Activation of NR2B and Autophagy Signaling Pathways Following Traumatic Brain Injury

Gregory E. Bigford
University of Miami, gbigford@med.miami.edu

Follow this and additional works at: https://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
https://scholarlyrepository.miami.edu/oa_dissertations/204

This Open access is brought to you for free and open access by the Electronic Theses and Dissertations at Scholarly Repository. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of Scholarly Repository. For more information, please contact repository.library@miami.edu.
UNIVERSITY OF MIAMI

ACTIVATION OF NR2B AND AUTOPHAGY SIGNALING PATHWAYS FOLLOWING TRAUMATIC BRAIN INJURY

By

Gregory E. Bigford

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

May 2009
ACTIVATION OF NR2B AND AUTOPHAGY SIGNALING PATHWAYS FOLLOWING TRAUMATIC BRAIN INJURY

Gregory E. Bigford

Approved:

Nirupa Chaudhari, Ph.D.  Terri A. Scandura, Ph.D.
Professor  Dean of the Graduate School
Physiology and Biophysics

Ellen Barrett, Ph.D.  W. Dalton Dietrich, Ph.D.
Professor  Professor
Physiology and Biophysics  Neurological Surgery

Robert W. Keane, Ph.D.
Professor
Physiology and Biophysics
Hyper-activation of N-methyl-D-aspartate receptors (NRs) is associated with excitotoxic cell death during secondary injury following traumatic brain injury (TBI). The efficiency of the NR is dependent on the location of receptors in membrane raft microdomains that provide a platform for coupling of NRs and effector proteins. In many neurodegenerative diseases, activation of the autophagy pathway has been suggested to contribute to glutamate excitotoxicity, but whether increased autophagy signaling contributes to pathology after TBI has not been defined. In these studies, I investigate whether membrane rafts mediate NR signaling and autophagy in cortices of adult male rats subjected to moderate TBI and in sham-operated controls. These studies demonstrate that membrane rafts of the normal rat cortex contain a novel multi-protein signaling complex that links the NR2B glutamate receptor and the autophagic protein Beclin 1. TBI caused a rapid disruption of this complex in which NR2B and pCaMKII were recruited to membrane microdomains. Alteration in NR2B-Beclin 1 association in membrane rafts resulted in activation of autophagy as demonstrated by increased expression of key autophagic proteins Beclin 1, ATG 5 and ATG 7, and significant increases in autophagic vacuoles in neurons of traumatized brains. Administration of the NR2B antagonist RO 25-6981
significantly blocked TBI-induced redistribution of NR2B signaling intermediates and Beclin 1 and delayed the increase in autophagy protein expression in traumatized cortices. Thus, stimulation of autophagy by NR2B signaling may be regulated by redistribution of Beclin 1 in membrane rafts after TBI.
DEDICATION

First and foremost to my parents, Felicia and Lennox, whose continual love, dedication, support, encouragement and freedom, have allowed me all the experiences in the world, and taught me the humility to touch others in my life the way they have touched me. Everything good that is in me, I owe to them.

To my brother Francis, whose life reminds me everyday to cherish what is in my mind, body, soul, spirit and my heart.

To God.
ACKNOWLEDGMENTS

To my mentor, Robert W. Keane, Ph.D., for educating me as a scientist, respecting me as an individual, and continually supporting me as a friend.

To my committee: Dr. Ellen Barrett, Dr. Nirupa Chaudhari, and Dr. W. Dalton Dietrich, whose continual commitment, support, and insight encouraged me to excel and challenged me to put forth my best.

To the members of the Keane Lab: Angela Davis, Ph.D. and Pablo Vaccari, Ph.D., for creating an enjoyable working environment.

To the members of the Bramlett/Dietrich Lab, who were always willing to contribute to my education and training as a scientist.

To Gianluca Del Rossi, Ph.D., for your encouragement as a supervisor, support, and most of all, for your friendship.

To Bradley Macintosh, Ph.D. and Tannin Schmidt, Ph.D., who have continually inspired me and whose friendships have continually uplifted me.

To Lascelles Kirby, RN, for giving me a brother and a family, and all of the comforts of home.

To Willie Buchser, Ph.D., Patrick Flynn, Ph.D., John Maher, Ph.D., and George Theodor, Ph.D. ‘For Life!’
## TABLE OF CONTENTS

### Chapter I

GENERAL INTRODUCTION and statement of the problem  

<table>
<thead>
<tr>
<th>I. PATHOPHYSIOLOGY OF TRAUMATIC BRAIN INJURY</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Overview of primary and secondary traumatic brain injury</td>
<td>1</td>
</tr>
<tr>
<td>B. Traumatic brain injury in the clinical setting</td>
<td>10</td>
</tr>
<tr>
<td>C. Animal models of traumatic brain injury</td>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. CELL DEATH MECHANISMS FOLLOWING TBI</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Glutamate Excitotoxicity</td>
<td></td>
</tr>
<tr>
<td>B. Autophagy as a programmed cellular response following TBI</td>
<td>22</td>
</tr>
</tbody>
</table>

| III. NMDA RECEPTOR SIGNALING AND MEMBRANE RAFTS | 30 |

### Chapter II (published manuscript)

ACTIVATION OF NR2B AND AUTOPHAGY SIGNALING PATHWAYS FOLLOWING TRAUMATIC BRAIN INJURY  

| Summary | 33 |
| Overview | 34 |
| Materials and Methods | 36 |
| Results | 43 |
| Consideration | 62 |

### Chapter III

GENERAL DISCUSSION  

| I. NR2B RECEPTOR AND TBI PATHOLOGY | 70 |
| II. NR2B-MEDIATED ACTIVATION OF AUTOPHAGY FOLLOWING TBI | 73 |
| III. MEMBRANE RAFTS IN NR2B AND AUTOPHAGIC SIGNALING | 75 |
| IV. AUTOPHAGY AND TBI PATHOLOGY | 76 |
| V. APPLICATION OF RO 25-6981 AS A TREATMENT FOR TBI | 78 |
Chapter IV

I. MODEL OF NR2B MULTI-PROTEIN SIGNALING COMPLEX AND AUTOPHAGIC ACTIVATION AFTER TBI 82

II. SUMMARY AND FUTURE DIRECTIONS 83

APPENDIX 86

REFERENCES 87
GLOSSARY OF ABBREVIATIONS

4E-BP1 – 4E binding protein 1

AD – Alzheimer’s Disease

AMPA - α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid

ANOVA – Analysis of variance

AP – Autophagosome

APP – Amyloid precursor protein

APV – 2R-amino-5-phosphonovaleric acid

ATG – Autophagy gene related protein

ATP – Adenosine triphosphate

AV – Autophagic vacuole

AVd – Degradative autophagic vacuole

AVi – Initial autophagic vacuole

BBB – Blood brain barrier

CaMKII/IV – Calcium/calmodulin kinase II/IV

CCI – Closed cortical impact

CGS – cis-4-phosphonomethyl-2-piperidine-carboxylic acid

CNS – Central nervous system

CPP – (3-[(R)-2-carboxypiperazin-4-yl]-prop-2enyl-1-phosphonic acid)

CR – Contralateral raft

CT – Computed tomography

DNA – Diriboneucleic acid

EAAC1 – Excitatory amino acid carrier 1
EAAT4 – Excitatory amino acid transporter 4
EAAT5 – Excitatory amino acid transporter 5
E/A – Epon-Araldite
EM – Electron microscopy
FADD – Fas-associated protein with death domain
GCO – Glasgow coma scale
GLAST – Glutamate-aspartate transporter
GLT1 – Glutamate transporter 1
GOS – Glasgow outcome scale
HD – Huntington disease
HRP – Horseradish peroxidase
ICAM-1 – Intercellular cell adhesion molecule 1
ICP – Intracranial pressure
IL-1 – Interleukin 1
IL-6 – Interleukin 6
LC3 – Light chain 3
MAP2 – Microtubule associate protein
mTOR – Mammalian target of rapimycin
MRI – Magnetic resonance imaging
mRNA – Messenger ribonucleic acid
NeuN – Neuronal specific nuclear protein
NMDA – N-methyl-D-Aspartate
nPIST – Novel isoform of a PDZ-domain containing protein
NR – NMDA receptor
NR1 – NMDA receptor 1 subunit
NR2A – NMDA receptor 2A subunit
NR2B – NMDA receptor 2B subunit
p70 S6K – p70 ribosomal protein S6 kinase
PBS – Phosphate buffered saline
PD – Parkinson’s disease
PDZ – PSD/Drosophila disc large tumor suppressor/Zonula occludens 1
PE – Phosphatidyl ethanalamine
PI3K – Phosphoinositide 3-kinase
PK (A/B/C) – Phospho-kinase A/B/C
PMSF – Phenylmethysulfonyl fluoride
PS1 – Presenilin 1
PSD – Post-synaptic density
PSD 95 – Post-synaptic density 95 protein
PTN – Phosphate Triton X-100 sodium chloride
PVDF – Polyvinylidene fluoride
ROS – Reactive oxygen species
SCI – Spinal cord injury
SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNpc – Substantia Nigra pars compacta
TGFβ - Transforming growth factor beta
TGN – Trans-Golgi-network
TI – Triton X-100 insoluble

TNFα - Tumor necrosis factor alpha

TNFR1 – Tumor necrosis factor receptor 1

TS – Triton X-100 soluble

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling

UVRAG – UV irradiation resistant associated gene

VCAM1 – Vascular cell adhesion molecule 1
Chapter I

GENERAL INTRODUCTION and statement of the problem

Traumatic brain injury (TBI) represent the leading cause of death and permanent disability among children and adolescents in the United States, afflicting approximately 1.4 million individuals at a cost of over $60 billion dollars annually (Thurman et al., 1999; Thurman 2001; Finkelstein et al., 2006; National Center for Injury Prevention and Control, 2008). The main cause of TBI in the United States is due to falls in senescence, motor vehicle crashes, struck by/against events, and assault. The prevalence is significantly greater in men than in women (Maas et al., 2008). TBI is a burgeoning socioeconomic problem worldwide and affects both high income and low/middle income countries (Ghajar, 2000; Maas et al., 2008). There are a multitude of classifications of TBI based on pathophysiological changes, both focal and diffuse in nature (Bramlett and Dietrich, 2004). As such, there is no accepted therapeutic treatment proven to confer protection against the damaging effects of TBI. Thus, the development of therapeutic strategies for the treatment of TBI may prove beneficial in contributing to improved functional outcome.

I. PATHOPHYSIOLOGY OF TRAUMATIC BRAIN INJURY

A. Overview of Primary and Secondary Traumatic Brain Injury

Central nervous system (CNS) injury results in pathophysiological changes that extend from minutes to years after the initial insult (Bethea and Dietrich, 2002; Bramlett and Dietrich, 2004). The functional deficits that result after TBI have been attributed to a multitude of pathological processes including
excessive neuro-excitation (Personn and Hillered, 1992; Reeves et al., 1997),
target-cell deafferentation (Povlishock and Christman, 1995) and cell death
(Yakovlev et al., 1997; Conti et al., 1998; Newcomb et al., 1999; Clarke et al.,
2000; Keane et al., 2001). The primary insult initiates a wide range of secondary
injury mechanisms that contribute to the pathogenesis of TBI (Graham, 1996;
Gennarelli and Graham, 1998).

TBI has been characterized as an acquired injury resulting from
mechanical impact, acceleration/deceleration, penetrating or blast injury
(Bramlett and Dietrich, 2004; Maas et al., 2008). Although TBI is a
heterogeneous injury, which may be classified in terms of mechanism and
severity (Maas et al., 2008), the general pathology can be classified in terms of
primary and secondary injury mechanisms. The primary phase is a result of the
direct, immediate mechanical disruption of brain tissue (Morales et al., 2005),
which may result in both focal and diffuse injury.

The mechanical deformation/compression of cortical tissue, results in focal
contusions, including those occurring directly beneath fractures, coup contusions
beneath the site of impact and countercoup contusions in distant and opposing
regions to the site of impact (Gennarelli and Graham, 1998; Gaetz, 2004). Focal
injury also includes epi- and sub-dural hemorrhage and hematoma (Adams,
1992; Graham, 1996), caused by laceration of dural, pial and cortical blood
vessels. Additionally, hemodynamic changes of focal reductions in local cerebral
blood flow (DeWitt et al., 1986; Dietrich et al., 1998) result in immediate cellular
changes that cause plasma membrane rupturing and failure of ATP driven ion channels (Maas et al., 2008) leading to necrotic cell death (Dietrich et al., 1994a). The shearing forces and tearing of white matter tracts and blood vessels (Adams et al., 1977, 1991; Gennarelli, 1993; Bramlett and Dietrich, 2004; Maas et al., 2008) caused by the initial impact result in diffuse brain injury (Adams et al., 1982b; Christman et al., 1994; Gentleman et al., 1995; Povlishock, 1992) that is characterized by diffuse axonal injury, vascular injury, hypoxic-ischemic injury and brain swelling (Adams, 1992; Graham, 1996; Graham and Genarelli, 1997). Moreover, primary axotomy caused by the impact lead to impaired axonal transport, axonal swelling and neuronal disconnection (Maxwell et al., 1997; Kelley et al., 2006). Pathological changes in the axolemma occur rapidly; as early as 5 minutes following TBI characterized by neurofilament compaction and mitochondrial abnormalities (Maxwell et al., 1988; Pettus et al., 1994).

Additionally, the primary insult initiates a wide range of secondary injury mechanisms that exacerbate and contribute to the pathogenesis of the injury (Graham, 1996; Gennarelli and Graham, 1998). A host of physiological changes including hypoxia/ischemia due to reduction in cerebral blood flow (Yamakami and McIntosh, 1989), swelling and edema (Ishige et al., 1987; Soares et al., 1992), axonal damage and deafferentation (Povlishock, 1992; Povlishock et al., 1992; Maxwell et al., 1997), impaired blood-brain barrier and energy metabolism (Povlishock et al., 1978; Dietrich et al., 1994b) and microvascular alterations (Dietrich et al., 1994a) mediate complex and poorly understood cascades of
molecular, cellular and biochemical events that may persist for days, weeks and months (Table 1).

<table>
<thead>
<tr>
<th><strong>Events Post TBI</strong></th>
<th><strong>Time After TBI</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Release of excitatory amino acids</td>
<td>Minutes to months</td>
<td>Choi, 1987&lt;br&gt;Katayama et al., 1990&lt;br&gt;Globus et al., 1995&lt;br&gt;Myseros et al., 1995&lt;br&gt;Zornow et al., 1995&lt;br&gt;Zauner et al., 1996&lt;br&gt;Obrenovitch and Urenjak, 1997&lt;br&gt;Bullock et al., 1998&lt;br&gt;Koura et al., 1998</td>
</tr>
<tr>
<td>Decreased local cerebral blood flow</td>
<td>30 minutes</td>
<td>DeWitt et al., 1986&lt;br&gt;Muir et al., 1992&lt;br&gt;Kochanek et al., 1995&lt;br&gt;Dietrich et al., 1996&lt;br&gt;Ginsberg et al., 1997&lt;br&gt;Dietrich et al., 1998&lt;br&gt;Jiang et al., 2000</td>
</tr>
<tr>
<td>Increased reactive oxygen species</td>
<td>30 minutes</td>
<td>Povlishock and Kontos, 1992&lt;br&gt;Bondy and LeBel, 1993&lt;br&gt;Hall, 1993&lt;br&gt;Hall and Braughler, 1993&lt;br&gt;Lewen and Hillered, 1998&lt;br&gt;Marklund et al., 2001a&lt;br&gt;Marklund et al., 2001b</td>
</tr>
<tr>
<td>Activation of microglia and astrocytes</td>
<td>Minutes to hours</td>
<td>Condorelli et al., 1990&lt;br&gt;Norton et al., 1992&lt;br&gt;Smith et al., 1995&lt;br&gt;Carbonell et al., 1999&lt;br&gt;Hill-Felberg et al., 1999&lt;br&gt;Maas et al., 2008</td>
</tr>
<tr>
<td>Increase in pro-inflammatory cytokines IL-1, IL-6, TNFα, TGFβ</td>
<td>Minutes to hours</td>
<td>Raghupathi et al., 1995&lt;br&gt;Fan et al., 1996&lt;br&gt;Allan and Rothwell, 2001&lt;br&gt;Kinoshita et al., 2002&lt;br&gt;Zhu et al., 2004</td>
</tr>
<tr>
<td>Activation and processing of caspase -3, -8, -9</td>
<td>1 hour (Peak 6 hours)</td>
<td>Yakovlev et al., 1997&lt;br&gt;Pike et al., 1998&lt;br&gt;Keane et al., 2001&lt;br&gt;Maas et al., 2008</td>
</tr>
<tr>
<td>Cytotoxic/vasogenic</td>
<td>Hours to weeks (may last)</td>
<td>Van Der Brink et al.,</td>
</tr>
</tbody>
</table>
Table 1. Time course of events contributing to secondary injury following TBI.

<table>
<thead>
<tr>
<th>Event</th>
<th>Time Course</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>edema and vascular engorgement</td>
<td>for months</td>
<td>1990 McIntosh et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unterberg et al., 2004</td>
</tr>
<tr>
<td>Glial scar surrounds cavitation</td>
<td>Hours to weeks</td>
<td>Cortez et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovolenta et al., 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fawcett and Asher, 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fitch et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fitch and Silver, 2008</td>
</tr>
<tr>
<td>Increases expression of Autophagic proteins</td>
<td>Hours to days</td>
<td>Diskin et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clarke et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liu et al., 2008</td>
</tr>
<tr>
<td>Wallerian degeneration</td>
<td>Delayed (incomplete), months</td>
<td>Bramlett and Dietrich, 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kelley et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vargas and Barres, 2007</td>
</tr>
</tbody>
</table>

Some of these molecular and cellular changes initiate inflammatory responses that greatly exacerbate long-term and widespread cellular damage leading to programmed cell death (Bramlett and Dietrich, 2004; Maas et al., 2008). For example, activation of microglia and astrocytes has been observed within minutes to hours after TBI (Maas et al., 2008). Moreover, injured tissue is infiltrated by macrophages, T-cell lymphocytes and leucocytes that adhere to injured tissue via upregulation of various cell adhesion molecules such as P-selectin, intercellular cell adhesion molecule (ICAM-1), and vascular adhesion molecule (VCAM-1) (Werner and Engelhard, 2007). This inflammatory response is followed by extravasation, release of pro-inflammatory cytokines, chemokines and bioactive lipids (Bazan, 2005 Bazan et al., 2005) resulting in the removal of damaged tissue. Up-regulation of pro-inflammatory enzymes tumor necrosis factor (TNF), interleukin-1β (IL-1β) and interleukin-6β (IL-6β) initiates the immune response and may contribute to cytotoxicity and apoptosis at the site of injury (Renno et al., 1995; Werner and Engelhard, 2007).
Another important component contributing to pathogenesis of TBI is the formation of the glial scar. Within hours after TBI and continuing for weeks, astrocytes produce microfilaments and neutropines, which are integral components of the forming scar tissue (Fabricius et al., 2006). The glial scar also consists of proteins that inhibit axonal growth such as chondroitin sulfate proteoglycans, NG2, neurocan, versican, brevican, phosphacan and platelet-derived growth factor receptor-α (Fawcett and Asher, 1999), which can bind to laminin, thus inhibiting the ability of the axon to grow (Bradbury et al., 2002; Morgenstern et al., 2002). Astrocytes then fill the cavity left by the primary injury (Fawcett and Asher, 1999). This process contributes greatly to the permanent damage observed in TBI pathology.

Other physiological and cellular changes that have been implicated in the pathogenesis of TBI are release of excitatory amino acids, dysregulation of Ca$^{2+}$ signaling, mitochondrial dysfunction, and gene activation (Conti et al., 1998; Newcomb et al., 1999; Eldadah and Faden, 2000; Keane et al., 2001; Bramlett and Dietrich, 2004; Maas et al., 2008). Early gene activation, and the processing of both initiator and effector caspases have been reported after TBI (Yakovlev et al., 1997; Pike et al., 1998; Keane et al., 2001; Maas et al., 2008). Morphological hallmarks of apoptosis have been observed ultrastructurally in neurons following TBI and numerous TUNEL positive cells have been identified in cortex, hippocampus and thalamus in a variety of animal models of TBI (Rink et al., 1995; Colicos and Dash, 1996; Clark et al., 1997; Fox et al., 1998; Kaya et al., 1999, Newcomb et al., 1999, Keane et al., 2001a). Additionally,
oligodendrocytes, astrocytes and microglia have been observed to undergo apoptosis after TBI (Conti et al., 1998; Newcomb et al., 1999), contributing to the overall pathology.

Specifically, mitochondrial dysfunction has been implicated in the ongoing cellular pathology following TBI, contributing greatly to cell death. Following both clinical and experimental TBI, structural damage in mitochondria are evident, including swollen mitochondria (Colicos and Dash, 1996), loss of cristae membranes (Pettus and Povlishock, 1996; Maxwell et al., 2003), as well as expanded matrix, and disrupted outer membrane (Lifshitz et al., 2003). There is a dysregulation of cellular respiration and energy metabolism (Lifshitz et al., 2004; Singh et al., 2006), and the concomitant uncoupling of glucose utilization and cerebral blood flow result in increased lactate and free radical production (Lifshitz et al., 2004), further contributing to protein and lipid peroxidation and overall TBI pathology (Singh et al., 2006). As well, glutamate and N-methyl-D-aspartate (NMDA) receptor mediated accumulation of intracellular $\text{Ca}^{2+}$ exceeds the buffering capacity of the mitochondria (Singh et al., 2006), resulting in mitochondrial membrane depolarization and a $\text{Ca}^{2+}$-activated permeability pore (Susin et al., 1998), lending to cristae disruption and mitochondrial swelling observed, as well as the release of apoptogenic proteins (Kroemer et al., 1998). The release of soluble cytochrome C, apoptosis inducing factor, and caspases from the mitochondria allow for the initiation of events resulting in characteristic apoptotic cell death (Rink et al., 1995; Colicos and Dash, 1996; Kroemer et al., 1998).
Recently, the programmed cellular response autophagy has been implicated in TBI pathology (Diskin et al., 2005; Erlich et al., 2007; Clark et al., 2008; Liu et al., 2008), and has also been reported to play a role following axotomy and excitotoxicity (Rubinsztein et al., 2005). It is understood to occur at basal levels, contributing to cell homeostasis (Shintani and Klionsky, 2004). The hallmark ultrastructural features of autophagy are double-membrane autophagic vacuoles (AV’s), and have been observed in dying neurons for many years (Clarke, 1990). Specifically, accumulation of AV’s, and the up-regulation of autophagy-related gene (ATG) proteins ATG12-ATG5 conjugate, Beclin 1, and LC3-II has been observed in different models of TBI (Diskin et al., 2005; Liu et al., 2008). These changes have been observed from hours to weeks after TBI (Erlich et al., 2006). Autophagy can be elicited by classic apoptotic stimuli in neurons, representing an alternative execution pathway (Xue et al., 1999; Yu et al., 2004).

Although proteins that participate in autophagy signaling are upregulated after TBI (Diskin et al., 2005; Clark et al., 2008; Liu et al., 2008), the exact role in TBI remains undefined. Several studies investigating autophagy in neurons suggest it as a protective mechanism in early stages of programmed cellular responses (Larsen and Sulzer, 2002; Liang et al., 1998), consistent with its evolutionary role as a cell autonomous nutritive-mechanism (Reggiori and Klionsky, 2002). In these circumstances, it may serve as a mechanism to recycle cellular components and discard injured cells (Shintani and Klionsky, 2004; Erlich et al., 2006). With greater cellular stress, and/or prolonged stages of injury, it
has been suggested that autophagy plays an active role in programmed cell
death (Yuan et al., 2003), possibly through removal of cellular organelles after
stress/injury (Larsen and Sulzer, 2002). Thus, whether autophagy plays a
protective or detrimental role after TBI remains unresolved.

Induction of autophagy is mediated by the evolutionarily conserved
protein, Beclin 1 (Liang et al., 1999; Kihara et al., 2001). Beclin 1 also regulates
trafficking of membrane glutamate receptors in neuronal subtypes (Yue et al.,
2002). Constitutive activation of the glutamate receptor disrupts Beclin 1
regulation, resulting in autophagic cell death and subsequent neuronal
degeneration (Yue et al., 2002), a mechanism similar to glutamate-mediated
toxicity observed following TBI (Faden et al., 1989; Palmer et al., 1993; Globus et
al., 1995; Bullock et al. 1998; Heintz and Zoghbi, 2000). Excitotoxicity following
TBI occurs through NMDA type glutamate receptors (NR) (Yi and Hazell, 2006)
and leads to both neuronal and glial cell death (Choi, 1988; Lipton and
Rosenberg, 1994; Matute et al., 2007). NRs demonstrate high Ca\textsuperscript{2+}
permeability resulting in activation of CaMKII, and triggering downstream cascades such as
production of reactive oxygen species, cytoskeletal alterations, mitochondrial
dysfunction and apoptosis (Choi, 1988; Tymianski, 1996; Tymianski and Tator,
1996). Whether NRs regulate Beclin 1, and whether excitotoxicity mediated by
glutamatergic signaling after TBI involves the activation of autophagy has not
been established. Additionally, NR distribution, trafficking and signaling has
been shown to involve membrane raft microdomains (Hering et al., 2003;
Bessho et al., 2005), and may be one possible mechanism for regulating the interaction of NMDA receptors with other proteins at synapses.

My thesis investigates neuro-excitation, NR signaling and activation of autophagy induced by moderate TBI. My studies describe the regulation of a novel multiprotein complex involving the NR2B receptor and Beclin 1. Membrane rafts provide a platform for the assembly of this complex, and thus regulate the activity of Beclin 1. Following moderate TBI, this protein complex is disrupted leading to increased autophagy that contributes to pathomechanisms and cell death.

Moreover, my studies investigate a therapeutic strategy that targets NR2B containing NR mediated signaling events. By using an NR2B receptor subtype specific antagonist, my experiments show that blocking NR2B signaling leads to disruption of the multiprotein complex, attenuation of autophagic processes, and significantly decreases in cell death. This study is the first characterization of a membrane raft specific multiprotein complex involving NR2B glutamate receptor and the autophagic protein Beclin 1, and demonstrates excitotoxic-signaling influences autophagic mechanisms that contribute to pathology following TBI.

B. Traumatic Brain Injury in the Clinical Setting

The diagnosis of TBI is based upon clinical symptoms and mechanism of injury (i.e. closed vs. penetrating). Clinical severity to evaluate level of consciousness is assessed using the Glasgow coma scale (GCS) (Teasdale and Jennett, 1974; Maas et al., 2008) that involves measuring eye, motor, and verbal components; level of injury scale, evaluating injury in defined body regions.
Moreover, the level of structural damage is evaluated primarily through computed tomography (CT) scan (Marshall and Gautille, 1990; Maas et al., 2008). The GCS is a universal classification system and consists of the sum score (range 3-15) of the three components, which are reported separately (Teasdale and Jennett, 1974). CT is useful in detecting intracranial lesions and haematomas, as well as skull fracture (Maas et al., 2008) after TBI. Underlying structural damage can be correlated with scores on GCS (Smits et al., 2007), which provides a useful tool in proper diagnosis and injury assessment. Since TBI is a slow, progressive pathology, follow-up CT’s are advisable for continued diagnosis. It has been reported that new lesions can be observed in ∼16% of patients with diffuse injury (Servadei et al., 2000). In later phases of TBI, magnetic resonance imaging (MRI) becomes increasingly informative, allowing for more sensitive detection of white matter lesions in patients with diffuse axonal injury (Huisman et al., 2004). In the more severely injured, deterioration of the GCS and development of progressive lesions as visualized by CT has been reported in 29-44% of patients (Morris et al., 1998; Juul et al., 2000; Maas et al., 2008). Monitoring intracranial pressure (ICP) has been shown to be helpful in diagnosis because raised ICP is related to poor behavioral outcome (Brain Trauma Foundation, 2007). However, effective ICP monitoring is only advantageous if it can lead to appropriate intervention.

Current clinical treatments target various injury processes including, excitotoxicity, Ca^{2+} mediated events, free radicals, mitochondrial dysfunction, inflammation and apoptosis (Bramlett and Dietrich, 2004), whereas other
treatment strategies have focused on enhancement of neuronal and glial regeneration (Kulbatski et al., 2008; Maas et al., 2008). Over 20 agents have been studied in phase III clinical trial (Maas et al., 2008) that target glutamate receptor antagonists, Ca$^{2+}$ channel blockers, free radical scavengers, and anti-inflammatory and anti-apoptotic agents (Morris et al., 1999; Vink and Nimmo, 2002; Bramlett and Dietrich, 2004). However, little success has been reported clinically because problems in data analysis such as injury location and severity of injury, strength of preclinical data, and understanding of the clinical window have confounded outcome measures (Bramlett and Dietrich, 2004; Maas et al., 2008). Promising outcome measures have been reported with the use of subunit specific glutamate receptor antagonists (Yurkewicz et al., 2005) that show improvement in behaviors assessed by the GCS. Other promising clinical strategies include the use of hypothermia, where recent meta-analysis reveals improvements in recovery of moderate TBI when hypothermia was maintained over 48 hours after injury (Dietrich et al., 1994c; Bramlett et al., 1995; Bramlett et al., 1997; Dietrich et al., 2004; Truettner et al., 2005; Peterson et al., 2008). Thus, a combinatorial treatment strategy of subunit specific glutamate receptor antagonists and hypothermia offers hope for improvement of functional outcomes following TBI.

**C. Animal Models of Traumatic Brain Injury**

Several experimental animal models of TBI have been used to study TBI because they produce several characteristics of the human TBI pathology, including focal and diffuse injuries and functional and behavioral deficits. The
The four most commonly used experimental models of TBI are the weight drop model, the impact acceleration model, the controlled cortical impact model, and the fluid percussion model (Table 2) (Morales et al., 2005), that produce various aspects of focal and diffuse injury observed in human pathology. Of these models, the midline fluid percussion model (Dixon et al., 1987; McIntosh et al., 1987) that was modified to a lateral injury over a single hemisphere in the rodent (McIntosh et al., 1989a), has become the most extensively used model, and produces, reliable, focal and diffuse characteristics of brain injury (Bramlett et al., 1997b; Graham et al., 2000; Thompson et al., 2005). In this model, a craniotomy is performed and the exposed dural surface is injured via impact of a bolus of physiological saline. Injury severity can be reproducibly adjusted by altering the force of the fluid pressure pulse transmitted through the saline reservoir. This force is regulated by the defined height of a pendulum arm. The mechanical force that is displaced onto the exposed brain, results in tissue deformation and a focal contusion (McIntosh et al., 1989; Bramlett et al., 1997b), and sheering forces (Thompson et al., 2005) at the gray/white matter interface contributing to the diffuse components of the injury.

<table>
<thead>
<tr>
<th>Model of Injury</th>
<th>Hallmark Pathological Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight drop</td>
<td>Focal contusion under the site of impact Local cortical cell loss Minimal remote cortical and hippocampal damage ↓motor function (up to 48 hrs) ↓cognitive function (up to 1 week)</td>
<td>Feeney et al., 1981 Chen et al., 1996 Tang et al., 1997</td>
</tr>
<tr>
<td>Condition</td>
<td>Effects</td>
<td>References</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
</tbody>
</table>
| Closed cortical                   | Focal hemorrhage  
Focal epi/subdural hematoma  
Cortical, hippocampal, thalamic cell loss  
Transient gross and fine motor impairment  
↓cognitive function                 | Dixon et al., 1991  
Sheff et al., 1997  
Raghupathi et al., 1998               |
| Impact acceleration               | Diffuse axonal injury  
Axonal swelling  
Cortical and hippocampal neuron damage  
Acute behavioral and cognitive deficits (no long term examination) | Marmarou et al., 1994  
Chen, 1996  
Folkerts et al., 1998  
Beaumont et al., 1999  
Povlishock et al., 1999               |
| Parasagittal fluid percussion    | Focal hematoma  
Focal epi/subdural hematoma  
Diffuse white matter damage  
Cortical, hippocampal, thalamic cell loss  
Behavioral and cognitive deficits (observed up to 1 year post injury)  
*Most clinically relevant*           | Dixon et al., 1987  
McIntosh et al., 1987  
Povlishock et al., 1992  
Pierce et al., 1998  
Thompson et al., 2005               |

**Table 2.** Experimental animal models of TBI.

In my dissertation, the parasagittal fluid percussion injury model was used to induce a moderate contusive injury to the exposed parietal cortex (Bregma levels -3.8mm, 2.5mm) (Zilles, 1985; Dietrich et al., 1994b). The immediate focal contusion and hemorrhage in the cortex is similar to what is observed in the human condition (Graham et al., 2000), and results in primarily necrotic cell death (Dietrich et al., 1994b; Rink et al., 1995). The contused tissue degenerates over time becoming a cavity lined with glia – *glia limitans*. There is progressive expansion of this cavity due to cell death observed up to one year after injury (Bramlett et al., 2002). Other selectively vulnerable brain regions
include the hippocampus, thalamus, medial septum, striatum, and amygdala (Cortez et al., 1989; Pierce et al., 1996; Smith et al., 1997a; Conti et al., 1998; Pierce et al., 1998; Raghupathi et al., 2003; Hallam et al., 2004). The continued, secondary injury results in a series of biochemical, molecular and structural changes that resemble necrotic, apoptotic and autophagic processes (Rink et al., 1995; Conti et al., 1998; Raghupathi et al., 2002; Clark et al., 2008; Liu et al., 2008). Moreover, the lateral fluid percussion model of TBI has been shown to reproduce several behavioral and functional deficits that resemble the human pathology. Various tests have been adapted to rodents to test reflexes, neuromotor, sensorimotor, and cognitive outcome measures following TBI (Thompson et al., 2005).

II. CELL DEATH MECHANISMS FOLLOWING TBI

A. Glutamate Excitotoxicity

Glutamate excitotoxicity has been described as neuronal cell death resulting from excessive glutamate receptor activation (Henchcliffe and Beal, 2007) that also contributes to glial cell dysregulation (Li et al., 1995; Park et al., 2004; Matute et al., 2007). Significant increases in the levels of extracellular glutamate are produced rapidly in traumatized brains in both human and animals (Faden et al., 1988, 1989; Katayama et al., 1990; Panter and Faden, 1992; Bullock et al., 1998). Glutamate binds to both ionotropic and metabotropic classes of glutamate receptors and initiates distinct signaling pathways resulting in different physiological outcomes. Specifically, activation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) kainate receptors triggers
rapid excitatory responses, allowing Na\(^+\) ion and K\(^+\) ion permeability. This results in membrane depolarization, in turn leading to the removal of voltage-dependant Mg\(^{2+}\) block from NMDA receptors and the subsequent activation of associated high conductance Ca\(^{2+}\) channels. Calcium activates a number of Ca\(^{2+}\) dependent enzymes that influences a variety of cellular components and initiates a series of cytoplasmic and nuclear processes that promote neuronal cell death. Ca\(^{2+}\) dependent activation of calpains and CaMKII, and downstream effects on c-Fos, c-Jun, and c-Myc, have all been shown to contribute to necrotic and apoptotic cell death. Furthermore, Ca\(^{2+}\)-dependent endonucleases degrade DNA and exacerbate the pathological effect. Moreover, second messenger cascades activating reactive oxygen species (ROS) and mitochondrial dysfunction also contribute to cell death (Lipton and Rosenberg, 1994; Lu et al., 2000). Ca\(^{2+}\) permeability is governed by asparagine residual (N598) in the NR1 subunit within the channel pore loop structure of the second membrane domain (Arundine and Tymianski, 2004). This domain also determines the voltage-dependent Mg\(^{2+}\) block of the NMDAR (Burnashev et al., 1992), control gating properties, potentiation and block by polyamines, inhibition by protons and Zn\(^{2+}\), and affinity to glutamate and glycine (Schneggenburger and Ascher, 1997; Traynelis et al., 1998). In this regard, glutamate activation of NMDA receptor pathways have been implicated and extensively in studies of TBI, as they mediate excessive Ca\(^{2+}\) influx that contributes to delayed excitotoxic cell death processes (Choi, 1987, 1988; Tymianski, 1996; Tymianski and Tator, 1996).
Functional NRs contain heteromeric combinations of the NR1 subunit plus one or more of NR2A-D subunits (Nakanishi et al., 1992; Hollman et al., 1994). NR2A and NR2B subunits are the major NR2 subtypes present in forebrain structures (Monyer et al., 1994) and are phosphorylated by a variety of protein kinases, including protein kinase A (PKA), protein kinase C (PKC), Cam kinase II and IV (CaMKII, CaMKIV), and members of the Src family of tyrosine kinases (Ron, 2004; Salter and Kalia, 2004). The phosphorylation state of NR subunits regulate the function of receptor ion channels, trafficking of the receptor between intracellular membranes and the cell surface, and the distribution of clustering of receptors in the neuronal membrane (Tingley et al., 1997; Fong et al., 2002; Scott et al., 2003; Salter and Kalia, 2004). NRs have been shown to promote neuronal survival, but also to contribute to cell degeneration and neuron loss in a variety of pathological conditions, including cerebral ischemia (Gao et al., 2005; Picconi et al., 2006), seizure (Stafstrom and Sasaki-Adams, 2003), and TBI (Arundine et al., 2003; Yurkewicz et al., 2005; Giza et al., 2006; Lei et al., 2006). The location of the NR activated on the neuron specifies the transcriptional response (Zhang et al., 2007). For example, synaptic NRs induce a coordinated up-regulation of pro-survival genes whereas extra-synaptic NRs induce expression of a putative calcium-activated chloride channel that kills neurons (Zhang et al., 2007). Recent work has also established that NR2A- and NR2B-containing subpopulations of NRs have differential roles in mediating neuronal survival and death (Liu et al., 2007). Activation of either synaptic or extra-synaptic NR2A-containing receptors promotes neuronal survival and exerts a
neuro-protective action whereas activation of either synaptic or extra-synaptic NR2B-containing receptors results in excitotoxicity and neuronal apoptosis, (Liu et al., 2007). It has also been suggested that differences in the degree of receptor activation accounts for the directly opposing effects on cell fate, with low or moderate glutamate stimulation of the receptor promoting cell survival and overactivation inducing neuronal damage (Bossy-Wetzel et al., 2004; Hardingham and Bading, 2003). Therefore, the precise nature of the early signaling events that determine neuronal fate, and the molecular mechanism that mediates neuronal survival or death after CNS injury remains undefined.

Both in vitro and in vivo models of traumatic neuronal injury have shown the extent of involvement of NRs in cell death. Stretch-injury models of either neuronal or astrocytic cultures result in decreases in Mg\(^{2+}\) blockade, large ionic currents and increases in intracellular Ca\(^{2+}\) (Zhang et al., 1996), and tearing of rat neuronal/glial co-cultures produce extensive cell death. These effects are significantly reduced by the general NR antagonist MK801 (Regan and Choi, 1994; Mukhin et al., 1997; Mukhin et al., 1998). Various animal models have also shown that treatment with NR antagonists reduce post-injury tissue damage and associated behavioral changes (Table 3). Several competitive antagonists of the glutamate/NMDA site, 2R-amino-5-phosphonovaleric acid (APV), (3-[(R)-2-carboxypiperazin-4-yl]-prop-2-enyl-1-phosphonic acid (CPP), and cis-4-phosphonomethyl-2-piperidine-carboxylic acid (CGS-19755) have been shown to attenuate metabolic dysfunction, decrease concentration of extracellular glutamate, reduce cerebral edema, and improve behavioral outcome following
lateral fluid percussion injury (Faden et al., 1989; Panter and Faden, 1992; Kawamata et al., 1992; Okiyama et al., 1997). However, these drugs are not permeable to blood brain barrier (BBB) permeability and limits their effectiveness in clinical settings. Un-competitive and non-competitive ion channel blockers, such as MK801, ketamine, phencyclidine and dextrophan have also been successful in reducing brain edema, restoring ionic homeostasis and metabolic function, and improving functional recovery after TBI (McIntosh et al., 1989b, 1990b; Shapira et al., 1990; Panter and Faden, 1992; Hamm et al., 1993; Smith et al., 1993; Golding and Vink, 1995). However, detrimental side effects, including increased cell vacuole size and psychotometic effects (Olney et al., 1989; Kornhuber and Weller, 1997) have limited their use clinically. Other NR antagonists that modulate the glycine or polyamine binding site and Mg$^{2+}$ blockade have also been tested, however, their effects have not been fully evaluated (Frankiewicz et al., 2000).

More recently, NR2 subunit specific antagonists have been evaluated for neuro-protective efficacy after TBI. TBI induces degradation of NR2A- and NR2B- subunits, thus contributing to an impaired ability for memory association following injury (Kumar et al., 2002). Additionally, NR2B antagonists have shown efficacy in neuro-protection in various neurodegenerative models (Nikam and Meltzer, 2002). For example, CP-101,606, a non-competitive NR2B subunit specific antagonist inhibits NMDA-induced injury and increases c-fos expression that alters cortical spreading depression in mice (Menniti et al., 2000). Interestingly, CP-101,606 treatment also had a significant positive effect in the
clinical setting that resulted in marked decreases in mortality rate and improved behavioral outcome on dichotomized Glasgow Outcome Scale (GOS) (Yurkewicz et al., 2005). There are other non-competitive NR2B antagonists on the market, including RO 25-6981 (Fischer et al., 1997; Mutel et al., 1998) and PD0196860 that show efficacy in blocking NR2B signaling but these have not been tested in models of TBI. Therefore, there is a need to develop and test new NR2B antagonist for improved functional outcomes following TBI.

<table>
<thead>
<tr>
<th>Antagonist Sub-category</th>
<th>Antagonist</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-competitive (block ion channel by binding site within)</td>
<td>Amantadine</td>
<td>University Health Network, Toronto, 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kalia et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Dextromethorphan</td>
<td>Wong et al., 1988</td>
</tr>
<tr>
<td></td>
<td>Ketamine</td>
<td>Harrison and Simmonds, 1985</td>
</tr>
<tr>
<td></td>
<td>Memantine</td>
<td>Robinson and Keating, 2006</td>
</tr>
<tr>
<td></td>
<td>Phencyclidine</td>
<td>Snell and Johnson, 1985 Farlow, 2004</td>
</tr>
<tr>
<td>Non-competitive (binds to allosteric sites in the channel)</td>
<td>Dizocilpine (MK-801)</td>
<td>Woodruff et al., 1987 Mcintosh et al., 1989b Mcintosh et al., 1990b Nakao et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Aptiganel (Cerestat, CNS-1102) – binds Mg$^{2+}$ binding site</td>
<td>McBurney, 1997</td>
</tr>
<tr>
<td>Glycine site (bind and block the glycine site)</td>
<td>7-chlorokynurenate</td>
<td>Hartley et al., 1990</td>
</tr>
<tr>
<td></td>
<td>5,7-dichlorokynurenate (DCKA)</td>
<td>Frankiewicz et al., 2000</td>
</tr>
<tr>
<td></td>
<td>1-aminocyclopropane carboxylic acid (ACPC)</td>
<td>Papp et al., 2002</td>
</tr>
<tr>
<td>Competitive (bind and block the glutamate binding site)</td>
<td>2-amino-7-phosphonoheptanoic acid (AP7)</td>
<td>Van der Bos et al., 1992</td>
</tr>
<tr>
<td></td>
<td>(3-[(R)-2-carboxypiperazin-4-yl]-prop-2-enyl-1-phosphonic acid (CPP)</td>
<td>Eblen et al., 1996</td>
</tr>
<tr>
<td></td>
<td>R-2-amino-5-</td>
<td>Klishin et al., 1995</td>
</tr>
<tr>
<td>Name</td>
<td>Functionality</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>phoshanopentanoate (APV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traxoprodil (CP-101,606)</td>
<td>*NR2B specific</td>
<td>Yurkewicz et al., 2005</td>
</tr>
<tr>
<td>Ro 25-6981</td>
<td>*NR2B specific</td>
<td>Fischer et al., 1997; Mutel et al., 1998; Lynch et al., 2001</td>
</tr>
</tbody>
</table>

Table 3. Experimentally used NMDA receptor antagonists.

Excitotoxicity induced by TBI also causes dysfunction of glia. In the normal brain, astrocytes play an integral role in regulating the amount/concentration of glutamate in the extracellular space. Five subsets of glutamate transporters have been cloned and identified: GLT1, GLAST, EAAC1, EAAT4, and EAAT5 (Danbolt, 2001). Both GLT1 and GLAST are expressed predominantly in astrocytes (Danbolt et al., 1992; Rothstein et al., 1994; Lehre et al., 1995; Reye et al., 2002), and GLT1 has been shown to represent ~90% of total glutamate transport activity in the forebrain (Yi and Hazell, 2006). GLT1 knockout mice show <5% glutamate transport activity compared to the wild-type (Tanaka et al., 1997), and glutamate exposure is significantly more neuro-toxic in astrocyte-poor rat cell cultures (Rosenberg et al., 1992), demonstrating the importance of functional re-uptake mechanisms involving glia. The decrease in post injury glutamate uptake, as well as decreases in both GLT1 and GLAST expression, has been observed both *in vitro* and *in vivo*. As such, dysregulation of astrocytes after TBI results in dramatic impairment of glutamate re-uptake mechanisms in the brain, and contributes to greater glutamate related toxicity. Moreover, glia are vulnerable to glutamate, as they express most subtypes of both ionotrophic and metabotrophic glutamate receptors (Belachew and Gallo, 2004; Matute, 2006; Matute et al., 2006). Hyperactivation of glial receptors results in cell death of
astrocytes, microglia, oligodendrocytes and Schwann cells (Matute et al., 2007). Taken together, excessive glutamate release after TBI results in a significant and progressive cell loss and subsequent functional deficits. Signaling cascades initiated by NRs contribute significantly to tissue damage after TBI, and it is possible that other yet undiscovered cellular processes regulated by NRs will add to this devastating process. Therefore, NR-mediated excitotoxic pathways remain an important area of study following TBI. My studies investigate NR2B receptor specific protein associations and signaling pathways and their contribution to cortical pathophysiology following TBI, and aims to identify subsequent cellular processes that may be possible targets for intervention.

B. Autophagy as a programmed cellular response following TBI

Macroautophagy (hereafter referred to as autophagy) is an evolutionary conserved pathway in which cytoplasm and organelles are engulfed within double-membrane vesicles, known as autophagosomes (Shintani and Klionsky, 2004). Genetic analysis has identified at least 25 autogenic (ATG) genes that code for proteins that regulate this complex process. In particular, the ATG protein Beclin 1, a member of the class III PI3 kinase complex (Liang et al., 1999; Kihara et al., 2001), has been shown to be necessary for the induction of autophagy (Liang et al., 1999; Petiot et al., 2000). Beclin 1 directs membrane dynamics during autophagy by mediating the localization of other key ATG proteins to the pre-autosomal (isolation) membrane (Kihara et al., 2001). Isolation membrane maturation and elongation involves two ubiquitin-like
pathways, where the ATG proteins that are included in this process are highly conserved (Figure 1) (Baehrecke, 2005; Qu et al. 2003; Yue et al. 2003).

**Integrated pathway for Autophagy**

![Integrated pathway for Autophagy](image)

**Figure 1.** Mechanism of autophagic vacuole formation. An initial signaling event results in Beclin 1 transporting ATG 5-ATG 12 to the trans-Golgi network (TGN) where the induction of autophagic membrane formation can occur. Secondary signaling events phosphorylate mTOR, favoring LC3-ATG 7 formation, which is necessary for the maturation and fusion of the autophagic vacuole.

As a catabolic process, autophagy provides a bio-energetically efficient alternative to *de novo* synthesis (Kundu and Thompson, 2005) where recycled components provide substrate that can be utilized for energy metabolism and protein synthesis (Meijer and Codogno, 2004; Eskelinen, 2005). First characterized in yeast, autophagy was found to be essential for survival during nutrient-limitation, functioning as a short-term response to nutrient and/or amino acid deficiency (Meijer and Codogno, 2004; Eskelinen, 2005). In fact, yeast
mutants defective in various autophagy genes do not survive starvation (Tsukada and Ohsumi, 1993; Thum et al., 1994). Genetic evidence of mammalian gene orthologues have made it possible to test such a role in mammalian cells. Serum and amino acid starved HeLa cells and growth factor deprived bone marrow cells lead to the activation of autophagy (Boya et al., 2005; Lum et al., 2005) and knock-down of several key autophagic proteins resulted in cell death in both cell types in their nutrient deprived condition. Further, cells survived for weeks on the energy provided by the catabolized substrates (including organelles) and were able to fully recover after 6 weeks in a nutrient-poor state (Lum et al. 2005). These studies indicate autophagy as a key response contributing to cell survival under these conditions.

During the initial (first) membrane conjugation step, ATG12 is activated in an ATP-dependent manner by ATG7 (E1-like enzyme), leading to the thioester bond between the two proteins (Ohsumi, 2001; Yang et al., 2005). ATG12 undergoes a conformational change and forms a new thioester bond with ATG10 (E2-like enzyme) and releases ATG7. Finally, ATG12 forms an isopeptide bond with ATG5 thereby releasing ATG10 (Ohsumi, 2001; Yang et al., 2005). The irreversible formation of ATG12-ATG5 conjugate binds ATG16, forming a multimeric complex (Kuma et al., 2002; Mizushima et al., 2003), and ATG16 is believed to stabilize the ATG12-ATG5 conjugate (Ohsumi, 2001). Thus, the multimeric complex is necessary for the elongation of the isolation membrane (Yang et al., 2005), but the exact molecular basis for membrane elongation is poorly understood.
The second conjugation step in the autophagic process involves ATP-dependent activation of ATG8 (LC3) by ATG7 (E1-like), an essential protein in autophagy. Following activation, ATG8 forms a thioester bond with a conjugating E2 enzyme, ATG3 (Ichimura et al., 2000). In the final step, LC3 interacts with phosphatidyl ethanoalamine (PE), an abundant membrane phospholipid (Ichimura et al., 2000; Yang et al., 2005). This lipidation reaction results in a conformational change in LC3 that is crucial in mediating membrane dynamics of autophagy (Ichimura et al., 2004; Yang et al., 2005). LC3 can be detected in 2 forms, LC3-I and LC3-II (Kabeya et al., 2000). LC3-I is ubiquitinated to form LC3-II, which is the form that binds to PE and the autophagosomal membrane. Therefore, LC3-II is routinely used as a marker for complete autophagosomal maturation and fusion (Kabeya et al., 2000). Moreover, autophagy may work together with the proteasome pathway to degrade misfolded proteins that have been retained in the endoplasmic reticulum, but the molecular interactions of these two degradative processes is poorly understood.

Autophagy signaling is tightly regulated by the target or rapamycin (TOR) pathway, or mammalian TOR (mTOR) pathway in mammals. mTOR is a serine/threonine kinase involved in most regulatory pathways related to nutrient conditions and energy metabolism (Yang et al., 2005). mTOR exerts an inhibitory effect on autophagy via two mechanisms: (a) transducing signals to downstream effectors controlling translation and transcription (Cardenas et al., 1999) and (b) by direct inhibition of ATG proteins, preventing autophagosome formation (Levine and Klionsky, 2004). Downstream, mTOR can activate protein
phosphates 2A (PP2A) (Jiang and Broach, 1999), eukaryotic initiation factor 4E binding protein 1 (4E-BPI) (Fingar et al., 2002), which have been shown to regulate various gene products involved in autophagy machinery, including ATG8 and ATG14 (Huang et al., 2000; Chan et al., 2001). Directly, activated mTOR phosphorylates ATG13, preventing its association with ATG1 (Kamada et al., 2000). ATG1-ATG13 is a regulatory complex promoting assembly of proteins in the autophagosomal membrane, including the LC3-ATG7 complex. Although the complexity of many of these signaling cascades and protein interactions have not fully been elucidated, it is evident that inhibition of mTOR is necessary for the continual maturation of autophagic membranes and contribute to the expression of autophagy related proteins.

Many other signaling pathways interact with the autophagic process. For example, the class I PI3K/PKB pathway negatively modulates autophagy by activating downstream effectors including mTOR (Yang et al., 2005). Moreover, the class III PI3K complex regulates Beclin 1, the transport protein necessary for the induction of autophagosome formation (Kihara et al., 2001). Additionally, autophagy is also described as a prominent feature of cell death during embryonic development and during regression of hormone-dependent tumors (Yue et al., 2003; Cecconi et al., 2007; LeBot et al., 2007; Qu et al., 2007). Analysis of several types of programmed cell death (PCD) suggests that autophagic (or type II) death may be distinct from apoptotic (type I) cell death. However, there is cross-talk between autophagy and apoptosis. In my thesis I
examine whether TBI induces autophagy and whether excessive excitotoxic activity may promote autophagy thus contributing to neuronal damage.

Autophagy has been observed in several neurodegenerative conditions including Huntington’s Disease (HD) (Kegel et al., 2000; Petersen et al., 2001), Parkinson’s Disease (PD) (Anglade et al., 1997), and Alzheimer’s Disease (AD) (Cataldo et al., 1994, 1995; Boland et al., 2008). In HD, there is an increased number of autophagosomes observed both in cell culture models and in vivo (Rubinsztein et al., 2005), where both double-membrane vacuoles as well as single membrane, late autophagosomes, were reported. Further, HD cells, containing intracellular aggregates (inclusions) also expressed greater LC3-II as observed by immunoblotting and immunohistochemistry, as well as inactivation of TOR (Ravikumar et al., 2004). There is also a marked increase in autophagy that is seen in dopaminergic neurons of substantia nigra parts compacts (SNpc) in PD (Rubinsztein et al., 2005). As well, overexpression of α-synuclein, the deposited protein in PD, in a neuronal cell line induces autophagy (Hsu et al., 2000). Autophagy may be activated in response to intracellular dysfunction, however, as dysfunctionality increases, autophagy may result in cell death. In AD, increased autophagy has been observed in vulnerable populations of neurons at early stages of AD as well as in various transgenic models of AD, including presenilin-1 (PS1) and amyloid precursor protein (APP) overexpression (Nixon et al., 2004a,b; Yu et al., 2004). In both of these models, increased levels of LC3-II and accumulation of autophagosomes have been observed ultrastructurally (Yu et al., 2004; Nixon et al., 2005; Nixon, 2007). Autophagy also
induces cell death following ischemic insult to the brain (Adhami et al., 2006; Adhami et al., 2007; Wen et al., 2008). Both biochemical and morphological evidence of autophagy has been observed in rat brains following ischemia, and cell death was attenuated when animals were treated with specific autophagy inhibitors (Wen et al., 2008). Interestingly, loss of autophagy has also been observed to lead to neurodegeneration in the CNS. ATG7 knockout mice, showed numerous behavioral deficits, neuronal loss and early mortality (Komatsu et al., 2006). Similarly, in rodent models lacking ATG5 and Beclin 1 gene products, increased cell death has been observed (Liang et al., 1998; Lum et al., 2005). As well, autophagy is a component of the lysosomal pathway, which, in neurons, is essential in regulating membrane and synaptic protein turnover, including glutamate receptor dynamics (Tai and Schuman, 2008). In this manner, autophagy contributes to neuronal dynamics in response to synaptic stimuli. Thus autophagy is critical for neuronal survival as well, and illustrates the importance of understanding its mechanism of activation and function under normal and pathological conditions.

Recently, autophagic processes have been observed following TBI and both ultrastructural and biochemical evidence has been reported. In controlled cortical impact (CCI) models of injury, ultrastructural evidence of autophagy was observed at 2h and 24h after injury, where LC3-II expression was significantly increased at 2, 24, and 48h after injury (Lai et al., 2007). Similarly in a fluid percussion injury model, a significant increase in autophagosomes is observed after 4h, and LC3-II accumulation is observed after 24h (Liu et al., 2008). Similar
changes have been observed in humans after TBI (Clarke et al., 2008). Further reports show that Beclin 1 is significantly increased 4h after CCI injury, and increased levels are maintained up to at least 3 weeks post-trauma (Diskin et al., 2005). Regulation of Beclin 1 has been shown to involve a complex of proteins including the membrane glutamate receptor GluRδ2 (at post-synaptic density) in cerebellar Purkinje cells (Yue et al., 2002). In the Lurcher mutant mouse, there is constitutive activation of GluRδ2, that disrupts Beclin 1 regulation, resulting in autophagic cell death and subsequent neuronal degeneration (Yue et al., 2002). Further, autophagic degeneration has also been observed in neurons in models of slow glutamate excitotoxicity in vitro (Matyja et al., 2005). These processes are analogous to glutamate-mediated toxicity observed following TBI (Faden et al., 1989; Palmer et al., 1993; Globus et al., 1995; Bullock et al., 1998; Heintz and Zoghbi, 2000). Excitotoxicity following TBI involved hyper-excitability of NRs (Yi and Hazell, 2006), that result in both neuronal and glial cell death (Choi, 1988; Lipton and Rosenberg, 1994; Lee et al., 1999; Matute et al., 2007). NRs have high permeability to Ca\(^{2+}\), and increased intracellular Ca\(^{2+}\) trigger downstream neuro-toxic cascades leading to formation of reactive oxygen species, cytoskeletal alterations, mitochondrial dysfunction and apoptosis (Choi, 1988, Tymianski, 1996; Tymianski and Tator, 1996). Whether NR2B containing NRs, which are enriched in forebrain structures, provide similar regulation of Beclin 1, and whether excitotoxicity mediated by glutamatergic signaling after TBI involves the activation of autophagy has not been established. My studies involve biochemical pharmacological analysis of NR2B, receptor signaling intermediates
and several key autophagic proteins to verify the link between NR2B receptor signaling and autophagy.

III. NMDA RECEPTORS SIGNALING AND MEMBRANE RAFTS

Membrane rafts have been shown to regulate the distribution of NRs in the plasma membrane, and to regulate their trafficking and signaling (Hering et al., 2003, Bessho et al., 2005). Membrane rafts are small, heterogeneous, dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes (Pike, 2006) and are ubiquitous features of mammalian cells (Figure 2). The high sterol and sphingolipid composition of membrane rafts give them a low buoyant density observed in density gradients (Simons and Ikonen, 1997; Simons and Toomre, 2000). Sphingolipids have saturated acyl tails, giving membrane rafts a tight, rigid organization in the liquid-ordered phase within the plasma membrane (Pike, 2004). Membrane rafts are very small (500 nm), but in response to various stimuli, can coalesce into larger units, clustering components and allowing for the interaction of proteins and subsequent signaling events (Brown and London, 2000). Membrane rafts are involved in various neuronal processes, including maintenance of synapses and dendritic spines (Suzuki et al., 2001; Suzuki, 2002; Hering et al., 2003; Fortin et al., 2004), protein trafficking and channel localization (Nabi and Le, 2003; Wong et al., 2004), neurotrophic factor signaling and nerve growth cone guidance (Paratcha and Ibanez, 2002; Guirland et al., 2004), and the formation of death receptor complexes (Lotocki et al., 2004; Keane et al., 2006) and various other signaling complexes (Schroeder et al., 1994).
Figure 2. Schematic diagram of membrane rafts. Membrane rafts exist as 10-200 nm complexes that form larger clusters via protein-protein and protein-lipid interactions (Pike, 2006). Membrane rafts have been reported to function in the endoplasmic reticulum, Golgi, and the mitochondria, in addition to the plasma membrane and take part in viral entry into the cell, cell signaling, and protein and lipid trafficking. Thus, membrane rafts provide a basis for novel therapeutic strategies targeting mechanisms that depend on rafts for activity.

With respect to brain trauma, redistribution of TNFR1 into membrane rafts and non-raft regions of the plasma membrane regulate the diversity of signaling responses initiated by these receptors in the normal brain and after TBI (Lotocki et al., 2004). NRs located in membrane rafts have been reported to mediate neuro-toxicity (Frank et al., 2004; Abulrob et al., 2005), whereas NRs outside of membrane rafts are responsible for glutamate-mediated growth cone guidance (Guirland et al., 2004). In this manner, membrane rafts may provide one possible mechanism for regulating the interaction of NRs with other proteins at synapses. However, the role of these microdomains in NR signal transduction after TBI has yet to be determined. My studies involve the isolation of cortical membrane rafts, the determination of NR2B, synaptic and autophagic protein localization and association to determine the involvement of these microdomains in NR2B specific signaling.
It is evident that TBI bears a significant social and economic weight in the United States and globally, and elucidating cellular and molecular mechanisms that contribute to its pathology is of great value. There is much evidence supporting that glutamate induced toxicity significantly contributes to cell death after TBI, exacerbating the slow, progressive nature of the pathology. Currently, glutamatergic signaling events activated after TBI are not fully understood. Recent evidence has also implicated autophagy as a process activated after TBI, however, its role or contribution to TBI pathology has also yet to be clarified. The current dissertation addresses whether membrane rafts provide a platform for interaction between NRs and autophagic proteins, and whether this interaction may mediate glutamatergic signaling and autophagic processes following TBI. Here I show that Beclin 1 is found in a multi-protein complex with NMDA (NR2B) type glutamate receptors in membrane rafts in the normal rat cortex. TBI induced a rapid re-distribution of Beclin 1 out of membrane rafts. An early increase in NR2B and signaling intermediate expression levels, followed by a significant increase in expression levels of key autophagic proteins was observed to occur through membrane raft domains. Moreover, biochemical and morphological changes observed after TBI were attenuated with an NR2B specific antagonists. These findings suggest that regulation of Beclin1 involved NR2B glutamate receptors in membrane raft domains, and constitutive activation of NR2B after TBI induces a re-distribution of Beclin 1 which leads to an up-regulation of autophagic processes, possibly contributing to patho-mechanisms following TBI.
Chapter II

ACTIVATION OF NR2B AND AUTOPHAGY SIGNALING PATHWAYS FOLLOWING TRAUMATIC BRAIN INJURY

Summary

Hyperactivation of N-methyl-D-aspartate receptors (NRs) is associated with neuronal cell death induced by traumatic brain injury (TBI) and many neurodegenerative conditions. NRs signaling efficiency is dependent on receptor localization in membrane raft microdomains. Recently, excitotoxicity has been linked to autophagy, but mechanisms governing signal transduction remain unclear. Here we have identified protein interactions between NR2B signaling intermediates and the autophagic protein Beclin 1 in membrane rafts of the normal rat cerebral cortex. Moderate TBI induced rapid recruitment and association of NR2B and pCaMKII to membrane rafts, and translocation of Beclin 1 out of membrane microdomains. Furthermore, TBI caused significant increases in expression of key autophagic proteins and morphological hallmarks of autophagy that were significantly attenuated by treatment with the NR2B antagonist Ro 25-6981. Thus, stimulation of autophagy by NR2B signaling may be regulated by redistribution of Beclin 1 in membrane rafts after TBI.
Overview

Activation of N-methyl-D-aspartate (NMDA) receptors (NRs) plays a critical role in glutamate excitotoxic damage in a variety of pathological conditions, including traumatic brain injury (TBI) (Arundine et al., 2003; Yurkewicz et al., 2005; Giza et al., 2006; Lei et al., 2006). Functional NR’s contain heteromeric combinations of the NR1 subunit with one or more of NR2A-D (Nakanishi et al., 1992; Hollman et al., 1994). NR2A and NR2B are the major NR2 subtypes present in forebrain structures (Monyer et al., 1994). These receptors interact with 185 or more proteins and form signaling complexes in the postsynaptic density (Grant, 2003). The NR complexes localize signaling molecules with Ca\(^{2+}\) influx and facilitate the activation of second messenger pathways that effect change in the channel (Lan et al., 2001), synapse (Lisman, 2001; Malinow and Malenka, 2002) and nucleus (Deisseroth et al., 1996; Hardingham et al., 2001). NR2B-containing receptors play a critical role in synaptic plasticity by activation and interaction with CaMKII at the synapse, a key component of the underlying signaling transduction pathway (Barria and Malinow, 2005). However, the mechanism regulating distribution and clustering of NR2B receptors in the neuronal membrane, particularly following injury remains undefined.

Redistribution of NR’s in membrane rafts is one possible mechanism for regulating the efficiency of NR signaling (Besshoh et al., 2005). Membrane rafts are heterogeneous, dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes (Pike, 2006), and have been implicated in
various neuronal processes, including maintenance of synapses and dendritic spines (Suzuki et al., 2001; Suzuki, 2002; Hering et al., 2003; Fortin et al., 2004), protein trafficking and channel localization (Nabi and Le, 2003; Wong et al., 2004; Besshoh et al., 2005), neurotrophic factor signaling, nerve growth cone guidance (Paratcha and Ibanez, 2002; Guirland et al., 2004), and the formation of death receptor signaling after TBI and spinal cord injury (SCI) (Lotocki et al., 2004; Keane et al., 2006; Davis et al., 2007). Thus, many important processes for neuronal function and viability could depend on localization of NR's in cholesterol-rich membrane rafts, however, this area has been little explored.

Intriguingly, one report provides evidence for a link between activation of the GluRδ2 receptor and stimulation of the autophagic pathway in dying lurcher Purkinje cells (Yue et al., 2002). In this excitotoxicity model, GluRδ2 interacts with a novel form of a post-synaptic density/Drosophila disc tumor suppressor/Zonula occludens-1 (PDZ) containing protein nPIST and the evolutionary conserved autophagy protein Beclin 1, thus activating the autophagy process. Autophagy is a highly regulated vesicular pathway for delivery of cellular proteins, membranes, and organelles to lysosomes for degradation and recycling (Shintanti and Klionsky, 2004). Here, we provide evidence that NR2B and signaling intermediates form protein interactions with Beclin-1 in membrane rafts in neurons in the normal rat cerebral cortex. TBI induced a rapid redistribution of Beclin 1 out of membrane rafts, altered association with NR2B signaling intermediates, and induced activation of the autophagy pathway. Thus, redistribution of Beclin 1 in membrane rafts and non-raft regions of the plasma
membrane may regulate signaling responses initiated by these receptors in the normal brain and after TBI.

**Material and Methods**

**Parasagittal Fluid Percussion Brain Injury**

All animal protocols were approved by the University of Miami Animal Care and Use Committee. Adult male Sprague Dawley rats (Charles River Laboratories, Raleigh, NC, USA) weighing 250-300 g (n=64, 8 per group) were surgically prepared under 3% halothane, 70% N₂O, and a balance of O₂, to achieve deep sedation. Animals were placed in a stereotaxic frame and a 4.8 mm craniotomy (3.8 mm posterior to bregma, 2.5 mm lateral to the midline) (Zilles, 1985; Dietrich et al., 1994b) was performed and an injury cap (18 gauge syringe hub; 8 mm length, PrecisionGlide needle, Becton Dickinson, Franklin Lakes, NJ, USA) was anchored over the exposed dura (Keane et al., 2001). Animals were allowed to recover for 24 h following craniotomy. The animals were then re-anesthetized and intubated endotracheally and mechanically ventilated using a Harvard rodent ventilator (Harvard Apparatus, Holliston, MA) at a mixture of 1.5% halothane, 70% N₂O, and a balance of O₂. Pancuronium bromide (0.5 mg/kg, intravenous (i.v.)) was administered every hour during the surgical procedure to facilitate mechanical ventilation. The femoral artery and vein were cannulated with a PE-50 cannula after sedation, for drug delivery and blood sampling for arterial blood pressure, arterial blood gases and serum glucose. Arterial blood gases were measured 15 minutes before and after TBI. For NR antagonist experiments, NR2B antagonist Ro 25-6981 (Sigma) or NR2A
antagonist NVP-AAM077 (Novartis Pharmaceuticals, Basel, Switzerland) (1 mg/kg, i.v.) was administered 30 minutes prior to injury (Liu et al., 2007). Rectal and temporalis muscle thermometers were used to maintain a core temperature of 37°C using self-adjusting feedback warming lamps.

A fluid-percussion injury device was connected to the injury cap. Animals were then subjected to moderate (1.7-2.1 atm) levels of TBI (Dietrich et al., 1994b; Hicks et al., 1996; Keane et al., 2001). After TBI, all animals were returned to their cages and allowed to recover from the surgical procedures. Sham-operated animals underwent all surgical procedures, but were not injured. If mortality or lung edema resulted from the injury, animals were excluded from analysis. For biochemical analysis, at the time of sacrifice, animals were deeply reanesthetized with halothane. Tissue samples were perfused and stored at 4°C, or snap-frozen in liquid nitrogen and stored at –80°C until the time of assay.

**Perfusion fixation**

Animals were anesthetized with an intramuscular injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Complete anesthetization was determined by the lack of a stereotypical retraction of the hindpaw in response to a nociceptive stimulus. Animals then received an intracardial injection of heparin (0.1 cc) and were perfused transcardially with physiological saline, followed by 300 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed and placed in 4% paraformaldehyde at 4°C for 48 hours, then transferred to 20% sucrose in 0.1 M PBS until they were ready to be sectioned.
Antibodies

Rabbit anti-ATG 5 polyclonal (1:1000), and rabbit anti-ATG 7 polyclonal (1:1000) were obtained from Abgent (San Diego, CA, USA). Rabbit anti-LC3 polyclonal (1:1000) was obtained from Abnova (Tokyo, Japan). Mouse anti-β-tubulin monoclonal (1:3000), mouse anti-caveolin-1 monoclonal (1:250), mouse anti-flotillin-1 monoclonal (1:250), mouse anti-Homer monoclonal (1:500), mouse anti-NR2B monoclonal (1:250), mouse anti-PSD 95 monoclonal (1:250), mouse anti-shank monoclonal (1:500), and mouse anti-Thy-1 monoclonal (1:1000) were obtained from BD Biosciences (San Diego, CA, USA). Mouse anti-pCaMKII monoclonal (1:1000), mouse anti-MTOR monoclonal (1:1000), mouse anti-pMTOR monoclonal (1:1000), mouse anti-p70 S6K monoclonal (1:1000), and mouse anti-p-p70 S6K monoclonal (1:1000), were obtained from Cell Signaling (Beverly, MA, USA). Mouse anti-Na⁺/K⁺ ATPase monoclonal (1:1000) was obtained from Chemicon International (Temecula, CA, USA). Rabbit anti-beclin-1 polyclonal (1:1000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cholera toxin sub-unit B-coupled to horseradish peroxidase (HRP) (5 µg/mL) was obtained from Sigma-Aldrich.

Immunohistochemistry

Animals were perfused with 4% paraformaldehyde solution as described above and brains were processed for cryostat sectioning (Leica SM 2000R sliding microtome). Sections (50 µm) were stored in free-floating cryostat media (30% ethylene glycol, 30% sucrose, 0.1 M PBS, pH 7.4) at 20°C then rinsed with 0.1 M PBS (pH 7.4) Tissue sections were blocked by treatment with 5% goat
serum (Vector Laboratories Inc., Burlingame, CA, USA) and 0.4% Triton X-100 (Sigma). Sections were incubated for 48 hours at 4°C with the primary antibodies. Primary antibody binding was detected with Alexa Fluor secondary antibody conjugates (1:200, Molecular Probes, Eugene, OR, USA). Controls lacking the primary antibody were run in parallel. Sections were coverslipped with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) for confocal analysis (Zeiss, LSM 510, scanning confocal microscope).

**Preparation of Lysate**

For immunoblot analyses and membrane raft isolation, sections of cerebral cortex (2 mm²) were dounce homogenized in PTN 50 extraction buffer (50 mM sodium phosphate, pH 7.4, 1% Triton X-100, 50 mM NaCl) containing protease inhibitors (1 mM aprotinin, 1 mM phenylmethysulfonyl fluoride (PMSF), 5 µg/mL leupeptin, 1 µg/mL pepstatin A) at a concentration of 100 µg/mL as previously described (Lotocki et al., 2006). Homogenate was centrifuged at 12,000 rpm for 2 min to pellet cellular debris. Supernatants were saved and stored at −80°C.

**Immunoblot Analysis**

Cortical lysates surrounding the injury epicenter were prepared from 15 min, 1, 4, 8, 24, and 48 h TBI animals and from 15 min and 48 h sham-operated controls. Protein levels were resolved on 10-20% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Applied Biosciences). Membranes were blocked (0.1% Tween 20, 0.4% I-block in PBS; Applied Biosciences). Membranes were incubated with primary antibodies followed by the appropriate
secondary antibody. Visualization of the signal was enhanced by chemiluminescence using a Phototope-HRP Detection Kit (Cell Signaling). To control for protein loading, the immunoblots were stripped with Restore™ Western blot stripping buffer (Pierce), and probed for β-tubulin as a protein loading control. MTOR and p70-S6K were used as internal protein standards.

Membrane raft isolation

Detergent-resistant membranes from adult rat cortices were isolated on the basis of their insolubility in Triton-X 100 at 4°C and their ability to float in density gradients (Lotocki et al., 2006). Cortical lysates were placed in a centrifuge tube and mixed with an equal volume of 80% sucrose. The samples were overlaid with 30% and 5% sucrose, respectively. The discontinuous sucrose gradient was centrifuged at 130, 000 X g_{avg} for 20 h and aliquoted into eight fractions, 0.60 ml each, with fraction 1 being the uppermost (lightest) fraction. The insoluble pellet (fraction 9) was re-suspended in 0.60 ml of PTN 50 extraction buffer plus protease inhibitors. Membrane raft containing fractions were identified by the raft marker proteins, caveolin 1, flotillin 1, Thy 1, GM 1, and Triton X-100 soluble fractions were tracked by the soluble marker protein Na⁺/K⁺ ATPase. Sucrose solutions were made using morpholino ethane sulfonic acid (MES) (25 mM, pH 6.5, 150 mM NaCl). All procedures for raft isolation were conducted at 4°C. Caveolin 1 was used as a protein loading control.

Immunoprecipitation in membrane raft and soluble fractions

Membrane raft-containing fractions were pooled and centrifuged at 130 000 X g_{avg} for 2 h to pellet the rafts. The membrane raft pellet was resuspended
in PTN 50 extraction buffer plus protease inhibitor. Raft and soluble fractions were pre-cleared with 50 µL of mouse or rabbit IgG Trueblot™ beads (eBioscience) for 1 hour. Beads were pelleted by centrifugation at 12,000 X g_{avg} for 30 seconds. The resulting supernatants were mixed with 5 µg/mL of anti-NR2B (BD Pharmingen) or 5 µg/mL of anti-Beclin 1(Santa Cruz) and rotated overnight at 4°C. Samples were incubated with 50 µL of mouse or rabbit IgG Trublot™ beads (eBioscience) and rotated for an hour. Beads were pelleted and washed six times in PTN 50 extraction buffer, resuspended in Laemelli loading buffer, and heated to 95°C for 3 min before analysis by immunoblotting using appropriate primary antibodies and HRP-conjugated mouse IgG Trueblot™ or rabbit IgG Trueblot™ (eBioscience) secondary antibodies (1:1000).

**Transmission Electron Microscopy**

Deeply anesthetized male Sprague-Dawley rats were perfused with saline followed by cold fixative, containing 4% paraformaldehyde, 0.1% gluteraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Regions of interest including ipsilateral cortical layer IV-VI of injured and control animals were grossly dissected under a microscope into ~ 1-mm tissue blocks and postfixed overnight, with 2% gluteraldehyde. Sections were then rinsed 3 times in 0.15 M phosphate buffer and fixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.4 for 2 h at room temperature followed by overnight fixation at 4°C. Sections were dehydrated using cold graded alcohols (25%, 50%, 70%, 95% and 100% ethanol), and then rinsed in propylene oxide. Tissues were penetrated overnight with a 1:1 mixture of propylene oxide and Epon-Araldite (E/A) with DMP-30 (0.1 ml DMP-30 to 5 ml
E/A). Sections were placed in fresh E/A with DMP-30 in embedding molds and placed in a 64°C oven overnight. Seventy nm thin sections were cut on a Reichert ultramicrotome, mounted on 200 mesh copper grids, contrasted with uranyl acetate and Sato's lead, then examined with a Phillips CM-10 electron microscope at 80 KV. Digital micrographs were acquired and contrast, density, and sharpness of final images were adjusted using Adobe Photoshop CS.

**Neuronal culture and glutamate treatment**

Neuronal cultures were obtained by dissociation of 16-17-day Sprague-Dawley rat embryo brains. The tissue was disrupted into a cell suspension by gentle trituration and the cells were grown on poly-L-lysine coated tissue culture dishes in N5 medium that contained 5% serum fraction that supports the long-term survival of neurons as described (Kaufmann and Barrett, 1983). Cultures were treated with 100 µM glutamate, or pretreated with 50-50,000 ng/ml Ro 25-6981 30 minutes prior to glutamate treatment. Cell viability was assayed by adding 50 µg (final concentration) of propidium iodide to neuronal culture for 20 minutes, and reading at 595 nm.

**Statistical Analysis**

**Immunoblot analysis**

Data are expressed as mean ± S.E.M. Quantification of bands corresponding to changes in protein levels was made using scanned densitometric analysis and NIH Image Program 1.62f. Between group differences in immunoblots were analyzed using one-way analysis of variance (ANOVA), followed by Tukey post hoc comparison. Cortical lysate and neuronal
cultures were normalized to β-tubulin, and membrane rafts were normalized to caveolin 1. *p ≤ 0.05 vs sham at 15 min.

**Autophagic vacuole quantification**

For electron microscopy, a copper grid (200 mesh) was placed over mounted tissue and sections were randomly sampled such that every fifth grid was analyzed. Therefore, 40 grid sections per mounted tissue sample were analyzed. Five neurons per grid sections were randomly chosen for the determination of the number of autophagic vacuoles present in each neuron. Autophagic vacuoles (AV’s) were identified by morphological criteria as described (Liu et al., 2008). Briefly, initial AV’s (AVi’s) were identified by their hallmark double membrane morphology. Mitochondria were distinguished from AVi’s by the presence of cristae and relative size (AV’s ~300-900 nm, mitochondria ~0.15-1 µm). Degradative AV’s (AVd’s) were identified as having a single membrane structure containing electron dense material. These AVd’s are different from damaged mitochondria on the basis of electron density and relative size. Quantification of autophagic vacuoles was by two-tailed student t-test. *p ≤ 0.05 vs sham at 15 min.

**Results**

**TBI acutely increases cortical NR2B and pCaMKII**

NR signaling at synapses is mediated by interaction with the scaffolding protein PSD 95 and other signaling intermediates (Kornau et al., 1995; Wu et al., 2005). Calcium influx through NRs activates CaMKII (Ulrich Bayer et al., 2006; Meng et al., 2003; Meng et al., 2004) that leads to downstream initiation of
multiple signaling cascades. In order to define whether TBI altered proteins in NR signaling cascades, cortical lysates from control and injured animals at various times after trauma were analyzed by immunoblotting procedures (Fig. 3A). TBI induced an acute increase in the levels of NR2B and pCaMKII in cortical lysates from injured animals at 1, 4h and 8h after TBI. However levels of NR2B and pCaMKII had returned to baseline levels thereafter. TBI did not affect the levels of PSD 95 in lysates at any time-point measured. Thus, TBI induces significant and dynamic changes in NR2B and pCaMKII expression, but does not significantly alter the levels of PSD 95.

**TBI induces an increase in Beclin 1 and key autophagic protein expression in the injured cortex**

We next determined whether TBI induced changes in signaling intermediates in the autophagy pathway. Beclin 1 mediates the localization of autophagic proteins to nascent membranes and is required for the induction of autophagy (Liang et al., 1999; Petiot et al., 2000). Pro-autophagic proteins ATG 5 and ATG 7 are necessary for autophagic vacuolization (Yu et al., 2004; Boya et al., 2005; Pyo et al., 2005; Komatsu et al.; 2006). The processed form of LC3 (LC3II) localizes in autophagosome membranes (Kabeya et al., 2000) and can be used as a marker for autophagic vacuole formation (Kabeya et al., 2000; Mizushima et al., 2001; Boya et al., 2005). To determine the temporal profile of expression of autophagic proteins induced by TBI, cortical lysates were analyzed for Beclin 1, ATG 5, ATG 7, and LC3II from the ipsilateral hemispheres and sham-operated animals (Fig 3B). Increased levels of Beclin 1 and ATG 7 were observed in traumatized cortices by 4 h following TBI, and elevated levels were
maintained until 48 h post trauma. Increases in the lipidated form of LC3 were observed as early as 1 h following TBI, and a significant increase in ATG 5 was observed by 24 hours post trauma. In contrast, the levels of pMTOR and p-p70 S6K, negative regulators of autophagy, decreased in lysates of injured animals at 24 and 48h after TBI (Fig. 3B). No changes were observed in the levels of mTOR, p70 S6K and β-tubulin that served as internal standards or loading controls. Thus, key proteins in the autophagic pathway are significantly up-regulated in the injured cerebral cortex, and negative regulators of the process are significantly down-regulated following TBI.
Figure 3. TBI induces changes in NR2B signaling intermediates and autophagy proteins in the injured cortex. **A.** Immunoblot analysis of NR2B, pCaMKII, PSD95. Rats were subjected to moderate TBI, and cerebral cortices were harvested at the indicated times after injury. Levels of NR2B and pCaMKII were increased at 1 and 4 h after injury. **B.** Immunoblot analysis of Beclin 1, LC3, ATG5, ATG7, pMTOR, p-p70 S6K, MTOR, p70 S6K. Levels of Beclin 1, LC3, ATG5 and ATG7 significantly increase after injury but with different time courses. In contrast, levels of the negative regulator of autophagy pMTOR and p-p70 S6K show significant decreases after trauma. *p ≤ 0.05 vs sham at 15 min. MTOR and p70 S6K were used as internal standards. β-tubulin was used as a loading control. n=8 for each group.
Beclin 1 is present in cortical neurons, and TBI induces alterations in protein expression pattern

Figure 4 shows confocal images of the cell type expression and regional distribution of Beclin 1 and LC3 in neurons in cerebral cortices of sham-operated animals and injured cortices at 24 h after injury. Sections were stained for Beclin 1, LC3 (red) and the neuronal markers NeuN and MAP2 (green). Beclin 1 immunoreactivity was seen in NeuN and MAP2 positive cells, indicating that Beclin 1 is expressed in neurons in the cortex of sham animals. Beclin 1 immunoreactivity was observed in a diffuse pattern in the perinuclear region of NeuN positive cells (red and merged, row 1), and less intense immunostaining was seen in MAP2 positive cells (red and merged, row 3). LC3 expression in cortical neurons of sham-operated animals was also detected using immunohistochemistry and confocal microscopy (red and merged, row 5).

Moderate TBI resulted in altered staining patterns of Beclin 1 and LC3 in cortical neurons (Fig. 4). At 24 h after injury, increased Beclin 1 immunoreactivity was present in neurons surrounding the injury epicenter and exhibited a punctate staining pattern in the cell soma (red and merged, row 2). Increased Beclin 1 expression was also present in dendritic processes of MAP2 positive cells and exhibited a punctate staining pattern (red and merged, row 4). Moreover, by 24 h after injury, increased LC3 immunoreactivity was seen in NeuN positive cells located around the injury epicenter (red and merged, row 6). LC3 immunoreactivity exhibited a punctate staining pattern similar to that observed for Beclin 1. Thus, TBI induces alterations in Beclin 1 and LC3 immunoreactivity consistent with formation of autophagic vacuoles.
Figure 4. Beclin 1 and LC3 autophagy proteins are present in cortical neurons, and TBI induces alterations in their expression patterns. Confocal images show cortical neurons near the injury epicenter of sham and injured brains at 24 h after TBI. Sections were stained for Beclin 1 and LC3 (red) and the neuronal markers NeuN and MAP2 (green). Beclin 1 immunoreactivity (red and merged, row 1 and 3) was observed in a diffuse pattern in the perinuclear region of NeuN positive cells and processes of MAP2 positive cells. Twenty-four hours after TBI, there was increased immunostaining of Beclin 1 in cortical neurons that was distributed in a punctate pattern in the cell soma and in processes (Rows 2 and 4). LC3 immunoreactivity was present as punctate inclusions in NeuN positive cells in sham-operated animals (red and merged, row 5) and showed increased staining 24 h after injury (red and merged, row 6). Scale bars = 10 µm.
Ultrastructural analysis of autophagic maturation after TBI

Induction of autophagy is manifested by the formation of autophagosomes (APs) that enclose cytoplasmic constituents. APs are double membrane vacuoles and are readily identified by electron microscopy (EM) procedures, and are hallmarks of autophagy. To establish whether TBI-induced increased expression of autophagic proteins resulted in autophagic maturation, cortical sections of sham and injured animals at 3 days after trauma were examined by transmission EM. Cortical neurons in sham-operated animals contained a small number of initial autophagic vacuoles (AVi) (Fig. 5A, B) and degradative vacuoles (AVd) (Fig. 5C). AVi’s and AVd’s were identified as double membrane structures (arrowheads) and were seen in cell processes and the axon trunk, as well as in perinuclear areas, thus indicating that autophagy is a constitutive physiological process in normal cortical neurons. Cortical neurons of rats at 3 days after TBI contained significantly more AVi’s and AVd’s (Fig. 5F). Moreover, large AV structures (Fig. 5 D,E) were frequently observed in injured cortical neurons ultrastructurally consistent with induction of autophagy after TBI.
**Figure 5.** Ultrastructural evidence for autophagy in normal cortical neurons and 3 days after TBI in adult male rats. Transmission electron micrographs of cortical neurons from sham-operated animals (A-C) and cortical neurons near the injury epicenter at 3 days after trauma (D-E). Arrows point to: isolation membrane (IM), initial autophagic vacuoles (AVi), degradative autophagic vacuoles (AVd), nucleus (Nu), autophagic vacuole cluster (AV cluster), and large autophagic vacuoles (large AV). Arrowheads point to double membrane vacuoles. (F) Quantification of number of AVi and AVd per cortical neuron in sham and injured brains at 3 days after trauma. *p ≤ 0.05 vs sham at 15 min. Scale bars = 500 nm. n=6 for each group.

**TBI alters Beclin 1, NR2B, and pCaMKII distribution in membrane rafts in the injured cortex**

To investigate whether membrane rafts are involved in NR2B signal transduction pathways after TBI, membrane rafts were isolated from sham-operated and traumatized cortices by discontinuous sucrose gradient centrifugation. The purity of membrane raft preparations was confirmed by immunoblotting gradient fractions with the membrane raft markers: flotillin 1, cholesterol-binding protein caveolin 1, GPI-anchored cell surface protein Thy 1, and sphingolipid ganglioside G\(_M1\), and Triton X-100 soluble (TS) fractions were identified by the non-membrane raft marker Na\(^+\)/K\(^+\)-ATPase (Fig. 6A). Low-density membrane raft fractions (fraction 2, labeled R) of gradients from both sham-operated and injured animals were enriched in flotillin 1, caveolin 1, Thy 1, and G\(_M1\), and the high-density Triton X-100 soluble fraction (fraction 8, labeled TS) was enriched in Na\(^+\)/K\(^+\)-ATPase. Although small amounts of NR2B, pCaMKII, PSD 95 and Beclin 1 were associated with membrane rafts in cortices of sham animals, most of these proteins were excluded from these microdomains (Fig. 6B). Association of NR2B and pCaMKII with membrane rafts was significantly increased by 1-8 h after TBI, but levels declined thereafter. In
contrast, there was a significant translocation of Beclin 1 out of membrane rafts, rapidly at 15 min and 1 h after TBI. Significant increases in NR2B, pCaMKII, and Beclin 1 were also observed in TS fractions of the gradient, exhibiting a similar temporal profile (Fig. 3 A,B) as that observed in cortical lysates (data not shown). Thus, a portion of NR2B, pCaMKII, PSD 95 and Beclin 1 are present in membrane rafts, and TBI signals recruitment of NR2B and pCaMKII to rafts, but rapid translocation of Beclin 1 out of rafts.

Figure 6. Partitioning of NR2B, signaling intermediates and Beclin 1 in membrane rafts in the normal and injured cortex. A. Density gradient profile of detergent-resistant membranes from cortices of sham-operated animals. Cortical membranes were extracted with 1.0% Triton X-100 at 4°C and separated on density gradients formed from 40%, 30%, and 5% sucrose. Eight fractions (from top to bottom of the gradient) were immunoblotted for the indicated proteins. Fraction 2 of the gradient was enriched for caveolin 1, flotillin 1, Thy 1 and GM1, indicating the membrane raft containing fraction (R), while fraction 8 contained the Na+/K+-pump, indicating the non-membrane raft or soluble fraction (TS). B. TBI induces recruitment of NR2B and pCaMKII into membrane rafts and translocation of Beclin 1 out of membrane rafts. Equal aliquots of the fractions
were subjected to SDS-PAGE, and the protein distribution was assessed by immunoblotting using specific antibodies against NR2B, pCaMKII, PSD 95, Beclin 1 and caveolin 1. * p ≤ 0.05 vs sham at 15 min. n=8 for each group.

**NR2B and signaling intermediates form a novel protein complex with Beclin 1 in membrane rafts that is disrupted after TBI**

When proteins are present in membrane rafts, association of those proteins is favored compared with that of proteins excluded from membrane rafts (Friedrichson and Kurzchalia, 1998). To assess the protein composition and association of signaling proteins in the NR2B signaling complexes, membrane rafts were isolated and immunoprecipitated with anti-NR2B antibody (Fig. 7A). In membrane rafts of cortices from sham-operated animals, NR2B was associated with PSD 95, Homer, Shank, and surprisingly with the autophagy protein Beclin 1. Within 15 min after TBI, the composition of the complex changed. There was a substantial loss of NR2B-associated adaptor proteins, PSD 95, Homer, and Shank, and the autophagic signaling protein Beclin 1. A transient association of pCaMKII was observed in the NR2B signaling complex at 15 min, 1 and 4 h after TBI. Anti-NR2B did not immunoprecipitate the raft-associated protein flotillin 1, demonstrating specificity in this membrane raft associated protein complex. In soluble fractions of cortical lysates of sham animals, anti-NR2B antiserum immunoprecipitated NR2B, but did not immunoprecipitate NR2B signaling intermediates PSD 95, Shank, Homer and the autophagic protein Beclin 1 (Fig. 7B), indicating that these proteins are not associated with each other in non-membrane raft fractions. In a reciprocal immunoprecipitation, from membrane rafts, using anti-Beclin 1 (Fig. 7C), NR2B was immunoprecipitated in the sham brain, thus serving as a positive control. Taken together, these findings show that
NR2B, PSD 95, Homer, Shank, and Beclin 1 form a novel complex in membrane rafts of cortical neurons in the normal brain. TBI induces translocation of NR2B into membrane rafts and Beclin 1 out of membrane rafts, altering formation of receptor-associated signaling complexes that may regulate different biological outcomes dictated by these complexes.

Figure 7. Membrane raft associated NR2B signaling complex is altered following TBI. A. Co-immunoprecipitation with anti-NR2B of membrane raft fractions of cortical lysates obtained from sham animals and at 15 min to 48 hr after TBI. NR2B immunoprecipitates were blotted for NR2B, PSD 95, Shank, Homer, Beclin 1, pCaMKII and Flotillin 1. In sham animals, anti-NR2B immunoprecipitated
NR2B, PSD 95, Shank, Homer, and Beclin 1, thus indicating association of these proteins in a multiprotein complex. TBI induced disassociation of this multiprotein signaling complex within 15 min. B. Co-immunoprecipitation using anti-NR2B demonstrated similar protein associations of NR2B signaling intermediates in cortical membrane rafts in the contralateral hemisphere of sham animals. However, the NR2B multiprotein signaling complex was not present in Triton X-100 insoluble fractions (TS) isolated from cortical lysates. C. Reciprocal coimmunoprecipitation with Beclin 1 of membrane rafts from sham and injured cortices and rafts obtained from injured animals. Anti-Beclin 1 immunoprecipitated Beclin-1 and NR2B in sham animals only, further indicating association of these proteins in a multiprotein complex in membrane rafts.

NR2B but not NR2A antagonist inhibits NR2B signaling and delays TBI-induced increases in Beclin 1 and key autophagic protein expression in the injured cortex

We next tested whether NR2B and NR2A-containing receptors exert differential roles in mediating NMDA-induced signaling and autophagy in traumatized brains using subunit specific NR antagonists: Ro 25-6981, which specifically blocks NR2B containing receptors (Fischer et al., 1997) and NVP-AAM077, which preferentially inhibits NR2A-containing receptors (Liu et al., 2004). As proof of concept, animals were injected intravenously with 1 mg/mL/Kg of antagonist 30 min prior to TBI whereas sham animals served as controls. Cortical lysates were prepared at various times after injury and immunoblotted for NR2B signaling intermediates (Fig. 8) and autophagic proteins (Fig. 9). Pretreatment with Ro 25-6981 inhibited the TBI-induced increase in NR2B and pCaMKII (Fig. 8A), whereas NVP-AAM077 had little or no effect (Fig. 8B), indicating a critical involvement of NR2B but not NR2A-containing NR subtypes in mediating TBI-induced excitotoxic effects.

To examine antagonist effects on the autophagic signaling pathway, Ro 25-6981 and NVP-AAM077 treated animals were subjected to TBI and cortical
lysates were analyzed by immunoblotting procedures (Fig. 9). Animals pretreated with the NR2B antagonist showed increases in Beclin 1, LC3 and ATG 7, but the rise in these protein levels were delayed when compared to the acute increase observed in untreated animals (Fig. 9A). Levels of ATG 5 and pMTOR did not change after TBI, whereas levels of p-p70 S6K showed a significant decrease at 24 and 48 h after TBI (Fig. 9A). Animals pretreated with the NR2A antagonists (Fig. 9B) demonstrated increases and time-courses of expression of autophagic proteins similar to those observed in untreated animals (Fig. 3B), except a significantly earlier decreases in pMTOR and p-p70 S6K were observed. These results support the idea that NR2B-containing NRs specifically mediate TBI-induced up-regulation of key proteins in the autophagic pathway.
Figure 8. NR2B antagonist but not NR2A antagonist inhibits TBI-induced increases in expression of NR2B signaling intermediates in the injured cortex. Representative immunoblots of cortical lysates from sham animals and injured animals injected intravenously with 1 mg/ml/kg of Ro 25-6981 or NVP-AAM077 at 30 min prior to TBI. A. Ro 25-6981 (NR2B antagonist) blocked TBI induced increases in NR2B signaling intermediates. B. NVP-AAM077 (NR2A antagonist) had no effect on expression of NR2B signaling intermediates, where protein profiles were similar to those in untreated animals subjected to TBI (Fig. 1). *p ≤ 0.05 vs sham at 15 min. n=6 for each group.
Figure 9. NR2B antagonist but not NR2A antagonist inhibits TBI-induced increases in expression of autophagic proteins in the injured cortex. Representative immunoblots of cortical lysates from sham animals and injured animals injected intravenously with 1 mg/ml/kg of Ro 25-6981 or NVP-AAM077 at 30 min prior to TBI. **A.** Pretreatment with the Ro 25-6981 (NR2B antagonist) blocked the TBI-induced increases in protein expression of autophagic proteins after TBI (**B**). NVP-AAM077 (NR2A antagonist) did not alter expression of autophagic proteins after TBI, where protein profiles in both signaling pathways were similar to untreated animals subjected to TBI (Fig. 1). *p ≤ 0.05 vs sham at 15 min. n=6 for each group.
NR2B but not NR2A antagonist alters the protein composition and association of NR2B signaling complexes with Beclin 1 in membrane rafts

As shown in Fig 7, a portion of NR2B in cortical tissue is associated with membrane rafts where it forms a signaling complex that includes NR2B, PSD95, Shank, Homer, and Beclin 1. TBI disrupts protein associations in this complex and induces translocation of Beclin 1 out of membrane raft fractions. To determine whether NMDA antagonists altered protein associations of NR2B and Beclin 1, membrane rafts were isolated from cortices of injured rats pretreated with Ro 25-691 and NVP-AAM077 and immunoprecipitated with anti-NR2B (Fig. 10). NR2B, PSD 95, Homer, Shank, and Beclin 1 were immunoprecipitated with NR2B in sham animals. However, pretreatment of injured animals with the NR2B, but not the NR2A antagonist, significantly delayed the disassociation of Beclin from NR2B signaling intermediates and blocked the translocation of Beclin 1 out of membrane rafts. Thus, it appears that the dissociation of NR2B signaling intermediates and Beclin 1 after TBI in membrane rafts is dependent on NR2B activity.
Figure 10. NR2B but not NR2A antagonist inhibits TBI-induced alterations of protein associations of NR2B signaling proteins in membrane rafts. A. Co-immunoprecipitation with anti-NR2B of membrane raft fractions of cortical lysates obtained from sham animals and animals pretreated with injected intravenously with 1 mg/ml/kg of Ro 25-6981 (A) or 1 mg/ml/kg of NVP-AAM077 (B) at 30 min prior to injury. Membrane rafts were isolated from animals at 15 min to 48 hr after TBI and immunoprecipitated with anti-NR2B. NR2B immunoprecipitates were blotted for the indicated proteins. In sham animals, anti-NR2B immunoprecipitated NR2B, PSD 95, Shank, Homer, Beclin 1, thus indicating association of these proteins in a multiprotein complex. Animals pretreated with Ro 25-6981 (NR2B Antagonist) did not show TBI-induced disassociation of this multiprotein signaling complex at 15 min after trauma as observed in untreated animals subject to trauma (Fig. 5A). However, animals pretreated with the NVP-AAM077 (NR2A antagonist) demonstrated similar pattern of association of NR2B signaling proteins as those observed in untreated sham and injured animals (Fig. 7).
NR2B antagonist blocks glutamate excitotoxic death and induction of autophagy in primary neuronal cultures

Glutamate excitotoxicity contributes to neuronal cell death in primary neuronal cultures (Regan and Choi, 1994) and following TBI (Faden et al., 1989). To determine whether NR2B antagonists blocked glutamate excitotoxicity, we pre-treated primary neuronal cultures with Ro 25-6981 followed by glutamate treatment (100 µM). Figure 11 A, B show a dose response of neuroprotective effects of Ro 25-6981. At concentrations of 50-10 x 10³ ng/ml, the Ro 25-6981 significantly blocked neuronal death. At doses higher than 50 x 10³ ng/ml, Ro 25-6981 was toxic. Thus, NR2B antagonist significantly blocks glutamate excitotoxicity of primary neurons. To investigate whether glutamate exposure induces autophagy protein expression in primary neuronal cultures, we treated cortical neurons with 100 µM glutamate and analyzed autophagy protein expression by immunoblot analysis. Significant increases in Beclin 1 and LC3 and lipidated LC3II were observed at 2-6 hours after glutamate treatment (Fig. 11C). Pre-treatment with the NR2B antagonist Ro 25-6981 blocked the expressional changes observed in Beclin 1 and LC3 treated with glutamate (Fig. 11D). Taken together, these results indicate that glutamate treatment of cortical neurons results in significant cell death and increases expression of autophagy proteins. NR2B antagonist Ro 25-6981 blocks cell death and the induction of autophagy proteins by glutamate treatment.
Figure 11. Glutamate treatment of primary neuronal cultures results in activation of autophagy and cell death that is blocked by the NR2B antagonist Ro 25-6981. 

A. Primary cortical neurons were treated with 100 µM glutamate for 8 hours resulted in cell death as assayed by propidium iodide. Ro 25-6981 (10 ng/ml – 25 µg/ml) administered 30 minutes prior to glutamate treatment significantly decreased glutamate induced cell death.

B. Immunoblots of primary cortical neurons treated with 100 µM glutamate for 8 hours resulted in significant increases in Beclin 1 and LC3II at 2-6 hours after glutamate treatment.

C. Ro 25-6981 (100 ng/ml) administered 30 minutes prior to glutamate treatment prevented increases in Beclin 1 and LC3II. *p ≤ 0.05 vs control. n=6 wells for each group.
Consideration

Our data show that in the normal cortex, a portion of the NR2B receptor, the adaptor proteins PSD95, Shank and Homer, and the autophagic protein Beclin 1 localize in membrane rafts forming a novel multi-protein complex. TBI induced a dissociation of the protein complex and caused a rapid increase in the levels of NR2B, a translocation of pCaMKII to membrane rafts, and a translocation of Beclin 1 out of membrane raft microdomains. These findings suggest that membrane rafts mediate trafficking and signaling of NR2B and Beclin 1 following TBI.

The role of membrane rafts in the signal transduction of NR’s has been established in vitro (Frank et al., 2004) and in rat models of ischemia (Hu et al., 1998; Hering et al., 2003; Besshoh et al., 2005), and NR’s located in membrane rafts mediate neurotoxicity (Frank et al., 2004; Abulrob et al., 2005). Recent reports demonstrate that NR activation can produce either neuronal survival or death-promoting actions (Liu et al., 2007; Zhang et al., 2007) and that this dual action can be modulated by receptor subunit composition (Liu et al., 2007). NR2B-containing receptors have been shown to mediate pro-survival effects in several neuronal populations (Takadera et al., 2004; Hetman and Kharebava, 2006) and to contribute to cytotoxic cell death and apoptosis (Liu et al., 2007). Our findings extend these observations and indicate that following TBI, the composition of NR2B receptor-associated signaling complex that is found in membrane raft microdomains, undergo changes that may contribute to the activation of signal transduction pathways involving autophagy.
Post-translational modifications of several complex-associated proteins occur in membrane rafts in traumatized brains. For example, influx of extracellular calcium through activated NR’s following TBI increases CaMKII phosphorylation, and pCaMKII has been shown to interact transiently with NR2B (Ulrich-Bayer et al., 2001; Meng et al., 2002). However, prolonged NR2B stimulation results in a transition from reversible to persistent binding of pCaMKII to NR2B (Ulrich-Bayer et al., 2006), thus causing phosphorylation of the receptor. NR2B phosphorylation (Meng et al., 2002) and subsequent internalization (Vissel et al., 2001; Aarts and Tymanski, 2004) occurs via membrane raft microdomains (Besshoh et al., 2005). Moreover, pCaMKII binding to NR2B decreases NR2B association with PSD95 (Gardoni et al., 2001) thus exposing the internalization motif on the receptor (Roche et al., 2001). Disruption of NR2B/PSD95 interaction contributes to receptor destabilization at the membrane. Therefore, the phosphorylated state of NR2B may contribute to its internalization via membrane raft microdomains following TBI.

The data reported here identify novel protein interactions among the evolutionarily conserved autophagic protein Beclin 1, the NR2B receptor, and the synaptic scaffolding proteins PSD95, Shank, and Homer within membrane raft microdomains in the normal cortex. These interactions provide a physical linkage between the NR2B receptor and Beclin 1. At the synapse, NR2B and PSD95 have a known interaction through their common PDZ ligand domain (Roche et al., 2001; Dong et al., 2004; Kim and Sheng, 2004; Sheng, 2007). PSD95 interaction with Shank via PDZ/SH3 domains, and Shank interaction with
Homer via proline rich motifs, contributes to synaptic organization, the regulation of protein interaction and cytoplasmic signaling pathways (Migaud et al., 1998; Sprengel et al., 1998), acting as a key modular subdomain of the post-synaptic specialization (Sheng, 2001). It is likely that Beclin 1 is associated with this NR2B protein complex via interaction with Homer through their common coil-coil domains, and that this interaction is facilitated within membrane raft microdomains.

TBI induced a rapid recruitment of NR2B into membrane rafts, but caused a translocation of Beclin 1 out of these microdomains. Co-immunoprecipitation of the NR2B signaling complex revealed that NR2B/PSD95/Shank/Homer/Beclin 1 interactions in membrane rafts were lost after TBI, suggesting that release of Beclin 1 or PSD95/Shank/Homer/Beclin 1 from the complex in response to excessive stimulation of NR2B following TBI may be a critical event required for activation of autophagy in neurons. In support of this idea is our observation that inhibition of NR2B receptor signaling by the NR2B antagonist Ro 25-6981 delays the triggering of intracellular cascades that lead to autophagy and NMDA- or ischemia-induced neuronal apoptosis. The inability of the NR2A antagonist NVP-AAM077 to block TBI-induced changes in NR signaling and autophagy is an additional indication that NR2B receptor pathways have specific biological effects. Moreover, in the Lurcher mouse, GluRδ2 is linked to the autophagy process through similar protein-protein interactions involving an isoform of PIST and Beclin 1 (Yue et al., 2002). Delineation of the precise mechanism by which NR2B leads to induction of autophagy will require additional studies aimed at
determining the properties of NR2B/PSD95/Shank/Homer/Beclin 1 complex. For example, it is not clear whether Beclin 1 is covalently modified in response to signaling through the receptor and whether this modification leads to release of one or several of these proteins from the complex. As well, Beclin 1 is a Bcl-2 interacting protein (Liang et al., 1998) and is found as a component of the class II PI3 kinase complex, which is involved in signal transduction pathways involved in both apoptosis and autophagy. The anti-apoptotic protein Bcl-2 directly binds to Beclin 1, attenuating autophagy-dependent cell death (Kihara et al., 2001; Levine and Yuan, 2005: Pattingre et al., 2005), as well as regulating autophagy under non-toxic conditions (Shimizu et al., 2004; Luo and Rubensztein, 2007), suggesting Bcl-2 may function as an anti-apoptotic and anti-autophagic protein.

Autophagy is a pathway for lysosome-mediated bulk degradation of subcellular constituents, and contributes to routine turnover of cytoplasmic components (Shintanti and Klionsky, 2004). This process has been implicated in a number of neurodegenerative pathologies (Larsen and Sulzer, 2002), and significant increases in Beclin 1 have been observed in TBI (Diskin et al., 2005; Clark et al., 2008; Liu et al., 2008). Whether increased Beclin 1 expression leads to the induction of autophagy or whether it contributes to apoptotic signaling is currently under debate. Several key autophagic proteins have a direct interaction with the apoptotic pathway, and the interplay between these processes remains unclear. Inhibition of autophagy can trigger apoptosis (Boya et al., 2005; Takacs-Vellai et al., 2005) and apoptotic signals can activate autophagy (Xue et al., 1999). Recent studies report that ATG5 interacts with the Fas-associated protein
with death domain (FADD), a component of the extrinsic apoptotic pathway, which leads to interferon-induced cell death (Pyo et al., 2005), and thus provide another point of convergence of apoptotic and autophagic mechanisms. ATG5 is cleaved following Fas receptor activation, which can lead to cell death through interaction with Bcl-xl promoting Bax-induced mitochondrial dysfunction and apoptosis (Yousefi et al., 2006). Alternatively, autophagy may be a process that promotes survival. Autophagy is constitutively active at low basal levels to perform homeostatic functions, contributing to nutrient and energy conservation, subcellular remodeling and protein turnover (Baehrecke, 2005; Levine and Yuan, 2005). Increased death has been demonstrated in rodent models lacking ATG5, ATG7, and Beclin 1 gene products (Liang et al. 1998; Lum et al., 2005). Specifically in the nervous system, loss of ATG7 has been observed to lead to neurodegeneration, confirming autophagy as a critical function for survival in neurons (Komatsu et al., 2006), and in this regard, may provide a mechanism of re-establishing homeostasis following injury. Moreover, pro-autophagic proteins ATG 5 and ATG 7 are necessary for autophagic vacuolization (Yu et al., 2004; Boya et al., 2005; Pyo et al., 2005; Komatsu et al.; 2006). The processed form of LC3 (LC3II) localizes in autophagosome membranes (Kabeya et al., 2000) and can be used as a marker for autophagic vacuole formation (Kabeya et al., 2000; Mizushima et al., 2001; Boya et al., 2005). Here we observed an increase in these and other key autophagic proteins early after TBI, and a significant decrease in the negative regulator of autophagy mTOR that may contribute to increases in the number of autophagosomes in neurons observed after trauma.
Glutamate excitotoxicity contributes to secondary injury following TBI (Faden et al., 1989). Our data demonstrates that glutamate induced cell death of primary neuronal cultures, activates the autophagy pathway. This activation is significantly attenuated by the NR2B antagonist Ro 25-6981 at time points measured, thus demonstrating a link between NR2B signaling, induction of autophagy and cell death. Further, as proof of concept, Ro 25-5981 was administered intravenously 30 min prior to the traumatic insult. We have shown that Ro 25-6981 substantially delayed the dissociation of the NR2B-Beclin 1 containing signaling complex, and substantially delayed the induction of autophagy in vivo, further supporting the link between NR2B signaling and autophagy. Since Ro 25-6981 has a half-life of dissociation of approximately 5 hrs (Mutel et al., 1998), it is likely that further studies are needed to determine whether repeated administration of the antagonist will completely block autophagy induction following TBI.

Our findings demonstrate that moderate TBI induces a rapid redistribution of a novel NR2B multi-protein signaling complex, where the key autophagic protein Beclin 1, is translocated out of membrane raft microdomains. Thus, the release of Beclin 1 or PSD95/Shank/Homer/Beclin 1 from the complex in response to excessive stimulation of NR2B following TBI may be a critical event required for activation of autophagy in neurons. Autophagy has been implicated as an active process in other models of TBI (Diskin et al., 2005; Clark et al., 2008; Liu et al., 2008), however, its role as a protective or detrimental process is still unclear. Understanding of glutamatergic signaling through NR2B, and its
relationship to autophagic activation, may provide insight into the effects of excitotoxicity on cellular processes after injury and lead to development of pharmacological management of pathological cell loss.

Acknowledgments

We thank Ana Gomez for help with transmission electron microscopy and Doris Nonner for preparation of rat primary neuronal cultures. This work was supported by grants from NIH/NINDS, NS30291 and NS42133.
Chapter III

GENERAL DISCUSSION

Numerous reports have extensively documented that glutamate excitotoxicity is an important component of the prolonged secondary injury cascade that contributes to pathology after TBI (Choi, 1987, 1988; Personn and Hillered, 1992; Reeves et al., 1997). Despite these observations, the results of clinical trials testing NR antagonists as neuroprotectants after TBI have been discouraging. The experiments presented in my thesis introduce several novel findings that identify a glutamatergic signaling platform and multiprotein complex in membrane rafts involved in the activation of the autophagic process following TBI. First, my data shows that in the normal cerebral cortex, neurons express a multiprotein complex consisting of the NR2B glutamate receptor, the postsynaptic scaffolding proteins PSD 95, Shank, and Homer, and the autophagic protein Beclin 1. Second, this NR2B complex is present in membrane raft microdomains, which provide a platform for the specific protein interactions involved in glutamatergic signaling. Third, moderate TBI induces alterations in protein associations of this complex that leads to activation of pro-autophagic proteins resulting in a significant increase in autophagic vacuole formation. Fourth, an NR2B-specific antagonist prevents TBI-induced alterations of the membrane raft-associated NR2B complex, and attenuates the activation of autophagic proteins in vivo. Fifth, treatment of primary neurons in culture with the NR2B antagonist significantly reduces glutamate-mediated activation of autophagic signaling and inhibited excitotoxic cell death in vitro. Thus, this study
shows for the first time that a membrane raft-associated NR2B signaling complex activates autophagy after TBI, and suggest that targeting this complex may serve as a possible therapeutic intervention to prevent pathology after TBI.

I. **NR2B RECEPTOR AND TBI PATHOLOGY**

My experiments show a significant increase in NR2B expression at 1 h and 4 h after TBI, suggesting an acute response of the traumatized brain to cope with excessive glutamate stimulation induced by the initial insult. NR2B mRNA and subunits are expressed in dendritic compartments and in the endoplasmic reticulum (Fukaya et al., 2003; Giza et al., 2006) and may provide the necessary substrates and machinery for the acute increase in NR2B expression observed in my studies. Glutamate excitotoxic effects are mediated predominantly through NR type glutamate receptors that cause a significant increase in calcium influx thus activating a myriad of intracellular signaling cascades responsible for the slower, progressive cytotoxicity (Choi, 1987, 1988). Results were identified in cortical (Choi, 1987; Marcoux et al., 1988) and hippocampal (Siman and Noszek, 1988) neuronal cultures, and in cerebellar slices (Garthwaite and Garthwaite, 1986) demonstrating that both the influx of extracellular Ca\(^{2+}\) and Ca\(^{2+}\) release from intracellular stores activate kinases (PKC), lipases (phospholipase) and result in free radical production leading to acute excitotoxic cell swelling and necrotic cell death. However, the precise mechanism responsible for NR activation remains controversial. For example, the receptor composition mediating glutamate excitotoxicity is not known. Moreover, the precise level of activation, the receptor location on the neuron and the signaling cascade
responsible for downstream effects remain undefined. Recent studies also using cortical neuronal culture show that hyperactivation of extrasynaptic NR2B-containing receptors results in excitotoxicity and increasing neuronal apoptosis (Liu et al., 2007). Moreover, Zhang and colleagues (Zhang et al., 2007) report that electrical stimulation of synaptic NRs induce coordinated up-regulation of pro-survival genes whereas stimulation of extra-synaptic receptors induce cell death in cultured hippocampal neurons. These studies suggest that the receptor subtype cellular localization influences the differential roles of NRs in promoting cell survival and death. The present study shows that the NR2B glutamate receptor is present in membrane rafts in cortical neurons, \textit{in vivo}, where it complexes with the autophagic protein Beclin 1. Moderate TBI induces alterations in protein associations of this complex in which Beclin 1 translocates out of membrane rafts and no longer associates with the membrane receptor, which we observe to direct the up-regulation of autophagic proteins and activation of autophagy. However, the exact localization of this NR2B receptor complex, and the role of autophagy in the overall pathology of TBI is still not fully understood. Further experiments that investigate sub-cellular localization, including electron microscopy and NR2B immuno-labeling, will be useful in further assessing synaptic and extra-synaptic localization, both \textit{in vivo} and \textit{in vitro}. It is possible that NR2B receptor complexes in membrane rafts are located in extra-synaptic sites that are stimulated by glutamate overload at the synapse. These data, may support similar findings of NR2B localization using both cortical and hippocampal cultures (Liu et al., 2007; Zhang et al., 2007) and help
differentiate whether NR2B signaling may result in a survival or death response. It is also possible that there are heterogeneous populations of NR2B containing receptors in both synaptic and extra-synaptic sites that have differential signaling and subsequent outcomes. In this manner, experiments involving co-immuno-labeling at the electron microscopic level, can provide information regarding distinct localization of different NR2B receptor complexes. Thus, it could be determined whether the NR2B-Beclin 1 complex reported in my studies, is found in synaptic or extra-synaptic regions, which may further provide evidence for the activation of autophagy as protective or detrimental following TBI.

Another important result in my studies is the finding that pCaMKII associates with NR2B within 15 min following TBI. NR2B hyper-activation results in excessive calcium-influx that phosphorylates and activates CaMKII that stabilizes interaction with NR2B (Ulrich-Bayer et al., 2006) and subsequent internalization (Vissel et al., 2001; Meng et al., 2003; Aarts and Tymanski, 2004). A similar internalization and/or decrease in NR2B expression was observed in traumatized lysates at 8 hours after TBI and thereafter. This result significantly highlights the consequence of calcium influx and calcium-mediating signaling in the alteration and signaling of the NR2B multi-protein complex after TBI. The importance of calcium signaling in conferring glutamate-mediated cytotoxic damage in cortical and hippocampal neuronal culture (Choi, 1987, 1988; Marcoux et al., 1988; siman and Noszek, 1988), and in cerebellar slices (Garthwaite and Garthwaite), have been well documented as previously discussed. The in vivo observations presented in my studies suggest a novel link
between NR2B specific and downstream Ca\(^{2+}\) events that activate the autophagic cellular process, and may represent a specific Ca\(^{2+}\)-mediated response that contributes to neurotoxicity.

II. **NR2B-MEDIATED ACTIVATION OF AUTOPHAGY FOLLOWING TBI**

My studies are in agreement with recent reports of the activation of autophagy after TBI (Diskin et al., 2005; Clark et al., 2008; Liu et al., 2008), but these studies have not linked autophagy activation to glutamate-mediated NR signaling cascades. My data show that TBI induces alterations in protein associations of a novel NR2B-Beclin 1 signaling complex by 15 min after injury, and by 4 hours after TBI there is an increase in expression of key autophagic proteins in injured cortical lysates. Blocking NR2B activation using the Ro 25-6981 antagonist, not only prevented the alteration in the NR2B-Beclin 1 signaling complex, but also significantly attenuated the change in expression of autophagic proteins. Therefore, glutamate-mediated activation of NR2B and subsequent signaling appears to initiate autophagic processes after TBI.

As described previously, my studies demonstrate a change in the distribution and localization of Beclin 1 in cortical neurons after injury. Immunohistochemical micrographs illustrate that Beclin 1 shows a diffuse perinuclear organization in the normal brain, but by 24 hours post TBI, Beclin 1 exhibits a punctate pattern of distribution. These results are similar to previous reports using *in vivo* TBI methods (Diskin et al., 2005). Beclin 1 is a peripheral membrane protein and localizes in various discrete protein complexes, including class III PI3 kinase complex (Kihara et al., 2001) and the Bcl-2 multi-protein
complex (Pattingre et al., 2005). Here, I show that Beclin-1 is present in specialized membrane raft domains, in a novel protein complex including NR2B. Thus, Beclin 1 is found in heterogeneous populations, which may contribute to the diffuse organization within neurons of the normal cerebral cortex. The proteins that interact with Beclin 1 contribute to the regulation and localization of the protein. When autophagy is hyper-activated, Beclin 1 behaves as a transport protein that facilitates transport of the ATG5-ATG12 protein complex to nascent membranes thereby enhancing autophagosome formation (Liang et al., 1999; Petiot et al., 2000; Kihara et al., 2001). In this manner, Beclin 1 localizes to autophagosomes that can be observed before vacuole degradation begins. I show here that Beclin 1 localizes and is transported via membrane raft microdomains 4 hours and thereafter injury, consistent with the timing of increased expression of Beclin 1 observed after TBI. Thus, it is possible that membrane raft microdomains may contribute in transporting newly synthesized Beclin 1 to aid in formation of autophagosomes. Further, Beclin 1 is present within the trans-Golgi network (TGN) (Kihara et al., 2001), endoplasmic reticulum and mitochondria (Germain and Shore, 2003; Pattingre et al., 2005), which are organelles that are localized in autophagic vacuoles when autophagy is hyper-activated (Kihara et al., 2001; Bampton et al., 2005; Tasdemir et al., 2008). Therefore, Beclin 1 expression in discrete cellular compartments that converge in the formation of the autophagosome, contribute to the punctate staining pattern observed after TBI.
III. MEMBRANE RAFTS IN NR2B AND AUTOPHAGIC SIGNALING

In response to stimuli, membrane raft microdomains of the plasma membrane include or exclude proteins to variable extents thereby favoring specific protein-protein interactions that modulate the activity of signaling cascades (Brown and London, 2000; Pike, 2006). Membrane rafts have been shown to mediate NR signal transduction (Frank et al., 2004) as they facilitate the interactions of the receptor with intermediate and effector proteins. Moreover, membrane rafts are involved in other cellular processes including membrane trafficking and endocytosis/ exocytosis at the synapse and thus regulate diverse signaling platforms between the synapse and other cellular compartments. Beclin 1 localizes with class III PI3-kinase complexes (Kihara et al., 2001) and Rab9 positive late endosomes (Itakura et al., 2008). A Beclin 1-binding protein UV irradiation resistant associated gene (UVRAG), a regulator of autophagosome formation (Peplowska et al., 2008) disrupts Beclin 1 coil-coil interactions with other proteins such as Bcl-2 (Noble et al., 2008). UVRAG has been shown to recruit both autophagic and endosomal machinery to the late endosome (Peplowska et al., 2008) that directs proteins to the trans Golgi-network (Kihara et al., 2001). In this manner, it is possible that Beclin 1 is released from this regulatory complex and trafficked via alternative endosomal pathways to local trans Golgi-networks where nascent membranes can be utilized for autophagosomal membrane elongation and maturation. Thus it is possible that disruption of Beclin-1 interactions with Homer in the traumatized brain could be mediated by UVRAG. However, further studies using site-specific
mutagenesis and coimmunoprecipitation experiments will be needed to test this possibility.

IV. AUTOPHAGY AND TBI PATHOLOGY

Genetic studies using various animal models have highlighted the importance of autophagy in physiological pathological events (Komatsu et al., 2006). The principal role for autophagy is the supply of nutrients for survival and is responsible for the recycling of amino acids, bulk protein removal and digestion of subcellular organelles contributing to homeostatic conditions (Baehrecke, 2005; Levine and Yuan, 2005). In response to an increase in various stimuli, autophagic processes are increased to maintain normal cellular conditions and function, and conservation of the cell. Moreover, constitutive autophagy, which occurs independently of nutrient stress, contributes to mouse liver homeostasis (Komatsu et al., 2006), major histocompatibility class II antigen presentation (Paludan et al., 2005), and cellular defense against invading streptococci (Nakagawa et al., 2004) and Mycobacterium tuberculosis (Güiterrez et al., 2004). However, the physiological function of autophagy, particularly in neurons remains largely unknown.

Recently it has been suggested that as the load of the stimulus persists, constitutive autophagy may initiate cell death processes leading to the demise of the cell (Rubinsztein et al., 2005). This idea of ‘autophagic stress’ (Chu, 2006) highlights the dysregulation of autophagy when excessive autophagic demand cannot be balanced by cellular reserves. With respect to TBI, it is possible that during the neuronal response to injury, there is an increased level of timing and
stimulation of autophagy therefore switching the dynamic balance from constitutive autophagy to induction of cell death. In support of this idea are my studies that show an early translocation of the autophagic protein Beclin 1, 15 min after TBI, and a subsequent upregulation of several key autophagic proteins, including ATG 5, ATG 7, and LC3 II, as early as 1 hour after injury. Moreover, electron micrographs illustrate ultrastructural evidence of the accumulation of autophagic vacuoles 3 days after TBI. This morphology is characteristic of certain populations of neurons undergoing developmental cell death, including the isthmo-optic nucleus of chicks, tail motoneurons of larval frogs, and moth motoneurons (Clarke, 1990; Kinch et al., 2003). In addition, increased autophagy vacuoles have been described in PD (Anglade et al., 1997; Zhu et al., 2003), AD (Nixon et al., 2005), Lewy body dementia (Zhu et al., 2003), HD (Sapp et al., 1997), prion disease (Sikorska et al., 2004), lysosomal storage diseases (Koike et al., 2005) and X-linked or toxic myopathies (Nishino, 2003; Suzuki et al., 2002). Therefore, it is possible that the “autophagic stress” induced by TBI results in autophagic dysregulation thus initiating cell death.

Although my studies support the idea of “autophagic stress” following TBI, they have not determined a causal link between autophagy and cell death. The dynamic shift in autophagy is important to clarify and understand, however, it will further be necessary to determine the contribution of autophagy to TBI pathology. Several factors may affect the role and outcome of this process. As mentioned, location of the initial signal may be relevant in whether this stress response is protective or contributes to cell death. Timing of stimulation may play an
important role as well, where autophagy may initially be a protective response, as reported in nutrient deprived conditions in the yeast (Tsukada and Ohsumi, 1993; Thum et al., 1994; Meijer and Codogno, 2004; Eskelinen, 2005) as well in amino acid deprived HeLa and growth factor deprived (interleukin-3-dependent) bone marrow cells (Boya et al., 2005; Lum et al., 2005). Continued or prolonged stimulation may deplete autophagically produced resources and shift the balance of autophagy and apoptosis, subsequently contributing to cell death. Stereological analysis of neuronal cell death and autophagy may contribute to our understanding of the role of autophagy following TBI. An extended time scale may also provide valuable information clarifying whether there is an initial protective role, and subsequent convergence with cell death. Further, the ability to establish a specific and viable block of autophagy in vivo may further consolidate the role of autophagy across an extended time frame, and more directly assess its role in TBI pathology.

V. APPLICATION OF RO 25-6981 AS A TREATMENT FOR TBI

My studies have found a novel glutamate signaling mechanism involved in the activation of autophagy in vivo and in vitro, and cell death in vitro. The NR2B specific antagonist Ro 25-6981 was utilized to determine the specificity of this signaling mechanism, where I have shown that NR2B specific signaling initiates the autophagic response. This is the first report that demonstrates the NR2B antagonist Ro 25-6981 may confer a protective effect from patho-mechanisms after TBI.
Ro 25-6981 has well been established as an NR2B specific antagonist (Fischer et al., 1997; Mutel et al., 1998; Lynch et al., 2001), and has helped to associate NR2B signaling in a number of CNS conditions and pathologies. As a pharmacological agent, Ro 25-6981 has been used to understand the role of NR2B synaptic transmission in cortical short-term plasticity (Chamberlain et al., 2008), and cortical spreading depression in the rat (Peeters et al., 2007), as well as the contribution of NR2B to both long-term potentiation and long-term depression and resultant spatial learning and memory in the hippocampus (Liu et al., 2004; Dong et al., 2006; Fox et al., 2006; Wang et al., 2006; Bartlett et al., 2007).

In addition, Ro 25-6981 has previously been reported as an effective in attenuating pathophysiological inflammatory and neuropathic pain in the CNS (Chizh and Headley, 2005; Gogas, 2006). Specifically, targeting NR2B signaling in cortical regions with Ro 25-6981 reduced pain perception that contributes to migraine headaches (Peeters et al., 2007) and supra-spinal nociception contributing to chronic pain (Li and Zhuo, 1998; Wei et al., 2001; Wu et al., 2005; Chazot, 2004). Similarly, in the spinal cord, Ro 25-6981 has been used to evaluate the contribution of NR2B signaling to acute spinal nociception (Boyce et al., 1999; Chazot, 2004; Pederson et al., 2008). Moreover, these findings reveal no significant side-effects, unlike other NMDA antagonists, demonstrating a possible clinical utility for the treatment of various pain conditions.

NR2B specific signaling has also been implicated in several neurodegenerative conditions, contributing to and potentiating pathological effects in Parkinson's
disease (Del Dotto et al., 2001; Marino et al., 2003), and triggering early degeneration in Huntington’s disease (Kuppenbender et al., 2000; Li et al., 2003; Li et al., 2004). Ro 25-6981 has shown therapeutic potential in certain animal models of these disorders (Loschmann et al., 2004), but have yet to be evaluated fully. In addition, NR2B signaling is involved in the pathology of ischemia/stroke, epilepsy and various mental disorders (Chazot, 2004; Gogas, 2006). NR2B antagonists including Ro 25-6981 and CP 101, 606 have offered promising results in models of ischemia (Di et al., 1997; Bullock et al., 1999), however, preclinical research has been limited and demonstrated less conclusive results.

A growing body of research has shown that excitotoxic effects after TBI involves NR2B signaling, however, due to the lack of significant clinical results using general NMDA antagonists, research using NR2B specific antagonists as a therapeutic intervention has been scarce. It has previously been reported that the use of the NR2B antagonist CP 101, 606 showed favorable results for patients as evaluated by stratified motor scores and dichotomized GOS (Yurkewicz et al., 2005), however, no further results have been reported. Here, I demonstrate for the first time that pre-treatment with Ro 25-6981 is sufficient to delay alterations in the NR2B signaling complex, delay and attenuate the initiation and activation of autophagy in vivo and in vitro, and significantly reduce cell death in vitro. These findings indicate that Ro 25-6981 may be a beneficial therapeutic agent for treatment of TBI patho-mechanisms. In further studies, it may be prudent to use multiple injections of Ro 25-6981 at various critical time points after TBI, alter dosage, or deliver the compound in combination with other
agents. As these strategies are tested, the potential of Ro 25-6981 effects on signaling and secondary cell death will more completely be discerned.
Chapter IV

I. MODEL OF NR2B MULTI-PROTEIN SIGNALING COMPLEX AND AUTOPHAGIC ACTIVATION AFTER TBI

The results in this dissertation support a model (figure 12) in which the NR2B signaling complex consisting of NR2B, PSD 95, Shank, Homer and Beclin 1, is constitutively expressed in membrane rafts in cortical neurons. The NR2B complex acts as a regulator of autophagy through interaction and binding with Beclin 1. Moderate TBI induces interaction between pCaMKII and NR2B in membrane rafts, leading conformational changes in the receptor causing the release of Beclin 1 from the regulatory complex. Next, autophagy is hyper-activated as evident by up-regulation of Beclin1, ATG 5, ATG 7, and LC3 II, and the down regulation of the negative regulator of autophagy, mTOR. Continued NR2B stimulation and persistent activation of autophagy results in the accumulation of autophagic vacuoles, which may result in “autophagic stress” that contributes to cell death observed in TBI pathology.
NR2B signaling complex alterations and induction of autophagy after TBI

**Figure 12.** NR2B multi-protein complex signaling and activation of autophagy. TBI results in alterations in the membrane raft associated NR2B signaling complex, where Beclin 1 is released from this association. Beclin 1 release leads to induction of autophagy, and subsequent upregulation of pro-autophagic proteins, including Beclin 1, ATG 5, and ATG 7. A concomitant NR2B receptor internalization is observed.

II. SUMMARY AND FUTURE DIRECTIONS

My studies reveal a relationship between glutamate mediated NR2B signaling and initiation and activation of autophagy *in vivo*, as well as cell death *in vitro*. A single injection of the NR2B specific antagonist, Ro 25-6981 significantly delayed the onset of autophagy *in vivo* and *in vitro* and significantly reduced cell death *in vitro*. These findings raise a number of intriguing questions: 1) Does NR2B-mediated activation of autophagy directly contribute to cell death following TBI? In these studies, autophagy and cell death have been
correlated, these studies do not yet show a causal relationship. Studies using pharmacological agents that specifically block autophagy after TBI should aid in establishing if cell death is also prevented. Although the NR2B specific antagonist Ro 25-6981 greatly attenuates autophagy, it is not known whether other processes are affecting by blocking NR2B signaling. 2) Does blocking NR2B signaling after TBI and the downstream activation of autophagy and cell death lead to significant tissue sparing and improved functional outcomes? These parameters can be evaluated by using various sensorimotor behavioral outcome measures that assess working memory and sensorimotor coordination. 3) What is the therapeutic window of administration of the NR2B specific-antagonist and does this drug show efficacy in human TBI?

Excitotoxic damage after TBI not only occurs in neurons, but also has contributes to the demise of glial cells. Therefore, it will be important to establish whether my findings can be extended to glutamate excitotoxicity of astrocytes, oligodendrocytes and microglia after TBI. Moreover, it will be important to determine whether other forebrain structures such as hippocampus and thalamus, in addition to cerebral cortex examined in my studies, express a similar NR2B multi-protein signaling complexes and activation of autophagy. My preliminary data shows that NR2B and Beclin 1 associate in membrane rafts in the hippocampus, and these proteins increased in expression at 24 and 48 hours after TBI. However, further work is necessary to validate these findings. Additionally, immunohistochemical micrographs of CA3 hippocampal neurons exhibited diffuse perinuclear immunoreactivity in the normal brain, similar to that
found in cortical neurons. Preliminary immunohistochemical micrographs of astrocytes and microglia show very weak Beclin 1 staining 24 hours after TBI. However, other studies have reported an increase in Beclin 1 immunoreactivity in astrocytes at 3 days after injury (Diskin et al., 2005; Zhang et al., 2008), and it is possible that mechanisms other than excessive glutamate regulate the more delayed autophagy activation in astrocytes.

In conclusion my studies describing glutamatergic signaling have added new information to our understanding of the molecular mechanism and cellular processes that contribute to pathology after TBI. My findings demonstrate a novel NR2B specific multi-protein signaling complex, and a specific mechanism of activation of autophagy after TBI. Moreover, my studies reveal the importance of membrane raft microdomains in mediating NR2B signaling, and regulation of autophagy in cortical neurons. Further knowledge regarding the role of NR2B signaling and autophagy after TBI will help to facilitate development therapeutic interventions for treatment of TBI.
Figure 13. Low magnification immuno-staining in cortical neurons. Confocal images show cortical neurons near the injury epicenter of sham and injured brains at 24 h after TBI. Sections were stained for Beclin 1 (red) and the neuronal markers NeuN and MAP2 (green). Beclin 1 immunoreactivity (red and merged, row 1 and 2) was consistently observed in a diffuse pattern in perinuclear regions of NeuN and MAP2 positive cells. Twenty-four hours after TBI, there was increased immuno-staining of Beclin 1 (red and merged, row 3) observed in the cell soma and processes of MAP2 positive cells (merged, row 3). Scale bar = 10 μm.
REFERENCES


Fong DK., Rao A., Crump FT., Craig AM. (2002) Rapid synaptic remodeling by protein kinase C: reciprocal translocation of NMDA receptors and calcium/calmodulin-dependent kinase II.


Autophagy is increased after traumatic brain injury in mice and is partially inhibited by the antioxidant gamma-glutamylcysteiny1 ethyl ester. Journal of Cerebral Blood Flow and Metabolism 28, 540-50.


Gregory E. Bigford was born in Toronto, Canada on September 14, 1978. In the fall of 1997, he began studies at the University of Toronto, in Physical Education and Health. In 2001, he graduated with an honors degree in this discipline. During his time as a student, he received a Juri V. Daniel departmental award and Golden Key Society award for academic achievement and excellence.

Following a year and a half of travels, in the fall of 2002, he moved to Miami to pursue a Ph.D. in Exercise Physiology at the University of Miami. He enrolled in a general neuroscience and a neuro-physiology course at the medical school, subsequently applied and was accepted into the Neuroscience Ph.D. Graduate Program, beginning in the fall of 2003. He joined the laboratory of his mentor Robert W. Keane, Ph.D., where he began his research toward his doctoral dissertation studying glutamatergic signaling and autophagy following TBI. During his time as a student he received the Lois Pope Life Fellowship, annually, and was awarded a travel grant for the Annual Biomedical Research Conference for Minority Students, held in 2006. He has published two abstracts in 2005 and 2007 as well as a first author manuscript detailing his dissertation research published in the Journal of Neurotrauma, 2009.

Gregory received his Ph.D. degree in May 2009.