Autophagy induction was observed as early as three hours following amino acid withdrawal. Cell death, measured by lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays occurred within 24 h following starvation and was accompanied by an upregulation in caspase-3 activity but not calpain-1. The cell death that occurred following AA starvation was significantly alleviated by
treatment with the autophagy inhibitor 3-methyl adenine but not with the broad spectrum caspase inhibitors. Thus, this study demonstrates that 3-methyladenine-sensitive autophagic cell death due to AA starvation in PC-12 cells is mechanistically and biochemically similar to, yet distinct from, classic caspase dependent apoptosis.

Excitotoxicity has been documented as one of the major biochemical pathways leading to cell death, we examined the role of autophagy induction and prolonged autophagy in our neuronal cell culture model. Excitotoxicity, a form of acute stress due to excessive release of glutamate, was mimicked by exposing primary rat cerebellar granule neurons to the excitotoxin N-methyl-d-aspartate (NMDA). Our results demonstrated that excitotoxic NMDA exposure induced autophagosome formation in both the cell bodies and the neurites in as early as 3 h post-treatment, as evidenced by autophagy protein marker LC3 immunostaining, beclin-1 immunoblotting and fluorescent labeling with the monodansylcadaverine (MDC) dye. We also observed the increased levels of the autophagy proteins Atg8 (LC3) and beclin-1 (Atg6) in our animal model of controlled cortical impact. Prolonged exposure of the cultures to NMDA (12-24 h) however, produced abnormal and aggregated autophagosomes. Co-treatment of neurons with autophagy inhibitor 3-methyl adenine (3-MA) and NMDA reduced the levels of autophagosome-associated form of LC3 (LC3-II) and suppressed NMDA-induced autophagosome formation. Importantly, NMDA-mediated neuronal death was also robustly suppressed by 3-MA. Biochemical analysis furthermore showed that the neuroprotective effects of 3MA were likely mediated through suppression of NMDA-induced caspase-3 activity and oxidative stress. We also observed significant increases in the levels of the processed form of LC3 (LC3-II) and the beclin-1/bcl-2 ratio in the ipsilateral cortex of rats subjected to controlled cortical impact at various time points, after injury. Both of these changes are indicative of autophagy-enabling
events following brain trauma. We thus conclude that autophagy and possibly autophagic cell
death might play a role following the manifestation of brain trauma and may be neuroprotective
when exploited initially but when prolonged results in autophagic cell death. Collectively, our
data strongly suggest that autophagy induction and later autophagic cell death might be a
significant component of either neuroprotection or later neuronal death following brain trauma.
CHAPTER 1
LITERATURE REVIEW

Autophagy: A General Overview

Autophagy is an intracellular phenomenon that is activated under conditions of intracellular and extracellular modalities of stress such as endoplasmic (ER) stress or nutrient deprivation/starvation. This phenomenon is characterized by the presence of double membrane cytosolic vacuoles called “autophagosomes.” The autophagosomes have been documented to engulf and sequester cytosolic organelles under conditions of stress and ultimately fuse with the lysosomes. The lysosomal hydrolases then breakdown the organelles and recycle the proteins and amino acids into the cell machinery for its survival in nutrient compromised conditions (Dunn, 1990a, b; Klionsky, 2004; Levine and Klionsky, 2004; Meijer and Codogno, 2004).

Autophagy has been documented to be a highly conserved phenomenon from yeast to mammals. A common unified nomenclature has been proposed to name the proteins involved in the process of autophagy. The genes involved in mammalian autophagy are denoted as ‘ATG-genes’ while the proteins are depicted as ‘atg-proteins’ (Klionsky et al., 2003). Also a very recent report suggests and outlines the guidelines for the interpretation and the use of assays used to study autophagy in higher eukaryotes (Klionsky et al., 2008). Thus the studies in autophagy are gaining momentum and becoming more structured.

Though autophagy has been documented to assist cell survival, prolonged periods of autophagy induction have been recently suggested to be responsible for another type of cell death called “autophagic cell death” (type II) (Clarke, 1990; Lockshin and Zakeri, 2004b). Autophagic cell death is considered as a programmed cell death as its execution involves a sequential activation of a number of enzymes and autophagy proteins. Growth factor or nutrient deprivation mediated autophagic cell death has been documented in neuronal cell culture systems (Xue et al.,
Autophagic cell death, a conserved integral pathway involved in mammalian cell development (Levine and Klionsky, 2004) is also activated under certain pathological conditions such as the neurodegenerative diseases (Stefanis et al., 2001). Also, the activity of autophagosomes during prolonged periods of autophagy induction might end in the sequesteration and degradation of seminal cell organelles such as the mitochondria resulting in cell death.

**Classification of Autophagy**

Autophagy is classified into 3 types: a) macroautophagy, b) microautophagy and c) chaperone-mediated autophagy. Macroautophagy: It is the main route for bulk protein degradation under conditions of stress and starvation. Macroautophagy is a multi-step process and involves the formation of a double membraned vesicular structure called the autophagosome and is presumably derived from the endoplasmic reticulum (ER) (Dunn, 1990a). Autophagosomes engulf cytoplasmic components including whole organelles and transport them to the lysosomes. Once it reaches the lysosome, the outer membrane of the autophagosome fuses with the membrane of the lysosome and matures into a structure known as the “autophagolysosome.” The vesicle transported into the lysosome delivers its contents following the disintegration of its membrane. The vesicular contents are then broken down into amino acids and recycled into the protein machinery of the cell to sustain survival under nutrient deprivation conditions (Fig. 1-1). Recently, mammalian proteins involved in the process of macroautophagy have been identified and unified under a single nomenclature. Some of the proteins well known include Atg8, Atg6, Atg7, Atg12 and Atg5 (Klionsky et al., 2003) demonstrates the processes involved in macroautophagy (Klionsky and Emr, 2000). Since macroautophagy is the predominant form observed, it is often referred to as autophagy.
Microautophagy: In this process internalization of the proteins is made directly through the lysosomal membrane by invagination of the membrane at different locations forming a multivesicular body. It is non-selective and has an inherently basic activation level in the cell. Thus microautophagy seems to be activated even under normal conditions unlike macroautophagy (Larsen and Sulzer, 2002).

Chaperone-mediated autophagy: This process is restricted to elimination of proteins that possess an amino acid sequence biochemically related to the pentapeptide Lys-Phe-Glu-Arg-Gln (KFERQ) during conditions of prolonged starvation (Chiang and Dice, 1988; Dice et al., 1990). The proteins are tagged by the heat shock cognate (hsc-73) protein of 73 kDa and binds to a lysosome membrane receptor LAMP-2a which facilitates the entry of the complex into the lysosome for degradation purposes (Terlecky and Dice, 1993; Cuervo et al., 1994; Cuervo and Dice, 1996).

**Autophagosome Formation**

Autophagy is characterized by the formation of autophagosomes. Formation of autophagosomes is a well regulated process involving a number of proteins, some of which is mentioned above. One of the main proteins which control the formation of these double membraned vesicles is the mammalian target of rapamycin (mTOR). The TOR proteins are assigned to a protein family termed the phosphatidylinositol kinase related kinases (PIKKs) and function as Ser/Thr protein kinases (Hunter, 1995; Hoekstra, 1997; Raught et al., 2001). This protein is sensitive to the nutrient levels in the cell and is a regulator of autophagy. Under normal/healthy conditions, mTOR is hyperphosphorylated and exerts an inhibitory influence on the activation of the autophagy proteins. Under starvation conditions, this inhibitory influence is lifted and the phosphorylation state of the Atg13 changes (hypophosphorylated) which then
effectively binds to the Atg1 protein and initiates a cascading set of events resulting in the induction of autophagy.

Autophagosome formation, once initiated after nucleation from the endoplasmic reticulum (ER), undergoes a series of steps towards its maturation. The process is similar to the ubiquitin-proteosome pathway. One of the autophagosome proteins, Atg12, is conjugated to Atg7 (E1-like) and then to Atg10 (E2-like) forming thioester intermediates through its COOH-terminal glycine. Finally, Atg12 conjugates to Atg5 via an internal lysine residue in the latter. Atg16 then binds to this conjugate non-covalently and dimerizes with another Atg12-Atg5-Atg16 complex to form a complex required for autophagosome formation. Recent reports illustrate the co-localization of the Atg12-Atg5 protein complex with LC3 (mammalian orthologue of Atg8) (Mizushima et al., 2001). Figure 1-2 underlines the different Atg proteins involved in the autophagosome elongation following cell stress conditions such as nutrient deprivation or anoxia (Gozuacik and Kimchi, 2004).

**Different Forms of Cell Death**

Cell death is a highly regulated event in eukaryotic cells that can be both essential and detrimental in different circumstances (Nelson and White, 2004). According to literature, cell death has been classified into three types: a) apoptotic (Type I), b) autophagic (Type II) and c) necrotic/oncotic (Type III) (Baehrecke, 2003; Liu et al., 2004). A recent report adds to this list with the introduction of a new form of cell death called “necroptosis” (Degterev et al., 2005). Although apoptotic and autophagic cell death are examples of programmed cell death, oncotic cell death is uncontrolled and involves an inflammatory response.

Apoptotic cell death is characterized by cell shrinkage and blebbing, nuclear fragmentation and no inflammation. Activation of caspases such as caspase-3 and caspase-9 has long been considered the hallmark for apoptosis. Classical apoptotic process does not involve inflammation.
and caspase activation is late (Lockshin and Zakeri, 2004a). Apoptosis has generally been considered the core biochemical pathway of programmed cell death and is conserved from nematodes to mammals (Metzstein et al., 1998; Green, 2005). With the recent advances in understanding the apoptotic pathway, it can be said that cell death due to apoptosis depends on the cell type and the death stimulus and that morphological features may not always correlate with the biochemical features (Stefanis, 2005). The apoptotic pathway can be divided into three biochemical pathways activating different effectors leading to cell death. The intrinsic pathway involves the release of factors such as cytochrome c from the mitochondria and recruitment of caspase-9 to the apoptosome (a large protein complex in the cytosol) where it is activated through homophilic interaction and dimerization before activating caspases downstream such as caspase-3, finally resulting in cell death (Degterev et al., 2003).

The second pathway essentially involves the death receptor wherein the extracellular ligands bind to the death receptor. This binding results in the formation of a death receptor protein complex, which recruits caspase-8 to the membrane. Caspase-8 then activates the effector caspases directly, leading to cell death. Though these pathways are activated differently, there is still some crosstalk that occurs between these pathways (Degterev et al., 2003).

Apoptotic cell death has recently been shown to occur under ER stress conditions. Studies have shown that ER stress is capable of indirectly activating both the extrinsic and the intrinsic pathways of apoptosis mediating cell death with caspase-12 being the primary caspase involved (Rao et al., 2004). Excess endoplasmic stress can be induced by the accumulation of unfolded or misfolded proteins which subsequently activates multiple caspase-associated pathways.

Although autophagy has been primarily thought to be a cell survival mechanism under conditions of stress and disruption of homeostasis, recent evidence suggests that it might play a
role in programmed cell death is gaining momentum (Baehrecke, 2005). Autophagic cell death has been observed in the non-feeding metamorphosis stages in insects and amphibians, such as D. melanogaster and Xenopus laevis. This form of programmed cell death is involved in eliminating the cells not required for the organism. The presence of autophagosomes in the dying cells represents one of the morphological manifestations in type II cell death. The cells also manifest caspase activation towards the later stages in the cell death process. The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is an important regulator of autophagy and is involved in the maintenance of homeostasis in the cell (Hay and Sonenberg, 2004). Inhibition of different classes of PI3K results in varying effects on autophagy. Inhibition of class I family of PI3K results in activation of autophagy while inhibition of class III family of PI3K inhibits autophagy (Baehrecke, 2005). Though there is mounting evidence suggesting autophagic cell death as a form of cell death, there is still divided opinion about its existence. There seems to be considerable interactions in the autophagic and apoptotic pathways and recent studies suggest autophagy may play a role in apoptosis either by preceding and eventually initiating it or by delaying its onset (Canu et al., 2005) or both pathways might be mutually exclusive (Gozuacik and Kimchi, 2004).

Oncosis or necrotic cell death is the most drastic set of events that can occur in programmed cell death. It refers to process by which a cell ruptures following swelling and releases its intracellular components into the cytoplasm. It is normally a result of a harsh mechanical or chemical insult to the cell that disrupts its homeostasis leading to the release of a variety of factors that assist in the cell death process. Massive inflammation results which either helps in the healing or elimination of the damaged tissue. Necrotic cell death can also substitute in cases where the apoptotic process is non-functional (Proskuryakov et al., 2003). Recent
reports have revealed the therapeutic benefits of this form of cell death in treating cancer (Zong et al., 2004).

**Traumatic Brain Injury**

Traumatic brain injury (TBI) has been one of the major health problems affecting young male adults between the ages of 15 to 24 and elderly people of both sexes over 75 years of age in the U.S.A. TBI is defined as acquired brain injury due to sudden mechanical trauma to the head region that causes brain damage (http://www.ninds.nih.gov/disorders/tbi/detail_tbi.htm) (Pineda et al., 2004b). The financial burden associated with TBI grosses to more than $56 billion a year with more than 5 million Americans alive today who have had a TBI resulting in a permanent damage to the brain. Survivors of TBI often suffer cognitive deficits and also changes in the behavior and communicative abilities. Some patients are also susceptible to secondary complication such as epilepsy, Alzheimer’s disease, Parkinson’s disease and post-traumatic dementia.

Traumatic brain injury (TBI) is a complex neurological disorder that involves a sequence of events leading to cell death in the brain. Cell death in TBI has been attributed to the primary mechanical injury followed by a cascade of protease activation leading to biochemical secondary injury. Trauma to the head can result in damage to the underlying soft tissue. Majority of brain contusions result in seizures and depending on the severity of the injury, the underlying brain tissue and meninges can suffer shear damage due to skull fractures. Brain trauma also results in damage to the vascular system and can result in hematomas which may, depending on its location have a detrimental effect on neuronal survival. The secondary biochemical injury occurs days to weeks after the primary mechanical injury. This occurs in the form of a variety of insults such as neurotoxic ischemia, proteolysis, oxidative stress and inflammatory cytokines mediated by activation of host immune system (microglia and lymphocytes) (McIntosh et al., 1998; Wang
et al., 2004; Ottens et al., 2006; Wieloch and Nikolich, 2006). Another major player is the disruption in Ca\(^{+2}\) ion homeostasis following injury. Following injury there is an increase in the intracellular calcium concentration which results in the activation of many proteases one of which is the cysteine protease calpain. Increases in Ca\(^{+2}\) levels also induce excessive release of the excitatory amino acid glutamate via activation of the glutamate receptor leading to glutamate excitotoxicity. These biochemical events have the potential to lead to prolonged depolarization accompanied with ATP depletion, compromise in the axonal integrity and also an increase in intracranial pressure (Kupina et al., 2003; Yi and Hazell, 2006). The well documented proteolytic events following TBI has been cell death resulting from the activation of calpains and caspases resulting in neuronal cell death in a multi-faceted manner (Raghupathi, 2004). Oncotic cell death is sudden and more drastic, occurring due to the mechanical injury to the brain tissue and is more immediate. Apoptotic cell death following brain trauma resulting from activation of caspases is a more delayed and secondary biochemical response to the primary mechanical injury. The evidence for activation of calpains and caspases protease has been well studied employing the cytoskeletal protein \(\alpha\)II-spectrin. TBI studies have demonstrated \(\alpha\)II-spectrin to undergo proteolysis by both calpains and caspases is \(\alpha\)II-spectrin (Pineda et al., 2004a; Czogalla and Sikorski, 2005). Thus, understanding the molecular and cellular mechanisms following TBI would help develop better therapies to arrest the neuronal and functional loss associated with the injury.

**Autophagy in Brain Injury and Homeostasis**

Recent studies have demonstrated an integral role for autophagy in maintaining the homeostasis and development of the mammalian brain. Separate studies have shown the importance of the autophagy protein Atg5 in the development of the central nervous system (Hara et al., 2006; Komatsu et al., 2006). The presence of autophagic vacuoles has been observed in dystrophic
neurons and implicated to be either beneficial or detrimental in the pathogenesis of neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease (Webb et al., 2003; Zhu et al., 2003; Li et al., 2007; Nixon, 2007; Sarkar et al., 2007b). Autophagy has also been demonstrated to be beneficial in the clearance of mutant aggregates in polyglutamine disease states (Williams et al., 2006; Floto et al., 2007; Sarkar et al., 2007b; King et al., 2008). Excitotoxicity resulting from excess release of the neurotransmitter, glutamate has been one of the major factors contributing to the pathology after brain injury (Ankarcrona et al., 1995; Ferrer et al., 1995; Portera-Cailliau et al., 1997). Excessive stimulation of the glutamate receptor in the spinal cord motor neurons and the organotypic hippocampal slice cultures has shown the induction of autophagosome formation and subsequent neuronal death. The presence of autophagosomes evidenced in the neurons made the authors speculate if excitotoxicity-associated autophagy induction results in autophagic cell death (Borsello et al., 2003; Tarabal et al., 2005). (Wang et al., 2006) demonstrated the induction of autophagy in Lurcher mice, a genetically engineered animal model of excitotoxicity, shows the accumulation of autophagosomes at the distal ends of the axons suggesting a breakdown in the retrograde transport of materials in the neuron. Studies have also demonstrated the induction of autophagy after mechanical or biochemical insult in neurons in the brains of experimental animals. Beclin-1 (Atg6), LC3 (Atg8), Atg7 and Atg5 have been the well documented autophagy proteins in studies associated with brain injury. (Diskin et al., 2005) demonstrated the up regulation of one of the key autophagy proteins beclin-1 (Atg6) near the site of injury in their rat model of closed head injury. Beclin-1 is a bcl-2 interacting protein that has been documented to be an important player in the induction of autophagy. Bcl-2, the anti-apoptotic protein has been shown to interact with beclin-1 via the BH-3 domain on beclin-1. Bcl-2 exerts a controlling effect on the activity of beclin-1.
Under normal homeostatic conditions, beclin-1 is bound to bcl-2 and hence not available to induce autophagy. Following a disruption in the cell homeostasis due to stress conditions or insults, beclin-1 interaction with bcl-2 is weakened and beclin-1 now becomes available to induce autophagy. Beclin-1, thus is available to complex with the Class III PI3K and the Vps34 protein to signal and activate autophagy proteins downstream. This interaction between these proteins is of utmost significance as it governs the switch between inducing autophagy or apoptosis (Pattingre et al., 2005; Pattingre and Levine, 2006; Erlich et al., 2007; Feng et al., 2007; Maiuri et al., 2007b). Elevated levels of beclin-1 was observed at early stages post-injury and maintained in the neurons for at least 3 weeks after injury. High levels of beclin-1 were also observed in astrocytes starting at 3 days following injury. A follow up study by the same group showed that the increased levels in beclin-1 were observed in neurons in response to the injury. They speculated that the overexpression of beclin-1 protein at the site of injury can enhance autophagy induction as a mechanism to discard injured cells and reduce the extent of damage to cells from the injured components (Erlich et al., 2006). Recently autophagy associated neuronal death has also been studied in neuronal injury models. (Uchiyama et al., 2008) demonstrated that hypoxia/ischemia brain injury in the neonatal brain results in energy failure, oxidative stress and unbalanced ion fluxes inducing elevated levels of autophagy in the brain neurons. Their results demonstrated caspase-dependent and independent cell death in the hippocampal pyramidal neurons with the accumulation of LC3-PE positive autophagosomes after ischemic brain insult. Significant neuroprotection of the pyramidal neurons was observed in mice deficient in ATG7, a gene involved in the regulation of autophagy, implying autophagic cell death (Koike et al., 2008). Autophagy has also been demonstrated in the brains of rodent models by the presence and accumulation of autophagy protein LC3 (Atg8) following traumatic brain injury (Liu et al., 2007;
Clark et al., 2008). Studies exploring the role for autophagy in brain trauma are still in its infancy. Further studies have to be conducted to elucidate the role of autophagy and autophagic cell death after brain injury.

Figure 1-1. Illustration of the sequence of events in the induction of autophagy and the formation of autophagosomes in eukaryotic cells.
Figure 1-2. Autophagy proteins (Atg) involved in the elongation of autophagosomes.
CHAPTER 2
MATERIALS AND METHODS

Animal Treatment

Appropriate pre-and post-injury management guidelines were maintained and these measures were done in compliance with guidelines set forth by the University of Florida (UF) Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health (NIH) guidelines detailed in the Guide for the Care and Use of Laboratory Animals. All experiments were performed using male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) that were aged 60 days and weighed between 240 to 270 g. Animals were housed in groups of two per cage and maintained on a 12 h light/dark cycle (lights on 7 AM to 7 PM). Food and water were available ad libitum.

Gel Electrophoresis and Electrotransfer

The cell lysates were collected at different time points after treatment with the appropriate media using lysis buffer containing 1% (v/v) Triton X-100, 5 mM EGTA, 5 mM EDTA, 150 mM NaCl and 20 mM Tris HCl (pH 7.4). The protein content was determined using DC Protein Assay (Bio-Rad, Hercules, CA) and the protein concentration was standardized to 1 μg/μL. Twenty micrograms of protein were subjected to SDS-PAGE gel electrophoresis on 4-20% or 6% Tris-glycine gels (Invitrogen, Carlsbad, CA) and then transferred onto PVDF membrane on a semi-dry electro transferring unit (Bio-Rad). Following the transfer, the membranes were blocked in 5% nonfat dry milk in 1X Tris buffered saline with Tween-20 (TBST) and probed overnight with primary antibody at 4°C. The following day, the membranes were washed with TBST and probed with either secondary peroxidase conjugated anti-rabbit or the biotinylated anti-mouse antibody. Immunoreactivity was detected by either using streptavidin alkaline phosphatase conjugate tertiary antibody or enhanced chemiluminescence (ECL) reaction.
Densitometric quantification of the bands was performed using ImageJ software (version 1.29x; NIH, Bethesda, MD).

**Immunoblot Analysis and Antibodies**

Immunoblotting membranes containing tissue protein were incubated overnight with the primary antibody at 4°C. On the following day, the membranes were washed three times with TBST and probed with the secondary antibody for an hour. Immunoreactivity was detected by using streptavidin alkaline phosphatase conjugate tertiary antibody. Monoclonal anti-mouse αII-spectrin (Affiniti Research Products, Ltd., UK) and anti-β actin (Sigma Chemical Co., St. Louis, MO), were used at a dilution of 1:4000 in 5% milk. Antibodies rabbit polyclonal anti-αII-spectrin and anti-caspase-3 specific spectrin breakdown product of 120 kDa (SBDP120) were made in-house. Anti-NeuN antibody was obtained from Chemicon Laboratories (Temecula, CA) and anti-LC3 antibody from Novus Biologicals (Littleton, CO). Peroxynitrite was purchased from Calbiochem (San Diego, CA). Antibodies anti-GAPDH (EnCor Biotechnology, Alachua, Gainesville), were used at a dilution of 1:1000 in 5% milk. Secondary biotinylated antibodies (Amersham Biosciences, United Kingdom) and streptavidin alkaline phosphatase conjugated tertiary antibody (Amersham Biosciences, United Kingdom) were used at a dilution of 1:3000 in 5% milk.

**Cell Culture and Amino Acid Starvation Treatment**

PC-12 cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), 10% heat-inactivated horse serum (HS), penicillin (60,000U/L) and streptomycin (60mg/L) and amphotericin B (250µg/ml) at 37°C with 5% CO2. For experimental purposes the cells were plated in complete medium then at 75-80% confluency they were washed twice with PBS and incubated in serum free medium (SFM) with or without maitotoxin (MTX, 0.3 nM), staurosporine (STS, 0.5 µM), or in amino acid deprived medium Earl’s balanced salt solution.
(EBSS), (a starvation media and autophagy inducer) for the different time points as discussed for each assay. MTX is a calcium channel opener known to induce oncosis while STS is a broad spectrum inhibitor of protein kinases inducing apoptosis.

**Primary Cultures and Treatments**

Cerebellar cultures were obtained from dissociated cerebella of 6-8 day old Sprague Dawley rat pups (Harlan Laboratories) and plated in Dulbecco’s modified eagle’s medium (DMEM) supplemented with extra glucose, 25 mM KCl, 10% fetal bovine serum on culture dishes (Nunc plates, Fisher). 1β-arabinofuranosylcytosine (10 μM) was added to the culture medium 22 hours after plating to prevent the proliferation on non-neuronal cells for 48 hours. On the 8th day following harvesting, the neurons were exposed to different treatment conditions and subsequent experimental end points. The neurons were treated with or without NMDA (200 μM) for different time periods and the cells were eventually lysed with triton based lysis buffer for protein immunoblots. The other treatment condition involved a co-treatment of NMDA with 3-methyladenine (3-MA, 10 mM). For fluorescent microscopy, the neurons were cultured on glass coverslips coated with poly-l-lysine and treated in a similar manner as the cultures on plates.

**MTT Assay**

Cell death was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PC-12 cells were plated in 96-well plates equally and incubated at 370C for 48 hours prior to treatment. At respective time points, the wells were incubated with 15 μL of the dye solution available in the kit (Cell Titer 96® Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI) for 4 hours. After incubation, 100 μL solubilizing solution available in the kit was subsequently added to solubilize the formazan crystals formed in the previous incubation. Following the solubilization of the formazan crystals, absorbance was read at 570nm.
Lactate Dehydrogenase Release Assay (LDH assay) of Cell Death

Lactate dehydrogenase release assay was performed to assess cell death by measuring the release of lactate dehydrogenase in the medium from damaged cells due to necrosis and secondary necrosis following apoptosis or autophagic cell death. The cells at 80% confluency were incubated in SFM (Control), MTX or STS in SFM or EBSS (starvation) media for a period of 5 days in a 24-well plate. Culture medium, 25 µL, was collected after 0, 3, 6, 12 h and 1, 2, 3 and 5 days and stored at -20°C in 96-well flat bottom plates. Detection reagent (CytoTox One Reagent, Promega) 25 µL was added to each well containing the culture medium and incubated for 10 minutes in the dark at room temperature. Fluorescence was measured (excitation wavelength: 560 nm and emission wavelength: 590 nm) using a fluorescent microplate reader (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA). Six replicates for each time point per experiment were assayed and three such experiments were performed. The arbitrary fluorescent unit values were plotted against time. In a separate set of experiments cells at 80% confluency were pre-incubated for an hour with and without the following inhibitors: 3-MA (10 mM; autophagy inhibitor), z-VAD-fmk (30 µM; caspase inhibitor), Boc-D-fmk (30 µM; caspase inhibitor) and SJA-6017 (50 µM; calpain inhibitor) before incubation in EBSS supplemented with the inhibitors. The samples were then tested by the LDH assay after 12 and 24 hours. Six replicates for each time point and for each treatment were assayed.

MDC Labeling of Autophagosomes and Nuclear Morphology Using Hoechst 33258 Staining

The PC-12 cells were stained with 0.05 mM monodansylcadaverine (MDC) in PBS after 3, 6, 12 hours and 1 day time points at room temperature (RT) for 10 minutes.26 The cells were washed 2X with phosphate buffered saline (PBS), mounted using antifade solution (Prolong Antifade, Molecular Probes) and immediately observed using the Zeiss fluorescence microscope.
To assess nuclear morphology, the cells were treated with the appropriate media for either 1 day (STS, starvation, control) or 3 hours (MTX). They were washed twice with 1X PBS and incubated with Hoechst 33258 (0.5 mg/mL) in PBS at room temperature for 10 minutes. Following incubation, the cells were washed twice with 1X PBS and observed under the Zeiss fluorescence microscope. Apoptotic cells were characterized by condensed nuclei morphology.

**Immunocytochemistry**

PC-12 cells were plated on collagen I coated coverslips and at 80% confluency the cells were treated with EBSS. At various experimental time points the cells were fixed in 4% paraformaldehyde for 10 minutes at 4°C, washed with PBS, blocked for 30 minutes in 5% normal goat serum (NGS) in TBST and then incubated overnight with LC-3 antibody (1:100) in 5% NGS at 4°C. The bound antibody was subsequently detected by incubation with goat anti-rabbit Alexa red-conjugated secondary antibody (1:3000). The cells were rinsed twice with 1X PBS and subsequently mounted using the mounting medium for fluorescence containing DAPI (Vectashield, Vector Laboratories, Burlingame, CA) and viewed under the Zeiss fluorescent microscope.

Cerebellar cells plated on coverslips were fixed using freshly prepared 4% paraformaldehyde solution for 10 mins at 4°C, washed in pure methanol and then permeabilized with 1X tris buffered saline tween (TBST, Sigma Laboratories, St. Louis, MO). Following TBST washing, the cells were incubated in 5% normal goat serum (NGS) at 37 oC for 30 minutes before incubating with the primary antibody microtubule associated light chain-3 (LC-3; Atg8; 1:1000) in 5% NGS overnight at 4 oC. On the following day, the coverslips were washed 3 times with 1X TBST and incubated with the Alexa Fluor (Molecular Probes, Carlsbad, CA) red or green-conjugated secondary antibodies (1:3000) for 1 hour at 37 oC. The coverslips were then
washed with 1X TBST and then mounted with the mounting medium Vectashield (Burlingame, CA) and observed under the microscope.

**Caspase-3 Activity Assay**

PC-12 cells were incubated in EBSS (starvation) and SFM (control) treatment conditions for set periods of time to determine the caspase-3 activity. Caspase-3 activity was performed using Apo-ONE® caspase-3 activity assay kit (Promega). The cells were incubated in the Apo-ONE® caspase-3 reagent for 2 hours and fluorescence was read at excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm.

To assay for caspase-3 activity, control, NMDA-treated and NMDA/3-MA co-treated granule neurons from three different wells (12 and 24 h) were scraped in a buffer containing 20 mM Tris-HCl (pH 7.4 at 4°C), 150 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, and 1% (vol/vol) Triton X-100 for 1 h. The cleared lysates were mixed with 50% (vol/vol) glycerol. Cell lysates were assayed with 100 µM acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-MCA; Bachem Bioscience), 100 mM HEPES, 10% glycerol, 1 mM EDTA, 10 mM dithiothreitol. Fluorescence (excitation, 380 ± 15 nm; emission, 460 ± 15 nm) was measured at 60 minutes using a fluorescent microplate reader (SpectraMax Gemini EM, Molecular Devices) as described previously (McGinnis et al., 1999).

**Materials**

Dulbecco’s modified eagle medium (DMEM), Earl’s Balanced Salt Solution [(EBSS) without calcium or magnesium fetal bovine serum and horse serum were obtained from Gibco Laboratories (Carlsbad, CA). Staurosporine (STS), maitotoxin (MTX), monodansylcadaverine (MDC), and 3-methyl adenine (3-MA) were purchased from Sigma-Aldrich Laboratories (St. Louis, MO). Prolong Antifade was from Molecular Probes (Eugene, OR) while the broad spectrum caspase inhibitors zVAD-fmk (Z-Val-Ala-Asp (OMe)-fluoromethyl ketone) and Boc-
D-fmk (Boc- Asp fluoromethyl ketone) and the calpain inhibitor SJA-6017 were purchased from Calbiochem (San Diego, CA). Serum free medium (SFM) was comprised of penicillin (60,000U/L), streptomycin (60mg/L) and amphotericin B (250μg/ml) in DMEM.

**Chemicals and Antibodies**

N-methyl D aspartate (NMDA), 3-methyladenine (MA), monodansylcadaverine (MDC) was purchased from Sigma Laboratories (St. Louis, MO). Prolong Antifade was purchased from Molecular Probes (Eugene, OR). Fetal bovine serum and Dulbecco’s modified eagle’s medium (DMEM) was from Gibco laboratories (Grand Island, NY). Antibodies rabbit polyclonal anti-αII-spectrin and anti-caspase-3 specific spectrin breakdown product of 120 kDa (SBDP120) were made in-house. Anti-β-actin antibody was purchased from Sigma laboratories (St.Louis, MO), anti-NeuN antibody was obtained from Chemicon Laboratories (Temecula, CA) and anti-LC3 antibody from Novus Biologicals (Littleton, CO). Peroxynitrite was purchased from Calbiochem (San Diego, CA).

**Experimental Paradigm of Traumatic Brain Injury**

A controlled cortical impact injury device was used to produce traumatic brain injury (TBI) in adult rats (Dixon et al. 1991; Pike et al. 1998). Rats divided into 2 groups received either TBI injury or sham-injury (n=5). Rats were anesthetized with 4% isoflurane in a carrier gas of 70% N₂O/30% O₂ followed by maintenance anesthesia of 2% isoflurane in 70% N₂O/30% O₂. A midline cranial incision was made, the skin and the underlying soft tissues reflected and a unilateral (ipsilateral to the side of impact) craniotomy was performed adjacent to the central suture, midway between the bregma and lambda to expose the cortical tissue. Brain trauma was produced by impacting the right cortex (ipsilateral cortex) with a 6-mm diameter impactor tip at a velocity of 4 m/s with a 1.6 mm compression for 150 ms. Sham animals (n=5) received the craniotomy without the impactor injury and naïve control animals were kept under the same
environmental conditions without receiving a craniotomy or injury. All procedures and postoperative care for the animals were conducted in accordance with the guidelines set forth for humane handling of the animals by the University of Florida Institute for animal care and use committee and the National Institute of Health.

**Tissue Collection Post Traumatic Brain Injury Procedures**

After the appropriate time points (2h, 6h, 1d and 2d) following injury, TBI (n=5) and sham-injured (n=5) animals were deeply anaesthetized using sodium pentobarbital (100 mg/kg, i.p.) and decapitated at the loss of the toe pinch reflex. Cortical brain regions ipsilateral and in close proximity to the cortical impact were rapidly dissected, snap-frozen in liquid nitrogen and stored at -80°C for further processing. Cerebral cortex tissues from different animals were pulverized and homogenized in a small mortar-pastel to a fine powder set over dry ice. The homogenized cortical tissue powder was then lysed for 90 minutes at 4°C with a 1% (v/v) Triton X-100, 5 mM EGTA, 5 mM EDTA, 150 mM NaCl and 20 mM Tris HCl (pH 7.4), 1 mM dithiothreitol (all chemicals from Sigma-Aldrich, St. Louis, MO) and a Complete Mini protease inhibitor cocktail tablet (Roche Biochemicals, Indianapolis, IN). Brain cortex lysates were then centrifuged at 15,000 r.p.m. for 10 minutes at 4°C. The supernatant was retained and collected at 4°C to prevent proteolysis. The protein content was determined using a DC Protein Assay (BioRad Laboratories, Inc., Hercules, CA, USA), after which the protein concentration was standardized to 1 μg/μL for immunoblotting analysis. Twenty micrograms of protein were subjected to SDS-PAGE gel electrophoresis on 4-20% or 6% Tris-glycine gels (Invitrogen, Carlsbad, CA) and then transferred onto PVDF membrane on a semi-dry electro transferring unit (Bio-Rad). Following the transfer, the membranes were blocked in 5% nonfat dry milk in 1X Tris buffered saline with Tween-20 (TBST) and probed overnight with primary antibody at 4°C.
Immunoblots were probed with an anti-MAP-LC3 (Novus biological, Littleton, CO) antibody or anti-bcl-2 (BD biosciences, San Jose, CA) or anti-beclin-1 (Santa Cruz biotechnology, Santa Cruz, CA) overnight at 4°C. Following the overnight incubation with the primary antibodies, the PVDF membranes were incubated with biotinylated anti-rabbit (for bcl-2 and MAP-LC3) and biotinylated anti-sheep (for beclin-1) for an hour in 5% nonfat dry milk in 1X Tris buffered saline with Tween-20 (TBST). Immunoreactivity was finally detected by using streptavidin alkaline phosphatase conjugate tertiary antibody.

**Statistical Analysis**

One-way ANOVA with Tukey post hoc test was used to draw comparisons between groups in the LDH assay. Data was plotted as means ± S.E.M. (standard error of the mean). Student’s t-test was performed to draw statistical comparisons between two treatment groups and a p<0.05 was considered to be statistically significant. Pairwise comparisons were made using the student’s t-test while one-way ANOVA with Tukey post hoc test was used to draw comparisons between multiple groups. Data was plotted as means ± S.E.M. (standard error of the mean). A value of p ≤ 0.05 was considered to be statistically significant.
CHAPTER 3
AMINO ACID STARVATION INDUCED AUTOPHAGIC CELL DEATH IN PC-12 CELLS:
EVIDENCE FOR ACTIVATION OF CASPASE-3 BUT NOT CALPAIN-1

Introduction

Cell death, a highly regulated and well orchestrated process, is classified into three types: apoptotic (type I), autophagic (type II) and necrotic/oncotic (type III) (Baehrecke, 2003; Liu et al., 2004). While mechanisms underlying apoptotic cell death have been well documented (Hengartner, 2000), autophagic cell death is still poorly understood. Autophagy occurs under stress conditions and aids in replenishing amino acids by degrading intracellular macromolecules (Meijer and Codogno, 2004). It is characterized by the presence of double membrane vesicles found in the cytoplasm termed “autophagosomes”. Autophagosomes sequester cytoplasmic constituents including organelles and transport them to lysosomes where they are degraded and the amino acids recycled for rebuilding cellular machinery. Deprivation of nutritional support factors for prolonged periods has been reported to culminate in autophagic cell death (Clarke, 1990; Lockshin and Zakeri, 2004).

Growth factor or nutrient deprivation mediated autophagic cell death has been documented in neuronal cell culture systems (Larsen and Sulzer, 2002; Xue et al., 1999). Autophagic cell death, a conserved integral pathway involved in mammalian cell development (Levine and Klionsky, 2004) is also activated under certain pathological conditions such as the neurodegenerative diseases (Stefanis et al., 2001). A recent study observed an upregulation of Beclin-1 (Atg6), one of the proteins required for the autophagic process, at the cortical site of injury in mouse brains following closed head injury (Diskin et al., 2005). Other recent studies suggest there is evidence for the protective role of autophagy in neurodegenerative disorders that are characterized by aggregate proteins such as Huntington’s, Creutzfeldt-Jakob and Alzheimer’s disease (Ravikumar et al., 2004; Sikorska et al., 2004; Yu et al., 2004).
It has long been believed that the two prime protease families involved in cell death are the calpains [calpain-1 (μ-calpain) and calpain-2 (m-calpain)], activated in both apoptosis and oncosis, and the caspases, especially caspase-3, believed to be strictly activated in apoptosis (Chan, 2004; Green, 2005; Wang, 2000; Liu et al., 2004). Recently there has been increased interest in caspase-independent cell death pathways (Stefanis, 2005). Apoptosis and autophagy appear to share certain common regulatory mechanisms and may interact in a variety of ways depending upon the cellular environment and treatments undertaken. Autophagy may play a role in apoptosis either by preceding and eventually initiating it or by delaying its onset (Boya et al., 2005; Canu et al., 2005). In other circumstances both pathways may be mutually exclusive (Gozuacik and Kimchi, 2004). Despite the progress made in understanding the precise molecular mechanisms in autophagy, the processes that regulate autophagic cell death and its relation to apoptosis are still poorly understood (Yu et al., 2004). In the present study we evaluated the activation of caspases and calpains following amino acid (AA) starvation-induced autophagic cell death in PC-12 cells.

**Results**

**Autophagy Is Induced under Amino Acid Starvation Conditions**

PC-12 cells incubated in serum free medium (SFM; control cells) or subjected to amino acid deprivation in Earle’s Balanced Salt Solution (EBSS; AA deprived cells) for 3, 6, 12 hours, were subsequently incubated with monodansycadaverine (MDC; 0.05 mM), a fluorescent marker for autophagosomes (Biederbick et al., 1995) and analyzed for autophagosomes using fluorescence microscopy (Fig. 3-1A). The starved cells showed MDC labeled autophagosomes as early as 3 hours and these persisted for at least 12 hours while the SFM treated cells showed little to no MDC incorporation. This is consistent with previously reported results (Dunn, 1990) demonstrating autophagy induction under AA starvation conditions. To further confirm
autophagy, immunostaining with LC-3, a mammalian homologue of yeast Atg8 which associates with autophagosome membranes (Kabeya et al., 2000) was performed (Fig. 3-1B). LC-3 expression appeared more intense in AA starved cells when compared to control cells at the various time periods examined. These results confirm induction of autophagy in PC-12 cells under AA starvation conditions.

**Prolonged Amino Acid Starvation Induces Cell Death and Evidence of αII-spectrin Breakdown**

Having established autophagy induction following AA deprivation, we hypothesized that prolonged AA starvation would eventually lead to autophagic cell death. To assess cell death, lactate dehydrogenase (LDH) release assay was performed and the fluorimetric readings at different time points were plotted (Fig. 3-2A). Amino acid deprived cells incubated in EBSS (Starv) were able to survive the initial periods of starvation. However, amino acid deprivation, when prolonged for 24 hours or more, resulted in an increase in cell death when compared to controls. The cell death time course was similar to that induced by STS treatment. In contrast cells treated with MTX showed rapid cell death peaking as early as 3 hours. Also observed was an upward trend in cell death in the control cells after approximately 80 hours of incubation. These results suggest that AA deprived cells are protected from cell death at earlier time points by the induction of autophagy but prolonged starvation results in cell death. To confirm the LDH data we also performed the MTT assay for cell viability. The cell death pattern observed among the amino acid deprived cells (Starv) was similar to that induced by STS treatment and coincided with the LDH data (Fig. 3-2B).

To study the possible activation of caspases and calpains in starvation induced autophagy, cell lysates for the different treatments were collected at various time points and analyzed by Western blot analysis. We examined the cytoskeletal protein, αII-spectrin, a well
established substrate cleaved by both calpains and caspases under cell death conditions. When compared to control, lysates from the AA starved cells showed an increase in spectrin breakdown products 120 kDa (SBDP120) and 150 kDa (SBDP150) day 1 through day 5 (Fig. 2C). While the observed SBDP150 band can be representative of either calpain (SBDP150) or caspase-3 (SBDP150i) mediated proteolysis, the SBDP120 proteolytic fragment is caspases-3 specific. The MTX and STS treated cell lysates used as positive controls for calpain and caspase-3 specific spectrin proteolysis showed calpain specific SBDP150 and 145 and caspase specific bands SBDP120 and SBDP150i, respectively (Fig. 3-2C).

**Activation of Caspase-3 and not Calpains in Amino Acid Starvation Mediated Autophagy**

Based on the αII-spectrin proteolysis pattern observed we further examined the roles of calpain-1 and caspase-3 in αII-spectrin breakdown and autophagy. The cell lysates obtained from the different treatments were probed with an antibody specific for SBDP150 which in our previous studies have shown to be calpain specific (Nath et al., 1996; Pike et al., 1998). There was no detectable increase in the SBDP150 levels in the starved cells even at the time points when cell death was observed. The MTX (3 hours) and STS (1 day) treated positive controls showed that the antibody is able to detect the calpain-mediated SBDP150 (Fig. 3-3A).

Subsequently, the antibody specific for activated calpain-1 was used to directly determine its possible activation in AA starved cells as compared to cells incubated in SFM. As expected, activated calpain-1 was clearly detected after MTX and STS treatment, as positive controls. In contrast, AA deprived treatment conditions showed the complete absence of calpain-1 activation (Fig. 3-3B).

Caspase-3 activation under AA starvation conditions was also assessed indirectly by measuring the levels of SBDP120 using a caspase-3 specific anti-SBDP120 antibody (Nath et al., 1996; Pike et al., 1998). Increases in SBDP120 levels were first observed to be significant by
densitometric analysis in AA starved cells at 12 hours and this significance persisted for the 2 days (p<0.005; Student’s t-test), the time limit of our study, when compared to the untreated controls. A significant increase in the intensity of SBDP120 was observed in STS treated cells when compared to controls and this increase was comparable to the intensities observed in the AA starved cells (1 day) (Fig. 3-4A).

The direct approach to examine caspase-3 activation in starved cells was by immunoblot analysis of the cell lysates for activated caspase-3. Activated caspase-3 bands (19 kDa, 17 kDa) were detectable in starved cells from 12 hours onward and densitometry analysis showed a significant increase (p<0.005) by day 2. The expression levels at day 1, while showing a notable upward trend was not statistical significant. The activated caspase-3 levels were comparable between the STS treated and AA starved cells (1 day) (Fig. 3-4B). To further substantiate the presence of caspase-3, following prolonged starvation, caspase-3 activity was assayed using the Apo-One® Caspase-3 assay. Caspase-3 activity in starved cells was significantly higher than the control cells (Fig. 3-4C). It peaked at day 1 following starvation which coorelates with the immunoblot data.

**Activation of Cathepsins Following Starvation**

Cathepsin B activation had previously been shown to translocate into the cytosol during apoptosis (Guicciardi et al., 2000). Canu et al. (2005) recently demonstrated that the cathepsin B translocation into the cytosol was not due to a compromise in the lysosomal membrane integrity or function but was rather cathepsin B independent. Since autophagy involves lysosomal enzyme activation, the activation of cathepsin B, a lysosomal hydrolase, during the process of starvation causing cell death was investigated. We observed activation of activation of cathepsin B at the
earlier stages of starvation (3 and 6 hours). This activation appeared to cease at the later time periods when cell death was observed (Fig. 3-5).

**Prolonged Amino Acid Starvation Induced Nuclear Morphology and Autophagosome Abnormalities**

To assess nuclear morphology as a measure of cell death following AA starvation, PC-12 cells were maintained under the different conditions for 1 day and then stained with the Hoechst 33258 nuclear DNA stain. Both STS treated and AA starved cells demonstrated the characteristic DNA condensation accompanied by the collapse and shrinkage of the nucleus. Control cells, on the other hand, maintained the well defined oval nuclear morphology of a healthy cell with diffused DNA. In the MTX treated cells a more non-specific disintegration of the DNA was observed (Fig. 3-6A). Furthermore, in the cells deprived of AA, MDC-positive autophagosomes were seen at day 1. This indicates that autophagy is still taking place in the cells even though cell death is occurring (Fig. 3-6B). The autophagosomes that were previously well defined punctuate stains (Fig. 3-1A) were observed as intensely stained aggregates mostly surrounding the shrunken nucleus at day 1. Cells incubated in SFM or treated with STS for the same time periods did not show a comparable density of MDC positive cells (Fig. 3-5B). These results are consistent with previous studies that have implicated apoptotic processes in cell death as a consequence of autophagic induction under certain stress stimuli (Canu et al, 2005; Hengartner, 2005).

**Amino Acid Starvation Mediated Cell Death is Suppressed by Autophagy Inhibitors but not Caspase or Calpain Inhibitors**

To further evaluate the role of autophagy and caspases and calpains in starvation-induced cell death, we incubated PC12 cells in EBSS with and without 3-methyladenine (3-MA; autophagy inhibitor), zVAD-fmk and Boc-D-fmk (two broad spectrum caspase inhibitors), and SJA-6017 (calpain inhibitor). The LDH assay performed at 12 hours and 24 hours post treatment
showed that starvation-induced cell death significantly decreased with 3-MA treatment ($p<0.05$) (Fig. 3-7A, B). Surprisingly, the protective effects of caspase inhibitor Z-VAD was marginal (less than 10%) and not statistically significant following prolonged AA starvation. The other broad spectrum caspase inhibitor Boc-D fmk failed to show any protection. Also, there was no protection from calpain inhibitor SJA-6017 confirming our previous results that suggest that calpain is not involved in cell death due to AA starvation. Also, the treatment with 3-MA (10 mM) alone and in combination Z-VAD resulted in a robust and significant protection against cell death following AA starvation. Cell lysates analyzed for SBDP120 by immunoblotting showed a complete loss of SBDP120 expression in cells treated with broad spectrum caspase inhibitors, MA treatment and a combination of both 3-MA with Z-VAD (Fig. 3-7C). Since cell death was strongly inhibited with the 3-MA treatment alone and considering the morphological data in Fig. 5 it appears that cell death occurring under AA starvation conditions in PC-12 cells is mediated through autophagy and can be classified as type II autophagic cell death.

**Discussion**

Autophagy has been described as a cellular response to stressful stimuli like starvation. One of its primary functions is to recycle amino acids from degraded proteins for cellular survival under nutrient deprived conditions (Cuervo, 2004; Melendez et al., 2003; Otto et al., 2003). PC-12 cells have been acknowledged as a neuronal cell line since they respond reversibly to nerve growth factor (NGF) stimulation and acquire sympathetic neuronal phenotype capable of producing cathecholamines as neurotransmitters (Xue et al., 1999). In this study we demonstrated autophagy induction in PC-12 cells under amino acid starvation conditions (Fig. 3-1). Also, we showed that prolonged starvation resulted in 3-MA sensitive autophagic cell death (Fig. 3-7) a process that shares certain common features with the apoptotic pathway (Figs. 3-4, 3-6).
Autophagosomes, double membrane vesicles, have been considered to be one of the hallmark features of autophagy. These autophagosomes induced under conditions of stress, are responsible for engulfing cytosolic organelles and fusing with lysosomes where the engulfed proteins are dismantled into their constituent amino acids. The amino acids are then recycled within the cell (Wang and Klionsky, 2003). Autophagosomes, previously reported to accumulate MDC, an autophagolysosome marker (Mizushima, 2004; Munafo et al., 2001), were detected as early as 3 hours in amino acid starved cells but were not found in the control cells or cells treated with either STS or MTX. While the autophagosomes were observed as punctuate stains at earlier time periods following starvation, these punctuate markers were later transformed into condensed aggregates around the perinuclear region of the cells. These changes in the morphology of the MDC positive autophagosomes were coincident with the increases observed in cell death and the breakdown of αII-spectrin (Fig. 3-2). Autophagosome formation was substantiated by the co-localization of MDC with a processed form, LC3-II, of microtubule-associated protein LC-3, a mammalian homologue of yeast Atg8 which also associates with outer membrane of the autophagosomes (Mizushima, 2004; Kabeya et al., 2000). The LC-3 labeling of phenotypically normal autophagosomes was likewise observed as early as three hours following starvation (Fig. 3-1B).

To determine the involvement of caspases and calpains, following AA starvation mediated cell death, the proteolytic fragments of αII-spectrin were studied. The αII-spectrin fragmentation pattern is used as a biomarker to specifically identify the role of calpains and caspase-3 in cell death (Nath et al., 1996). Calpain-1 was of interest due to its role as one of the prime proteases responsible for oncotic cell death and to a lesser extent of apoptotic cell death (Wang, 2000). Calpain-1 has also previously been found to induce cleavage and release of
apoptosis inducing factor (AIF), in a caspase independent fashion, triggering the apoptotic pathway (Polster et al., 2005). In examining the role of calpains in AA starvation mediated cell death, there was no increase of calpain-specific spectrin breakdown product of 150 kDa (SBDP150) (Fig. 3-3A). This was confirmed by the lack of increase of the activated form of calpain-1 (Fig. 3-3B). Cells treated with MTX, as positive control for oncotic cell death, showed strong calpain activation. These observations pose some interesting questions and the need for further investigation as the cellular concentration of calcium appears to be adequate to activate these proteases. It should be noted that the EBSS starvation media does not provide extracellular Ca2+ (or Mg2+) and this may play a role in the lack of calpain activation under starvation conditions. This may suggest that the release of internal stores of Ca2+, if involved, is insufficient to generate sustained calpain activation in AA starvation induced cell death.

To address the involvement of caspases in cell death after prolonged AA starvation, additional tests were performed. The caspase-3 activity assay showed there was considerable caspase-3 activity (Fig. 3-4C) and this was confirmed by immunoblots for caspase-3 mediated SBDP120 and activated caspase-3 (Fig. 3-4A,B). These results initially suggested caspase-3 activation may play a role in autophagic cell death. In addition, nuclear morphology changes highlighted by Hoechst 33258 staining were comparable between the AA starved cells and those treated with STS (a known apoptosis inducer) at the same time point the aggregated MDC positive autophagosomes were observed in the cells (Fig. 3-6). While cathepsin B activation was observed at the early stages in the cytoplasm following starvation, the leaking of the lysosomal protease into the cytoplasm was limited to starvation periods upto 6 hours but not later suggesting that the lysosomal hydrolase is not a participant in the cell death process (Kroemer and Jaattella, 2005). Also the lysosomal membrane integrity appears to remain intact, as the
protease was not observed in the cytosol following prolonged periods of starvation. Taken together, these data imply that starvation induced autophagic cell death shares common features with classic apoptosis.

The LDH assay studies of inhibitor treated cell cultures showed a significant decrease in cell death in the presence of the inhibitor 3-MA. The data also showed a robust decrease in the levels of caspase-3 mediated SBDP120 following 3-MA treatment suggesting that autophagy precedes caspase-3 activation. These findings appear to be in agreement with a previous study which had demonstrated inhibition of apoptotic markers following 3-MA treatment (Canu et al., 2005). The two broad spectrum caspase inhibitors, zVAD and Boc-D, however, did not show significant protection from cell death under starvation conditions within the time frame examined. This is consistent with previous findings (Xue et al. 1999), which demonstrated that treatment of nerve growth factor deprived sympathetic neurons treated with pan-caspase inhibitors prevented the morphological changes that are characteristic of apoptosis, but did not prevent cell death nor did they affect autophagic activity. Our data shows that both broad spectrum caspases inhibitors fully suppressed the formation of SBDP120 band (Fig. 3-7C). The results suggest there is an activation of caspase-3 in autophagic cell death, but the inhibition of caspase-3 is not sufficient by itself to protect against this form of cell death. While we and others have demonstrated that concurrent with caspase-3 activation there is calpain activation in most forms of classical apoptosis including STS treatment (Fig. 3-4) and NGF deprivation in PC-12 cells (Nath et al., 1996), our results demonstrated that autophagic cell death following AA starvation does not activate calpain nor does it seem to be required (Fig. 3-3). The data suggests a subtle but important differences between apoptosis (type I) and autophagic cell death (type II).
Conclusion

In summary, prolonged AA starvation induces autophagic cell death in a rat neuronal-like cell type (PC-12). While beneficial early following stress activation, at some point, autophagy unchecked will begin to damage the cellular mitochondria and other vital organelles necessary for its continued survival resulting in cell death. The neural cell autophagic cell death paradigm described in this study will allow for further exploration of this form of cell death in neurological disorders such as stroke and traumatic brain injury, and neurodegenerative conditions like Alzheimer’s disease.
Figure 3-1. Autophagosome staining with MDC and LC3 after amino acid starvation of PC-12 cells. A) In the PC-12 cells, MDC (50 µM) accumulates as a punctate pattern (arrows) predominantly in the cytoplasm under amino acid (AA) starvation conditions (starv; EBSS treated) beginning as early as 3 hours. Inserts highlight portions of the cells expressing the MDC positive autophagosomes. Control (Ctrl) cells are incubated in SFM. Photomicrographs are at 400X magnification and the scale bar is 20 µm. B) DAPI (blue) co-localized with LC3 (red), a known autophagosome membrane marker under conditions of AA starvation and control conditions at various time points. Photomicrographs are at 400X magnification and the scale bar is 20 µm.
Figure 3-2. Amino Acid starvation induces cell death. A) i) Quantification of the LDH (lactate dehydrogenase) assay of cell death. The graph depicts cell death under control (SFM; □) and starvation conditions (EBSS; ●) from 0 to 120 hours post treatment, with MTX (▲) and STS (○) acting as the positive controls for calpain and caspase-3 mediated cell death, respectively. The expressed values are the mean ± S.E.M. (n=6). B) Cell death was also confirmed using MTT assay. The expressed values are the mean ± S.E.M. (n=4). C) Representative blots (n=3) of total αII-spectrin and its breakdown products (SBDP) observed under amino acid starvation and control conditions from 3 hours to 5 days. D) Positive controls are for calpain (MTX) and caspase-3 (STS) mediated spectrin breakdown products. Spectrin breakdown products of 120 kDa, 150 kDa and 145 kDa are denoted as SBDP120, SBDP150 and SBDP145, respectively. SBDP150i is the breakdown product mediated by caspases.
Figure 3-3. Evidence for the lack of calpains under starvation conditions in EBSS. A) Representative immunoblot (n=3) for spectrin breakdown product 150 kDa (SBDP150), a breakdown product that is calpain specific. MTX and STS incubated cells (3 hours, 1 day respectively) served as positive control for calpain mediated spectrin breakdown. Cell lysates were obtained at 3 hours and 1 day under AA starvation treatment (Starv) and control conditions (Ctrl). There was no SBDP150 for calpain found for any of the time points. B) Representative blots (n=3) of activated calpain-1 (76 kDa) under starvation and MTX treatment conditions. No activated calpain-1 was found under AA starvation treatment. MTX (3 hours) and STS (1 day) incubated cells served as positive controls.
A. Levels of SBDP120

B. Levels of activated Casp-3
C.

Figure 3-4. Evidence for caspase-3 activation under amino acid starvation conditions. A) Densitometric analysis of immunoblots of SBDP120 indicates caspases play a statistically significant role in spectrin breakdown under amino acid starvation (Starv) versus control (Ctrl) conditions as evaluated from 12 hours to 2 days. Staurosporine (STS) treated cells represent positive control for caspase-3 mediated spectrin breakdown. Student’s t-test was performed to evaluate statistical significance, *p<0.005; mean ± S.E.M; n=3. B) Densitometric analysis of activated caspase-3 showed significant increases under amino acid starvation (Starv) versus control (Ctrl) conditions by 2 days. STS treated cells represent positive control for activated caspase-3 expression (17 and 19 kDa). Student’s t-test was performed to evaluate statistical significance, *p<0.005; mean ± S.E.M; n=3). β-Actin acts as a protein loading control indicating even protein loading. C) Caspase-3 activation time course in AA starved cells and control (SFM) cells (n=3). Statistical comparisons were made using student’s t-test and p<0.05 was considered significant.

Figure 3-5. Activation of cathepsin B following starvation. Representative immunoblot shows that the cleaved activated form of Cathepsin B (30 kDa) from whole lysates of cells peaks at 3 and 6 hours following starvation prior to returning to normal. Cells treated with SFM were employed as controls (n=2).
Figure 3-6. Hoechst 33258 nuclear and MDC staining of cells under different treatment conditions. A) Hoechst 33258 immunostaining was performed to observe the nuclear morphology at day 1 for cells incubated under amino acid starvation (Starv), control (Ctrl), STS and MTX (3 hours) treatment conditions. Apoptotic nuclear morphology is represented by STS (1 day) treated cells while MTX (3 hours) treatment represents positive control for necrosis. Arrows indicate condensed DNA. B) MDC stained autophagosomes are still observed in cells under starvation conditions after 1 day (EBSS) when compared to cells incubated in SFM (Ctrl), STS and MTX (3 hours). Photomicrographs were taken at 400X magnification and scale bar represents 20 µm. Insert represent (2X) the region of cells showing abnormal autophagosomes.
Figure 3-7. Effects of caspase, calpain and autophagy inhibitors on AA starvation induced autophagic cell death and SBDP120 formation. A) and B) Lactate dehydrogenase cell death assay results at 12 (A) and 24 (B) hours after incubation with the caspase inhibitors Z (zVAD, 30 μM) and B (Boc-D, 30 μM), the calpain inhibitor SJA (SJA-6017, 50 μM), and the autophagy inhibitor MA (3-MA, 10mM) and the combination of caspase and autophagy inhibitors MA+Z (3-MA, 10 mM + zVAD, 30 μM) in starvation media S (EBSS). The fluorescent measurements are expressed in arbitrary units. The plates were read at excitation wavelength 560 nm and emission wavelength 590 nm. The values are mean ± SEM (n=6) and one-way ANOVA with Tukey post-hoc test was performed to evaluate statistical significance (***p<0.006; **p<0.001; *p<0.01). C) Representative immunoblot demonstrates the efficacy of the inhibitors on caspase-3 activation by their effect on SBDP120 at day 1 (n=3). β-Actin acts as a protein loading control indicating even protein loading.
CHAPTER 4
ACUTE NMDA TOXICITY IN RAT CEREBELLAR GRANULE NEURONS IS
ACCOMPANIED BY AUTOPHAGY AND 3-METHYLADENINE-SENSITIVE LATE
ONSET AUTOPHAGIC CELL DEATH

Introduction

Autophagy is an intracellular pathway that is activated in response to cell stress. This is a phenomenon where the cytoplasmic organelles in the cell are engulfed by double membrane vesicles called the autophagosomes and delivered to the lysosomes where the organelles are broken down by lysosomal proteases and the amino acids recycled back into the cell machinery to aid cell survival under conditions of stress (Dunn, 1990a; Klionsky, 2004). Some of the key proteins identified to be involved in this process are Atg4, Atg8, Atg12 and Atg5 (Klionsky et al., 2003). Autophagy has been reported to be vital in the development of the central nervous system (Hara et al., 2006; Komatsu et al., 2006). It has also been documented to be constitutively active in the healthy neurons and aid survival (Boland and Nixon, 2006).

Programmed cell death among neurons in the central nervous system is a regulated process. Depending upon the nature of the insult, the neurons undergo apoptotic (type I) or autophagic (type II) cell death or oncosic/necrotic (type III) (Baehrecke, 2003; Liu et al., 2004). Acute excitotoxic insults that result from using glutamate in primary culture has been shown to induce both oncosic and apoptotic cell death in neurons (Ankarcrona et al., 1995; Nath et al., 1998). Increased excitation of the glutamate receptors by its ligand has been shown to cause an imbalance in the ionic gradient in the neurons, resulting in an increase in the calcium and sodium levels intracellularly leading to oncosis. At the same time, this excessive activation has also been demonstrated to activate the endonucleases causing internucleosomal DNA fragmentation, thus resulting in apoptosis in neurons. Though extensive studies have been conducted on apoptotic
cell death mechanisms, the biochemical mechanisms involving autophagic cell death is poorly understood (Hengartner, 2000; Debnath et al., 2005; Gozuacik and Kimchi, 2007). Autophagic vacuoles have been shown to accumulate in affected neurons of several neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease. Wang et al., (2006) recently demonstrated the induction of autophagy was associated with axonal degeneration in Purkinje cells in Lurcher mice. More recent experimental evidence has also shown the upregulation of autophagy protein Beclin-1 (Atg 6) following closed head brain injury in rats (Diskin et al., 2005).

Excitotoxicity via N-methyl-D-aspartate (NMDA)-receptor overactivation, is one of the documented hallmark events that occur following acute brain injury. Hence we sought to examine if autophagy is a general response during excitotoxic NMDA challenge by using rat cerebellar granule neuronal cultures in vitro. In addition, we also addressed whether autophagic cell death contributes to NMDA excitotoxicity.

Results

Acute NMDA Exposure Induces Autophagy in Cerebellar Granule Neurons in Culture

Rat cerebellar granule neurons were treated with or without NMDA (200 μM) in serum free medium (SFM) to achieve excitotoxic and control conditions, respectively. To assess the possible induction of autophagy following acute NMDA exposure, the neurons were stained with antibody against microtubule associated light chain-3 (LC3) protein, a known autophagosome protein marker (Kabeya et al., 2000). Neurons subjected to NMDA exposure exhibited increased punctate LC3 immunostaining as early as 3 hours that persisted out to 12 hours when compared to the neurons under control conditions at 8 hours. Co-immunostaining with anti-NeuN antibody, a protein marker of mature neurons was employed to demonstrate that the increase in the LC3 positive autophagosomes was indeed found in neurons following NMDA treatment (Fig. 4-1).
However, in most neurons examined 24 hours post-NMDA exposure, LC3 positive staining was no longer punctate but rather showed a more localized accumulation in LC3 positive bodies suggestive of aggregated autophagosomes in the cell bodies of the neurons. Similarly, the signal from the fluorescent dye monodansylcadaverine (MDC) used to label the autophagosomes also showed a strong increase in autophagosome formation in the NMDA cell cultures 6-12 hours after treatment. The MDC staining displayed the punctate pattern of autophagosomes in both the cell bodies and the neurites after 6-12 hours of NMDA exposure. This punctate pattern of MDC staining was replaced by a more intense accumulation of what appeared to be aggregated autophagosomes in the cell bodies by 24 hours (Fig. 4-2). The observation of LC3 and MDC-positive “abnormal autophagosomes” at 24 hours coincided with the terminal degenerative phase of NMDA-induced neuronal death (see Fig. 4-6 later). This observation might also be suggestive of a breakdown in the recycling machinery involving autophagosomes.

**Autophagy Protein Marker Beclin-1 is Up-Regulated Following Early Phase NMDA Exposure**

Having established the induction of autophagy in neurons exposed to NMDA, we sought to study protein levels of the autophagy protein marker beclin-1 (Atg6). We performed immunoblots on cell lysates obtained from cultures following treatment with or without NMDA at different time periods. The beclin-1 levels appear to be increased in the NMDA-treated neurons when compared to controls at time periods ranging from 3h to 24h (Fig. 4-3A). Intriguingly, a decrease in the beclin-1 band intensities was observed at later time periods (12 and 24h) of NMDA exposure with the beclin-1 levels being robustly increased at early time periods of 3h and 6h. However, graphical representation of the band intensities quantified using Image J software, yielded a significant increase in beclin-1 protein levels after NMDA exposure when compared to controls (Fig. 4-3B).
**Autophagy Inhibitor 3-Methyladenine (3-MA) Effectively Suppresses NMDA-Induced Autophagy**

Having observed NMDA-induced autophagy, we examined the effects of autophagy inhibitor 3-Methyladenine (3-MA) for its ability to suppress the process of autophagy under excitotoxic conditions. Cell lysates from either neurons treated with NMDA or a combination of NMDA and 3-MA were LC3 immunoblot analyzed for the LC3 to LC3-II conversion. Immunoblot data demonstrated a reduction of the LC3-II band in the NMDA+3-MA co-treated neuronal cultures as compared to cultures treated with NMDA alone (Fig. 4-4A). As expected, densitometric analysis showed no change in the LC3-I band intensity levels while the LC3-II intensities in the NMDA+3-MA co-treated cultures were significantly lower than that observed in the NMDA-treated at both 12 and 24 hours (Fig. 4-4B). Consistent with this observation, there was a robust reduction in the immunostaining of autophagy marker LC3 after co-treatment of NMDA and 3-MA in cultures compared to the cultures treated with or without NMDA alone (8 and 12 hours) (Fig. 4-5A). This reduced level of expression was further confirmed using MDC staining. The MDC positive autophagosomes were relatively sparse in cerebellar cultures co-treated with NMDA and 3-MA when compared to cultures treated only with NMDA. We also noted that the aggregated form of autophagosomal bodies observed at 16 hours post-NMDA exposure were also absent the neuronal cell cultures co-treated with 3-MA (Fig. 4-5B).

**Cell Death in NMDA-Treated Neurons was Alleviated by 3-MA**

Since sustained and unrelieved autophagy has been documented to lead to autophagic (type II) cell death, additional studies were carried out to determine whether NMDA-induced autophagy contributed to cell death in the later stages of NMDA excitotoxicity. Neuronal cultures were treated with or without NMDA (200 μM) and a third set was co-treated with NMDA and 3-MA (10 mM). Cell death was assayed by measuring the lactate dehydrogenase...
(LDH) enzyme release into the medium. The LDH release plotted over a 24 hours showed an increase in the LDH release in NMDA-treated cultures compared to controls. This increase in LDH release following NMDA exposure was significantly alleviated when the cultures were co-treated with 3-MA. The levels of LDH release between NMDA and NMDA+3-MA co-treated cultures were significantly different at 6 hours through 24 hours (p<0.05) (Fig. 4-6A).

Representative phase contrast images of the neurons at 16 hours following NMDA-treatment demonstrated unhealthy neurons with retracted neurites and shrunken cell bodies. The NMDA-treated neurons showed apoptotic cell morphology when compared to controls. In contrast, neurons co-treated with NMDA and 3-MA showed a dramatic sparing of both neurite and cell body morphology (Fig. 4-6B).

**NMDA-Induced Caspase-3 Activation is Suppressed by 3-MA**

In our previous study (Sadasivan et al., 2006), we had demonstrated the activation of caspase-3 under conditions of prolonged amino acid starvation-induced autophagy in PC-12 cells. Here, we tested our hypothesis that the neuroprotective effects of 3-MA may have been achieved through caspase-3 suppression. To assess caspase-3 activation, we examined the proteolysis of an endogenous caspase-3 substrate (the breakdown of αII-spectrin) and by employing a caspase-3 enzymatic assay (Nath et al., 1996; Nath et al., 1998). The αII-spectrin breakdown profile using total anti-αII-spectrin antibody showed an increase of the caspase-3 generated spectrin breakdown product of 120 kDa (SBDP120) at 24 hours following treatment of cerebellar neurons with NMDA in culture. Increases in the calpain-generated SBDP150 and SBDP145 were also observed at 24 hours with NMDA-treated cultures (Fig. 4-7A).

Staurosporine (STS) treated cultures were used as positive controls for caspase-3 activation and SBDP120 generation. To further confirm that the 120 kDa band was caspase-3 generated, immunoblots were analyzed using anti-SBDP120 specific antibody developed previously in-
The blots confirmed the appearance of the SBDP120 at 24 hours in the NMDA-treated cultures and that it was not found in the controls. More importantly, 3-MA co-treatment suppressed the increased SBDP120 levels to near normal (Fig. 4-7A). Consistent with our hypothesis, densitometric analysis of the immunoblots showed a significant reduction in the caspase-3 mediated SBDP120 levels in NMDA and 3-MA co-treated cultures as compared to NMDA-treated cells (Fig. 4-7B). To assay the caspase-3 protease activity directly, the caspase-3 preferred substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was incubated with protease inhibitor-free cell lysates under various conditions. Caspase-3 activity was significantly increased in NMDA-treated cultures at 12 and 24 hours when compared to control cultures. On the other hand, this caspase-3 activity was significantly reduced by 3-MA co-treatment when compared to NMDA-treatment alone (12 and 24h) (Fig. 4-7C).

**NMDA Mediated Protein Nitration in Cerebellar Neurons is also Attenuated by 3-MA**

NMDA exposure has previously been documented to induce oxidative stress in neurons thus contributing to neuronal death (Arundine and Tymianski, 2004; Sanganahalli et al., 2006). We hypothesized that autophagy inhibition (with 3-MA) might also intervene NMDA-toxicity through this pathway. In our experiments oxidative stress due to NMDA was measured by identifying protein nitration at the tyrosine residue. To assess the oxidative modification of proteins by NMDA, we probed protein lysates from various conditions mentioned above with the anti-nitrotyrosine antibody in immunoblots. Multiple nitrated protein bands of different molecular weights with varied intensities were observed to be elevated following NMDA-treatment (data not shown). A nitrated protein of 70 kDa (p70), the most prominent protein observed in the NMDA-treated cells, was thus chosen for densitometric quantification (Fig. 4-8A). Nitrated protein density values plotted over a time scale showed a significant increase at 24
hours following treatment with NMDA when compared to controls (Fig. 4-8A). In cultures co-
treated with NMDA and 3-MA, protein nitration was attenuated to control levels (Fig. 4-8B).

Cerebellar granule neurons were also treated with free radical generating peroxynitrite
with or without 3-MA to study the effects of the autophagy inhibitor, whether it was indeed able
to alleviate cellular oxidative stress. An increase in cell death measured by LDH release over
time was observed in peroxynitrite-treated cultures compared to peroxynitrite and 3-MA co-
treatment. In addition, densitometric quantification of the peroxynitrite-induced nitrated protein
(p70) levels at 24 hours was also significantly suppressed by 3-MA co-treatment (see Fig. 4-8
inset).

Discussion

In this study, we demonstrated that acute NMDA exposure of cerebellar granule neurons
induces autophagy, as established by the autophagy protein markers LC3 and beclin-1(Figs. 4-1
and 4-3) and the fluorescent dye MDC (Fig. 4-2). We documented that while initial NMDA
challenge leads to the induction of autophagy and classical autophagosome formation (dispersed
and punctate vesicles); prolonged exposure of NMDA resulted in abnormal aggregated
autophagosomes in the cell bodies of neurons suggesting the commencement of autophagic cell
death. Autophagy inhibitor 3-MA not only efficiently suppressed the NMDA-induced autophagy
but also provided significant neuroprotection against prolonged exposure to NMDA (Figs. 4 and
5). Apoptosis-linked protease, caspase-3 activation and oxidative stress following prolonged
NMDA treatment were both observed to be alleviated with 3-MA co-treatment (Figs. 7 and 8).

Autophagy induction occurs in the central nervous system under conditions of
stress/starvation or protein aggregating neurodegenerative diseases (Ravikumar et al., 2002;
Webb et al., 2003; Codogno and Meijer, 2005; Komatsu et al., 2006). This study has shown that
acute excitotoxicity by NMDA exposure can act as a stressor to induce autophagy in cerebellar
neurons. Glutamate excitotoxicity has previously been documented as one of the pathways of cell death following experimental traumatic brain injury (Ankarcrona et al., 1995; Ferrer et al., 1995; Portera-Cailliau et al., 1997). (Erlich et al., 2006) demonstrated an increase in beclin-1 expression in mice following traumatic brain injury suggesting that autophagy is upregulated around the regions of injury to support the cells under duress and help disposing off injured components. Recently, there has also been suggestive evidence for the involvement of autophagy in chronic neurodegenerative diseases such as Parkinson’s disease and Huntington disease (Dickson, 2007; Rubinsztein et al., 2007; Sarkar et al., 2007a).

LC3 is a well known autophagy marker that exists as a pro-form which is processed at the C-terminal (removal of 5 amino acids) to expose the glycine residue, which is then conjugated to the phosphatidyl ethanolamine (PE) tail. The PE-conjugated LC3 then attaches itself to the outer membrane of the autophagosomes where it assists in the formation of mature autophagosomes. An increase in the LC3 immunostaining and the monodansylcadaverine (MDC) positive autophagosomes was observed following NMDA treatment as compared to control sets (Figs.4-1, 4-2). The NMDA treatment also increased the levels of LC3-I when compared to the controls at the earlier time periods (3 and 6 h). This transient enhancement in LC3-I protein levels in comparison to control was an indication of an enhanced capability of the cells to launch an autophagic response (data not shown). Unexpectedly, there was no apparent increase in LC3-II levels following NMDA treatment measured by immunoblots at early periods. This suggests that there may be a sub pool of LC3-II that once generated was translocated to the outer membrane of the autophagosomes. At this time, the degradation and recycling of the autophagosomes inside the neurons seem to function normally, thus maintaining a constant flux of the LC3-PE. This might explain the increase in the LC3 immunostaining and MDC-positive autophagosomes
observed, not accompanied with a similar increase in the LC3-PE levels through immunoblots (Kuma et al., 2007; Mizushima and Yoshimori, 2007).

Evidence from studies has the induction of autophagy and subsequent neuronal death in spinal cord motor neurons and organotypic hippocampal cultures, following glutamate receptor-mediated injury (Borsello et al., 2003; Tarabal et al., 2005). According to another study, a buildup of autophagosomes could be observed in the axonal terminals of neurons in Lucher mice, a mouse model of excitotoxicity (Wang et al., 2006). The authors speculated that the autophagosomes observed at the terminals or distal ends of the axon may be due to a breakdown of the retrograde transport. We extend their findings by demonstrating that NMDA in cultured neurons resulted in robust autophagosome formation throughout the cell bodies and neurites.

Since prolonged autophagy has been shown to result in autophagic cell death (type II), we hypothesized that this form of cell death may be a crucial component to NMDA excitotoxicity (Fig. 4-9). To test this hypothesis we employed autophagy inhibitor 3-MA and examined whether autophagy inhibition could alleviate NMDA-mediated neuronal death. The results showed effective inhibition of the processing of the LC3-I to the lipidated LC3-II protein, the latter being important in the stabilization of the autophagosomal membrane (Fig. 4-4, 4-5). Also there was a strong decrease in the LC3 immunostaining and the MDC-positive autophagosome staining with 3-MA co-treatment following NMDA exposure. The treatment of the neurons with 3-MA in this study resulted in significant protection against NMDA-induced cell death (Fig. 4-6). Thus, our data strongly suggest autophagic cell death to be a component of NMDA-mediated excitotoxic cell death.

Apoptotic and autophagy pathways are intricately balanced in the cell (Yu et al., 2004; Gonzalez-Polo et al., 2005; Pattingre and Levine, 2006). In our experiments we observed that
NMDA-induced caspase-3 activation and breakdown of spectrin was 3-MA sensitive. This observation strongly suggests that NMDA-induced 3-MA sensitive autophagy precedes caspase-3 activation as illustrated in our schematic (Fig. 4-9). Another well documented component of the pathology following NMDA exposure has been the generation of reactive oxygen species (ROS) (eg. nitric oxide radicals) which contributes to dysfunctional mitochondria and subsequent cell death (Bonfoco et al., 1995; Castilho et al., 1999; Barsoum et al., 2006; Nicholls et al., 2007). NMDA-exposure induces increase in the intracellular calcium ions which result in ER stresss and mitochondrial stress that result in the generation of reactive oxygen species (ROS). Interestingly, previous reports (Liu and Lenardo, 2007; Scherz-Shouval and Elazar, 2007) have suggested that oxidative stress is essential for the generation of autophagy, providing a potential positive feedback (Fig. 4-9). The use of 3-MA not only inhibits autophagy but also alleviates the oxidative stress generated due to acute exposure to NDMA. The effects of 3-MA may be due to its ability to inhibit autophagy at the earlier stages which later may be capable of inducing activation of caspase-3 and ROS. In this study it was demonstrated that (i) prolonged NMDA exposure (24 hours) enhanced protein nitration and that (ii) such increased protein nitration was suppressed significantly by autophagy inhibitor 3-MA (Fig. 4-8). Thus, these findings place NMDA-induced autophagic pathway ahead of NMDA-induced oxidative stress as illustrated in Figure 4-9. Taken together, the data suggest that NMDA excitotoxicity initially induces early autophagy as a self-defense mechanism which when prolonged results in the manifestation of abnormal autophagosome vesicles, leading to “autophagic cell death” which would involve caspase-3 activation and oxidative stress (Fig. 4-9).

**Conclusion**

In summary, this study highlights that autophagy is robustly induced in neurons subjected to excitotoxic NMDA exposure in a simple culture paradigm. In addition, it has also been
demonstrated for the first time that inhibition of autophagy protects against NMDA neurotoxicity in neurons by alleviating the associated oxidative stress and caspase-3 activation. Importantly, the data presented in this study, when taken together, strongly suggest that autophagic cell death might be a significant component of NMDA excitotoxicity. Further studies are now being carried out in our laboratory will examine whether autophagy and autophagic cell death play a significant role in an animal model of excitotoxicity.
Figure 4-1. NMDA excitotoxicity results in the induction of LC3-positive autophagosomes in rat cerebellar granule neurons. Representative fluorescent micrographs of cerebellar granule neurons in culture following treatment with NMDA (200 μM) show an increase in the MAP-LC3 (red) staining at time periods 3, 8 and 24 hours. NeuN (green) was used to stain mature neurons Arrows (yellow) represent the increased LC3 staining of autophagosomes in the cell bodies of the neurons co-localized with neuronal marker NeuN (green) while arrow heads (yellow) indicate the increase in the LC3-positive autophagosomes along the axon. Red arrows represent the LC3 staining of aggregated autophagosomes in the neuronal cell bodies at 24 hours. Neurons in the boxed regions have been magnified. All images were taken at 400X magnification. Scale bar represents 20 μm.
Figure 4-2. NMDA exposure induces the formation of MDC-positive autophagosomes in cerebellar granule neurons. Representative fluorescence micrographs of granule neurons incubated with monodansylcadaverine (MDC) show an increase in the labeling of the autophagosomes in both the neurites and the cell bodies. Arrows and arrow heads (yellow) indicate the normal punctate autophagosome staining at 6 and 12 hours in the cell bodies and neurites, respectively. Red arrows indicate the aggregated autophagosomes in the cell bodies following prolonged exposure to NMDA (200 μM) (12 to 24 hours). All images were taken at 400X magnification. Scale bar represents 10 μm.
Figure 4-3. NMDA exposure of neurons results in an increase in the beclin-1 levels in vitro. A) Lysates were obtained at different time periods 3 and 6 hours of neuronal cultures treated with or without NMDA (200 μM). These lysates were analyzed by immunoblots and probed with the anti-beclin-1 antibody (n=3). B) Quantification of the autophagy protein beclin-1 bands in the immunoblots was plotted. The band intensities were normalized against the loading control. Significant increases in the band intensities of the beclin-1 levels were observed after NMDA-treated neuronal cultures as compared to controls. The expressed values are means ± S.E.M. (n=3; *p<0.05). GAPDH was used as a loading control.
Figure 4-4. Autophagy inhibitor 3-MA suppresses LC3-II formation. A) Lysates were obtained at 12 and 24 hours from neuronal cultures treated with or without NMDA (200 μM) and a combination of NMDA+3-MA (200 μM+10 mM). These lysates subjected to immunoblotting were probed with anti-LC3 antibody. Representative immunoblot demonstrates a reduction in the LC3-II band in the neurons treated with 3-methyladenine (3-MA) (n=3). β-actin was used as a loading control. B) LC-3 immunoblot band intensities were quantified and plotted. A significant reduction in the LC3-II band was observed in NMDA/3-MA co-treated neuronal cultures compared to cultures treated with NMDA alone. The expressed values are means ± S.E.M. (n=3; *p<0.05 compared to NMDA).
Figure 4-5. NMDA-induced autophagosome formation is inhibited by 3-MA. Representative fluorescence micrographs A) show an increase in anti-LC3 immunostaining (red) localizes around DAPI (blue; nuclear stain) following NMDA (200 μM) exposure in cerebellar neurons compared to controls and NMDA+3-MA co-treated cultures. Arrows and arrow heads (yellow) indicate the punctate staining in the cell bodies and axons/neurites, respectively; in neurons following NMDA exposure for 8 and 12 h. Images are taken at 400X magnification. Scale bar represents 10 μm. B) Monodansylcadaverine (MDC; 0.05 mM) labeling of autophagosomes in neurons increase following acute exposure to NMDA (200 μM) compared to control conditions and co-treatment of NMDA and 3-MA. Arrows (red) indicate the accumulation of the autophagosomes in neuronal cell bodies at 12 and 16 hours following NMDA treatment while arrow heads (yellow) and arrows (yellow) indicate the presence of punctate autophagosomes in the existent neurites and cell bodies respectively. The arrows (red) in the image at 16 hours are indicative of the accumulation of the autophagosomes in the cell bodies of neurons following prolonged NMDA exposure. Images are taken at 400X magnification. Scale bar represents 20 μm.
Figure 4-6. Autophagy inhibitor 3-MA protects neurons against NMDA excitotoxicity. A) Representative phase contrast images indicates the changes in the morphology of neurons following treatments with NMDA (200 μM), NMDA (200 μM) + 3-MA (10 mM) and controls. Arrows (yellow) indicate surviving cell body morphology from apoptotic cell bodies (red arrows). Arrow heads (yellow) indicate healthy neurites while red arrow heads indicate degenerating neurites. Images are taken at 400X magnification. Scale bar represents 20 μm. B) LDH release recorded and plotted after incubating cerebellar neurons in culture in NMDA (200 μM) (◊), NMDA+3-MA (10 mM) (■) and controls (○). The expressed values are means ± S.E.M. (n=6; *p<0.05 NMDA vs. controls and #p<0.05 NMDA vs. NMDA+3MA).
Figure 4-7. NMDA-induced caspase-3 activation is suppressed by 3-MA. A) Representative immunoblot of αII-spectrin breakdown profile shows the presence of the caspase-3 specific spectrin breakdown product (SBDP) of 120 kDa in NMDA-treated cultures after 24 hours compared to the controls and NMDA+3-MA (n=3). Representative immunoblot probed with anti-SBDP120 shows a similar profile of the breakdown product in NMDA-treated cultures after 24 hours following treatment but not in controls or NMDA+3-MA co-treatment (n=3). B) Densitometric analyses of the immunoblots probed with anti-SBDP120 show a significant increase in the spectrin breakdown product of 120 kDa (SBDP120) after 24 hours following NMDA treatment in cerebellar neuronal cultures but not in controls or NMDA+3-MA co-treated cultures. The expressed values are means ± S.E.M. (n=3; *p<0.05). C) Caspase-3 enzymatic assay was determined using the caspase substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) incubated with protease-inhibitor free lysates obtained from cultures treated with or without NMDA and NMDA+3-MA co-treatment. An increase in the caspase-3 enzymatic activity was observed in NMDA-treated as opposed to controls or NMDA+3-MA co-treated cultures. The expressed values are means ± S.E.M. (n=3; *p<0.05 compared to control-treated; #p<0.05 compared to NMDA-treated).
Figure 4-8. Protein nitration in cerebellar granule neurons following NMDA-treatment is alleviated by 3-MA. A) Representative blot probed with anti-nitro-tyrosine, shows the presence of strong nitrated proteins of approximately 70 kDa size under NMDA-treated conditions at 24 hours post-treatment (n=3). Lysates obtained from cultures incubated in SFM at 3 and 24 hours were employed as controls. Densitometric quantifications of the nitrated protein (70 kDa) were plotted on a time scale under NMDA exposure conditions at 3, 6, 12 and 24 hours. Control values obtained at 3 and 24 hours were plotted. Values were plotted as the means ± S.E.M. (n=3, *p<0.05 vs. controls). B) Quantification of the nitrated protein levels among lysates treated with NMDA showed a significant increase in the values compared to NMDA+3-MA co-treated cultures at 24 hours. The values are expressed as means ± S.E.M. (n=3, *p<0.05 vs. controls; #p<0.05 vs. NMDA-treated). (Inset) Top panel: Autophagy inhibitor 3-MA treatment reduces oxidative damage in peroxynitrite treated cerebellar granule neuronal cultures. Quantification of the intensity values of the nitrated protein at 70 kDa in the Peroxynitrite (0.5 mM, OONO-) +3-MA co-treatment was significantly reduced compared to peroxynitrite (OONO-) treatment alone. The values are represented as means ± S.E.M. (n=3, *p<0.05). Bottom panel: Lactate dehydrogenase (LDH) release was assayed following treatment with a combination of peroxynitrite (0.5 mM, OONO-) and 3-MA (10 mM) (▲) and peroxynitrite -treatment (△) alone. The values are plotted as means±S.E.M. (n=6, *p<0.05).
Figure 4-9. Schematic representation of the involvement of autophagy and autophagic cell death in neurons following excitotoxic NMDA challenge.
CHAPTER 5
CHANGES IN AUTOPHAGY PROTEINS IN A RAT MODEL OF CONTROLLED CORTICAL IMPACT BRAIN INJURY

Introduction

Autophagy is an intracellular phenomenon that has been documented to sustain cell survival under conditions of stress, by lysosomal breakdown of the cytosolic organelles and proteins and recycling the amino acids into the cell machinery. It is characterized by the presence of double membrane cytoplasmic vesicles called the autophagosomes which sequester the cytosolic components before fusing with the lysosomes where the lysosomal hydrolases breakdown the sequestered organelles. A number of proteins that intricately regulate autophagy have been reported (Klionsky et al., 2003) Some of them include beclin-1 (Atg6), Atg4, MAP-LC3 (Atg8), Atg7, Atg12 and 5. Recently there has been tremendous interest in identifying the role of autophagy in the central nervous system. Autophagy has been shown to play an integral role in the neurodevelopment process (Hara et al., 2006; Komatsu et al., 2006). Other studies have delved into the potential neuroprotective roles of autophagy in clearing protein aggregates in neurons in animal models expressing polyglutamine phenotype disorders (Floto et al., 2007; Sarkar et al., 2007a; King et al., 2008). Recent studies have also studied the alterations in the autophagy protein expression proteins and involvement of autophagy following brain trauma (Zhu et al., 2003; Diskin et al., 2005; He et al., 2007; Koike et al., 2008). In this study, we demonstrate that the autophagy protein MAP-LC3-II is upregulated following controlled cortical impact, a more severe form of brain injury and these increases are in the cortical regions in close proximity to the injury. Also, we develop a correlation between the levels of the autophagy protein beclin-1 and the anti-apoptotic protein bel-2 at various time periods following the cortical injury in rats.
Results

Autophagy Induction Increases After Brain Injury in the Cortex

Autophagy induction has been studied using the expression levels of the autophagy protein MAP-LC3. To demonstrate the increases in autophagy induction after cortical impact injury we probed the cortical lysates obtained from the ipsilateral side of the injury with anti-MAP-LC3. We demonstrated increases in the conversion of the LC3-I to LC3-II as opposed to sham-injured or naïve animals (Fig. 5-1A). Quantification of the densities of the protein bands revealed a significant increase \((p \leq 0.05)\) in the levels of LC3-II protein as compared to LC3-I in traumatized animals as compared to sham-injured or naïve animals (Fig. 5-1B).

Beclin-1 Levels are Increased Following Brain Injury

Beclin-1 (Atg6) is an autophagy protein shown to be involved in the regulation of autophagy. To determine if it was affected in acute brain trauma, cortical lysates from the ipsilateral side of the injury were probed with anti-beclin-1. Also, effects of the brain trauma on the protein levels of anti-apoptotic protein, bcl-2 was studied. We demonstrated that beclin-1 levels are increased in a time-dependent manner. The levels start to go down 1d and 2d post-injury. The anti-apoptotic protein bcl-2 levels also seem to fluctuate in the cortex region close to the injury. As observed with the beclin-1 levels, the levels of bcl-2 drop towards 1d and 2d post-injury (Fig. 5-2).

Immunoblot Detection of Beclin-1/Bcl-2 Ratio After Brain Injury

The ratio of beclin-1 to bcl-2 levels has been demonstrated to be significant in the regulation of autophagy. Densitometric quantification of the band intensities of the beclin-1 and bcl-2 do not show any significant differences when plotted individually (Fig. 5-3A,B). The mean ratios of the band intensities of beclin-1/bcl-2 demonstrate a significant increase at days 1 and 2.
(p≤0.05) following traumatic brain injury (TBI). This increase was significantly higher than the ones observed in sham-injured animals (Fig. 5-3C).

Discussion

Autophagy has been shown to be neuroprotective in neurodegenerative diseases such as Parkinson’s disease, Huntington’s disease and Alzheimer’s disease (Webb et al., 2003; Nixon, 2007; Sarkar et al., 2008). There have also been reports about the relevance of autophagy in traumatic brain injury events such as closed head trauma and ischemic stroke (Diskin et al., 2005; Adhami et al., 2006). Here we corroborate the findings that autophagy is indeed induced after brain trauma, in an acute rat brain trauma model of controlled cortical impact. Our model of brain trauma is a more acute form of the injury and hence an important finding with clinical relevance in severe forms of brain contusions and other penetrating brain injuries. Through our studies we demonstrated increases in the processing of autophagy protein MAP-LC3 and also an increase in the ratio of beclin-1/bcl-2 following the brain trauma.

MAP-LC3 has been documented to be one of the most reliable markers to study autophagy induction (Mizushima, 2004; Mizushima et al., 2004). Increase in the processing of the LC3-I protein form to LC3-II has been documented to be one of the hallmarks of autophagy induction. In our results we demonstrated a similar increased processing of the pro-form of MAP-LC3 protein into the LC3-II form which is known to be associated with the autophagosomal membrane in the traumatized animals as compared to sham-injured animals (Fig. 5-1). Other proteins of interest were the autophagy protein beclin-1 (Atg6) and its interacting anti-apoptotic protein bcl-2.

Beclin-1 is a bcl-2 interacting protein that has been documented to be an important player in the induction of autophagy. Bcl-2, the anti-apoptotic protein has been shown to interact with beclin-1 via the BH-3 domain on beclin-1. Bcl-2 exerts a controlling effect on the activity of
beclin-1. Under normal homeostatic conditions, beclin-1 is bound to bcl-2 and hence not available to induce autophagy. Following a disruption in the cell homeostasis due to stress conditions or insults, beclin-1 interaction with bcl-2 is weakened and beclin-1 now becomes available to induce autophagy. This interaction between these proteins is of utmost significance as it governs the switch between inducing autophagy or apoptosis (Pattingre et al., 2005; Pattingre and Levine, 2006; Feng et al., 2007; Maiuri et al., 2007a; Maiuri et al., 2007b). Here we demonstrated in our experiments that though beclin-1 was qualitatively increased in traumatized animals compared to sham-injured animals, there was however no statistical significance. A similar observation was reported with the levels of bcl-2 in the traumatized and sham-injured animals (Fig. 5-2). However the ratio of beclin-1/bcl-2 showed a statistically significant increase in the TBI animals compared to sham animals at days 1 and 2 post-injury. The speculation is that the overexpression of beclin-1 protein at the site of injury can enhance autophagy induction as a mechanism to discard injured cells and reduce the extent of damage to cells from the injured components (Erlich et al., 2006).

Also, a decline in the levels of beclin-1 and bcl-2 was observed at later time points in the study (Fig. 5-3). This possibly might be because of the loss of neurons in the brain following the injury which coincided with the reduced protein levels. Also, the reduction in the bcl-2 levels noticed might suggest a transition in the cell death pathways from oncotic to apoptotic as well as reduced amounts of protein to interact with beclin-1. Thus, the free beclin-1 is now available to induce autophagy. Recently, autophagy-assosiated neuronal death has also been studied in neuronal injury models. Uchiyama and colleagues demonstrated that hypoxia/ischemia brain injury in the neonatal brain results in energy failure, oxidative stress and unbalanced ion fluxes inducing elevated levels of autophagy in the brain neurons (Uchiyama et al., 2008). Due to
commonalities between the pathologies in the two different brain injury etiology, comprehensive studies need to be conducted to elucidate the role of autophagy and autophagic cell death in the acute forms of brain trauma.

**Conclusion**

Our studies thus indicate autophagy induction in a rat model of controlled cortical impact brain injury by demonstrating increases in the levels of autophagy protein MAP-LC3-II and ratio of beclin-1/bcl-2.
Figure 5-1. Increased levels of MAP-LC3-II are observed following controlled cortical impact. A) Representative immunoblot of cortical lysates probed with anti-MAP-LC3 (n=5). GAPDH has been used as control for even protein loading. B) Densitometric quantification of the LC3-II bands denote a significant increase in MAP-LC3-II levels in the traumatized animals compared to sham animals (p≤0.05)
Figure 5-2. Beclin-1 levels increase following cortical injury. Representative immunoblots with cortical lysates probed with anti-beclin-1 and anti-bcl-2 (n=5). GAPDH is used as control for protein loading.
Figure 5-3. Increases in the ratio of beclin-1/bcl-2 indicate autophagy induction. A, B & C) Densitometric quantification of the bands presented in the immunoblots indicates a significant increase in the ratio of beclin-1/bcl-2 representing autophagy sustainence n traumatized animals compared to sham-injured animals (n=5; p≤0.05).
CHAPTER 6
CONCLUSION

Summary and Scientific Applications

Our main working hypothesis for this project was to elucidate the role of autophagy in a rat model of experimental controlled cortical impact (CCI). Results from our studies and also studies emerging from other labs have demonstrated the importance of autophagy in regulating the neuronal homeostasis in the central nervous system. Autophagy has been documented to play a critical role in cell survival following conditions of cell stress ranging from nutrient deprivation to cell injury. Though its beneficial effects in cell maintenance have been well documented, evidence for it contributing to a novel form of cell death ‘autophagic cell death’ (type II) is emerging. The popular belief is that autophagy is beneficial when it is induced initially following cell stress, but when prolonged left unchecked can result in the sequestration and in turn removal of cellular organelles that might be important for cell survival as discussed in our literature review in Chapter 1.

In our studies, we used a combination of biochemical protein assays and immunofluorescence techniques to study autophagy and autophagic cell death. Chapter 3 describes our work in establishing the working tools for studying autophagy in a neuronal cell line, PC-12 cells. Amino acid starvation induced autophagy in PC-12 cells and this involved the activation of cysteine proteases caspase-3 but not calpain-1. Calpains have been shown to cleave the autophagy protein Atg5 that suppressed autophagy and activated the apoptotic cell death pathway. Cell death observed in these cells following prolonged nutrient starvation was accompanied with the presence of monodansylcadaverine (MDC) and LC3 positive autophagosomes. Though apoptotic markers were observed, autophagy inhibitor 3-methyladenine (3-MA) was able to rescue cell death. This led us to believe that though beneficial
initially, autophagy when prolonged can result in a form of cell death that shows the presence of autophagosomes as well as is biochemically similar yet morphologically different to apoptosis.

We further extended our findings in PC-12 cells to a neuronal cell culture paradigm of excitotoxicity in the CNS. We harvested rat cerebellum and cultured granule neurons that were subsequently exposed to the excitotoxin NMDA as described in Chapter 4. We showed that NMDA exposure induced autophagy as early as 3 hours, detected by immunostaining with MAP-LC3 and also the presence of MDC-positive autophagosomes and beclin-1 immunoreaction. We demonstrated that cell death resulting from excitotoxic injury due to NMDA exposure increased at 1 day. This cell death was accompanied by morphological changes in the cell bodies and axons of the neurons and an increase in the presence of MDC-positive autophagosomes. As observed with the PC-12 cells, caspase-3 activity was increased following NMDA exposure. As documented previously, NMDA exposure also induces oxidative stress. According to a recent report, oxidative stress positively reinforces the induction of autophagy (Scherz-Shouval et al., 2006). We observed that 3-methyladenine, a pharmacological inhibitor of autophagy not only inhibited autophagy (evident by the loss of the LC3-II band), but also was efficient in alleviating the cell damage due to oxidative stress and the resulting possible autophagic cell death. Thus, we hypothesized that excitotoxic stress induced autophagy and oxidative stress which when prolonged resulted in 3-MA sensitive autophagic cell death.

We further explored the changes in autophagy proteins in our experimental model of controlled cortical impact (CCI) which is an animal model of severe traumatic brain injury (TBI). We screened for the presence of autophagy proteins MAP-LC3 (Atg8) and beclin-1 (Atg6). Following TBI, autophagy was induced close to the site of the injury in the cortex, evident by the increased levels of autophagy-related proteins. Also, an increase in the beclin-
1/bcl-2 ratio suggested the induction of autophagy after the injury (Pattingre et al., 2006). A similar induction of autophagy could not be observed in the underlying hippocampus, suggesting that autophagy might play a more critical role around the site of the injury possibly by clearing the cell debris and arresting the probable factors that might lead to a secondary wave of biochemical injury. In a recent study, rapamycin (autophagy inducer) was reported to be neuroprotective in an animal model of traumatic brain injury when injected at 4 hours following injury by the inhibition of the mTOR signaling (Erlich et al., 2007). As we hypothesized, autophagy definitely plays a role in neurotrauma, though further studies have to be conducted to further elucidate whether its neuroprotective or culminates in autophagic cell death.

**Future Directions**

Our work coupled with others has demonstrated a potential role for autophagy after brain injury. Proteomic studies from our lab has further reinforced this thought, as the autophagy protein Atg8 was shown to be upregulated following experimental TBI. The observation that autophagy is oxidative stress further opens the door to investigate the role of antioxidants as therapy options to either induce autophagy or arrest it. A temporal profile demonstrating the involvement of autophagy in experimental brain injury paradigm would help understand the dual roles of autophagy involving cell survival or autophagic cell death.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Shankar Sadasivan was born and raised in the business capital of India, Mumbai. He completed his high school education in 1995 and embarked on a career in science when he joined the Prin. K.M.Kundnani College of Pharmacy, Worli, Mumbai, to earn a B.Pharm. degree in Pharmaceutical Sciences. After completing his studies in pharmaceutical sciences, he became a registered pharmacist in the state of Maharashtra, India. He worked for a year in the Research and Development department as a scientist at Zandu Pharmaceuticals, Mumbai. He came to the U.S.A. in the year 2000 as a M.S. candidate in the field of pharmacology. He acquired his Master of Science diploma in 2003 from the Massachusetts College of Pharmacy and Health Sciences, Boston, MA. His research work focused on the investigating the therapeutic potential of precursors to the club drug gamma hydroxybutyric acid (GHB), gamma butyrolactone (GBL) and 1,4-butanediol (BD) in a cerebral ischemic model of transient and permanent middle cerebral artery occlusion (MCAO) in rats.

He came to the University of Florida in the year 2003, admitted as a doctor of philosophy student to the Interdisciplinary Program (IDP) in the college of Medicine. During his years in the program, he has published several research papers in peer-reviewed journals and was awarded the Bryan W. Robinson Neurological Foundation Grant-in-Aid Achievement Award in 2005. He was also nominated and awarded the Outstanding International Student Award for Research by College of Medicine in 2007. He joined the Departments of Neuroscience and Psychiatry, where he completed his doctoral degree under the mentorship of Dr. Kevin K Wang, in the fields of autophagy and neurotrauma as part of the Evelyn F. and William L. McKnight Brain Institute.