RESTORATION OF AUTOPHAGY DECREASES NEURONAL CELL DEATH AFTER TRAUMATIC BRAIN INJURY

By

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ABSTRACT OF THE DISSERTATION

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AFTER TRAUMATIC BRAIN INJURY

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Dissertation Director:

Dr. Radek Dobrowolski

and

Dr. Edward Bonder

Traumatic brain injury (TBI) is the leading cause of death and disability in children and young adults in United States. Around 5.3 million people are estimated to be living TBI-related disability in United States alone (https://www.ninds.nih.gov). TBI is often associated with accumulation of aggregated proteins and neuronal cell death, underscoring importance of degradation pathway such as autophagy. In our study, we attempted to understand the role of autophagy in TBI-related neuronal cell death. Using lateral fluid percussion injury model in rats, we found that autophagy flux was inhibited after TBI. Importantly, we discovered that treatment with 18 amino acid TAT-Beclin1 peptide increased autophagy flux and significantly

reduced neuronal cell death after TBI. Further investigation revealed the role of integrated stress response (ISR) mediated by ER stress markers ATF4, CHOP and GADD34, as well as transcription factor TFEB as mediators of neuronal cell death after TBI. TAT-Beclin1 peptide induced autophagy decreased ISR in neurons and inhibited TFEB signaling, likely due to increased AKT activity. Using LFP injury model in rats as well as cortical neuron stretch injury *in vitro*, we also found that impaired autophagy in TBI leads to sequestration of Wnt signal mediator Dishevelled-1 (DVL-1) in autophagosomes. Sequestration of Dvl-1 likely contributes to decreased Wnt signaling, increased GSK3 activity, and phospho-Tau buildup. Furthermore, we report that TAT-Beclin1 induced autophagy prevented Dvl-1 sequestration, decreased pTau buildup and reduced neuronal cell death. Based on these findings, we postulate that autophagy flux is inhibited after TBI, and induction of autophagy by TAT-Beclin1 is beneficial in decreasing TBI-related neuronal cell death.

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cells and its sophisticated machineries. My interest in microscopy was even more enhanced with lectures from microscopy class by Dr. Bonder on resolution and optics.

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I would like to dedicate this work to my loving parents

Mr. Kartik and Mrs. Narayani Mandal.

&

My Sons Vedant and Nikhil Saqcena

Chaitali Mandal Saqcena

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List of Abbreviations

ATG   Autophagy related gene

CK   Casein Kinase

CLEAR   Coordinated Lysosomal Expression and Regulation

Dvl-1   Dishevelled

GSK   Glycogen synthase kinase

ISR   Integrated stress response

LC3   Light chain 3

mTOR   Mechanistic target of rapamycin

PAS   Pre-autophagosomal structure

PI3P   phosphatidylinositol-3-phosphate

PE   phosphatidylethanolamine

SCI   Spinal Cord Injury

TBI   Traumatic Brain Injury

TFEB   Transcription factor EB

Chapter 1: Introduction

Eukaryotic cells have developed primarily two ways to degrade unwanted or damaged proteins and organelles: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system. Proteasome system is generally employed for protein degradation. Autophagy (self-eating) degrades proteins, lipids, nucleic acids, and polysaccharides in acidic lysosomes. Autophagy is evolutionally conserved from yeast to plants and mammals. Autophagy can be further classified into three distinct pathways: microautophagy, chaperone-mediated autophagy, and macroautophagy [5]. In this dissertation, macroautophagy will be more generally referred to as autophagy.

Christian de Duve first observed and discovered two important components of autophagy: autophagosomes and lysosomes, earning him Noble Prize in 1974. In early 1990s, Yoshinori Ohsumi discovered autophagy and many of the autophagy-related genes (ATG) in *S. cerevisiae,* unveiling the unknown mechanism of autophagy and opening a new field of research, for which he was awarded Noble Prize in 2016. In yeast, almost 27 ATG genes have been identified that regulate autophagy [6]. Orthologs of a large majority of these ATG genes have been discovered to perform similar roles in mammals, insects, worms, and plants underscoring the significance of autophagy in the survival of eukaryotes and higher life forms [7]. Autophagy, being a degradative pathway required for turnover of proteins and organelles, occurs at a basal level in cells for normal maintenance of cellular and organismal function. A large body of research has yielded important insights on the pleiotropic roles of autophagy in diverse biological functions, such

as in development, homeostasis, and aging, as well as in the pathophysiology of diseases [8, 9].

Autophagic Organelles

Autophagy is a cellular process that takes place at basal level, but can be further induced to increase the rate of degradation by external or internal stimuli such as starvation, stress, growth factor depletion, hypoxia, or pharmacological agents [10]. PI3K/mTOR pathway is a well-documented negative regulator of autophagy [11]. However, other pathways that impact autophagy bypassing mTOR signaling have also been described: inositol signaling (IP3) [12], calcium/calpain pathway [13], and lysosomal calcium/ calcineurin pathway [14, 15].

The process of autophagy is dependent on both cellular organelles as well as protein complexes. Major organelles that specifically participate in autophagy process are autophagosomes, amphisomes, autolysosomes, and lysosomes.

Autophagosomes: These are double-membraned vesicles that primarily function to carry cargo on microtubule to lysosome for degradation. The biogenesis of autophagosomes has been an intriguing question and many recent findings suggest that autophagosomes are formed *de novo.* Autophagosomes acquire components necessary for their formation such as membrane and other membrane bound proteins. In various studies it has been reported that autophagosome membrane may originate from plasma membrane, Golgi apparatus, endoplasmic reticulum-mitochondria contact site, or endoplasmic

reticulum [16-18]. The membrane is characterized by the presence of phosphatidylinositol-3-phosphate PI3P and Atg8/LC3 [19].

Amphisomes: These are autophagic vesicles formed by the fusion of autophagosomes and endosomes. Autophagosomes can fuse with early or late endosomes, and multiple endosomes can fuse with a single autophagosome. Amphisomes have membrane characteristics of both autophagosomes and membrane proteins of early or late endosome [20]. The amphisomes eventually fuse with lysosomes for cargo degradation.

Autolysosomes: These are short-lived organelles that form by the fusion of lysosomes and autophagosomes, and the membrane characteristics are very similar to that lysosomes [21].

Lysosomes: mediator of autophagy and cellular signaling
First discovered by Christian de Duve in rat liver lysates, lysosomes are degradation center of the cell where almost all macromolecules are degraded. Lysosomes are single-membraned vesicles housing nearly 60 hydrolase enzymes, which function optimally at pH of 4 to 5. Thus, maintenance of pH is critical in functioning of lysosomes [22, 23]. The acidic pH of lysosome is maintained by vacuolar-type ATPase (V-ATPase) that transports proton across the lysosomal membrane from cytoplasm into the lumen by hydrolysis of ATP [24]. The hydrolase enzymes are lytic in nature, and therefore rupture of lysosomes are toxic to cells

causing necrotic cell death [25]. Partial leakage of the lysosomal membrane reduces lysosomal function and can induce programmed cell death[26]. Cathepsin is a lysosomal enzyme that is implicated in programmed cell death upon lysosomal leakage [27].

Lysosomes regulate degradation, recycling of macromolecules, and interestingly regulate a number of signaling pathways. Lysosomal membrane serves as a platform for over 45 membrane proteins [28]. Some of the proteins that have been extensively study are lysosomal associated membrane protein (LAMP1, LAMP2), mTOR complex, ragulator, Rag a/b, Rag c/d, Rheb GTPase [29], as well as ion channels and transporters for H^+, Ca^{2+}, Na^+, K^+, and Cl^- [30]. Lysosomes also store intracellular calcium, and release of calcium is important for many signaling events and membrane trafficking [31, 32].

Lysosome is central hub for sensing nutrient in the environment and signal transduction for induction of autophagy or endocytosis through TFEB, identified as a master regulator of lysosomal biogenesis and autophagy. TFEB is a transcription factor whose nuclear translocation is regulated by combined action of mTOR kinase and calcineurin phosphatase. The activity of calcineurin on TFEB is mediated by calcium efflux from lysosomal mucolipin channel [33]. Lysosome is not just a degradation center for autophagosome fusion and cargo degradation but a regulator of autophagy process itself.

Protein complexes that are involved in autophagy are: Two ubiquitin like conjugation systems each made up of ATG protein complex such as ATG5-ATG12 and LC3 (Light chain 3, ATG8) – PE (phosphatidylethanolamine) [7, 34]. Another

important protein complex that plays important role in the formation of autophagosomes is class III phosphoinositide 3-kinase (PI3KC3), the complex is made up of Vps34, Beclin1(Atg6), Atg14L and Vps15 [35].

Steps in autophagy process
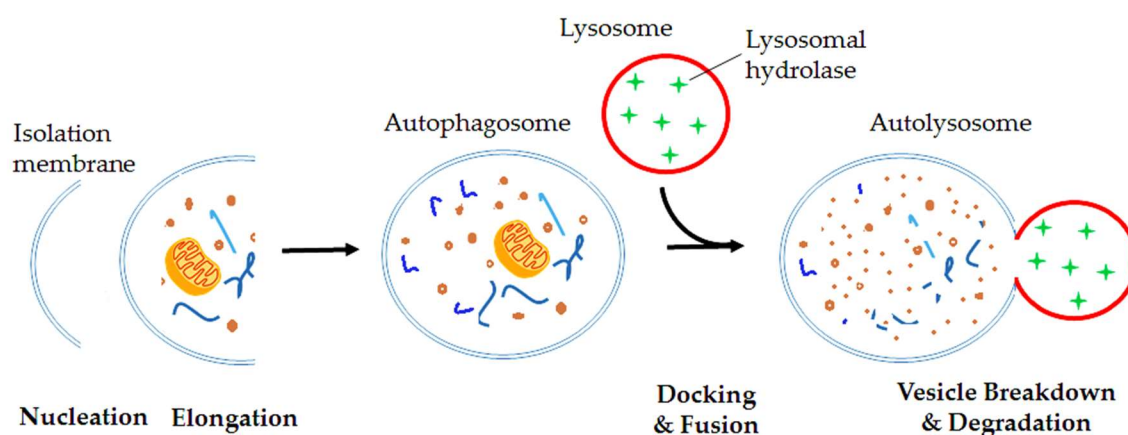
Figure 1.1: Formation of autophagosome and degradation of cargo. Autophagy process can be broadly divided into three different phases: first phase is the initiation of phagophore formation, followed by completion of autophagosome, and in the final phase fusion of autophagosome to lysosome occurs for the degradation of the cargo. The general steps in autophagy are illustrated in figure 1.1 (Melendez A and Levine B, 2009).

Initiation of autophagosome: The initiation of the phase begins with the formation of pre-autophagosomal structure, PAS, which serves as an active site for recruitment of all Atg machineries. The initial structure is derived from the tubular

extension of ER that is rich in PI3P [36], and ER plays an important role as the source of this initial nucleation of membrane [37, 38]. Initiation of nucleation of proteins on PAS begins with the assembly of ULK1/Atg1 complex which comprises of ULK1, FIP200, Atg13 and Atg101 [39, 40]. Activation of the complex requires phosphorylation of ULK1 by mTOR and autophosphorylation [41]. Another major complex important for nucleation on PAS is phosphatidylinositol 3-kinase class 1 (PI3KC1), which modifies the membrane for recruitment of the Atg complexes, and PI3K class 3 (PI3KC3) which forms two distinct complexes: complex I being important for autophagy and complex II for the endosomal pathway [42]. PI3KC3 complex I consists of Vps34, Vps15, Atg6/Beclin 1 and Atg 14, and produces phosphatidylinositol-3-phosphate on PAS which helps in the recruitment of more Atg machineries and aids in the conjugation reaction for expansion of the membrane and recruitment of Atg8 [36, 43]. Atg9 along with the PI3KC1 guide the localization of the two conjugation complex on PAS [44]. The initiation of nucleation and PAS formation is followed by expansion of the membrane to complete the autophagosome formation.

Expansion and completion of the autophagosome vesicles: The expansion of the nucleation membrane requires addition of lipid bilayer to the existing nucleation structure. Some studies have shown association of Atg9 with PAS and mitochondria, suggesting cycling of Atg9 between the PAS and mitochondria. Thus, Atg9 likely functions as a carrier for lipid bilayer. The two conjugation reactions important for the completion of autophagosome biogenesis has been

described above. The Atg5-Atg12 conjugation complex forms multimeric Atg12-Atg5-Atg16 complex, which localizes at PAS and is critical for the expansion of autophagosome. This multimeric complex is an irreversible conjugation [44, 45]. The second conjugation system Atg8-PE is required for the proteolytic cleavage of arginine at the C-terminal of Atg8 followed by conjugation of phosphatidylethanolamine (PE) to the exposed glycine residue of Atg8 by Atg7 (E1-like ligase enzyme); this is a reversible post translational modification. PE modification on Atg8 gives it a characteristic of membrane protein and localizes it to PAS [46, 47]. As autophagosome formation is completed, the Atg and other protein complexes required for vesicle formation are dissociated. This uncoating of the vesicle induces fusion of autophagosome to lysosome. Thus, the coating of Atg and other protein complexes on autophagosome acts as an inhibitor for fusion reaction preventing premature fusion of cargo to lysosome [5].

Fusion of autophagosomes with lysosomes: Uncoating of the Atgs from autophagosome prompts the double membrane vesicle for fusion with lysosomes. The mature autophagosome associates with microtubule for lysosomal fusion but such association with microtubule does not happen with incomplete autophagosomes [48]. Mature autophagosomes are targeted to lysosomes in Dynein-dependent movement on microtubule towards the minus end. [49, 50]. Membrane proteins, such as Rab7 [51], autophagosomal SNARE, and Syntaxin17 [52, 53], on the outer membrane of the autophagosomes associate with PI3P-rich mature autophagosomes to facilitate and cause fusion with lysosomes. SNAP29

and Atg14, part of PI3KCIII complex1, causes stable binary t-SNARE complex with Syntaxin17 and SNAP29, thereby prompting for tethering with lysosomal v-SNARE, VAMP8, and fusion to lysosomes [54]. In this final step, the cargo is delivered to hydrolase enzyme-rich lysosomes for degradation.

Bulk vs. selective autophagy

Bulk autophagy: Molecular mechanism for autophagy does not differ between bulk and selective autophagy. Bulk autophagy, however, is independent of selective machineries such as adaptor proteins and autophagic receptors.

Selective Autophagy: One of the functions of autophagy is the quality control of proteins and organelles, for which autophagosomes should be able to distinguish between functional and misfolded, damaged proteins and organelles. Thus, autophagy needs to be selective in cargo recognition and degradation. Cells have evolved different tags such as ubiquitin [55] and acetylation [56], as well as adaptor proteins and autophagic receptors to selectively recognize and package cargos. Most of the damaged organelles are ubiquitin tagged and are recognized by autophagic receptors such as P62, NBR1, NDP52, and OPTN (Optineurin) [55].

Autophagy has different nomenclature based on the specificity of cargo for degradation such as Mitophagy (degradation of mitochondria), Aggrephagy (degradation of protein aggregates), Pexophagy (degradation of peroxisomes), Lipophagy (degradation of lipids), Xenophagy (degradation of intracellular

bacterial pathogens), ER-phagy (degradation of ER), Ribophagy (degradation of ribosomes), and Zymophagy (degradation of secretory granules) [57].

Inhibitors of autophagy: There are many chemical small molecule inhibitors of autophagy that are available to modulate autophagy such as 3-Methyladenine (inhibits Class III PI3K), Spautin (inhibits Beclin1), E64d (inhibits cysteine protease), Pepstatin A (inhibits aspartic proteinase), PIK-III (inhibits VPS34), etc. Genetic manipulation of key autophagy genes, such as knocking out Atg5 or Atg7, can also inhibit autophagy [58, 59]

Chemical inducers of autophagy: Pharmacological agents that can upregulate autophagy and mediate degradation and clearance of organelles and proteins are rapamycin (mTOR inhibitor), lithium (decrease inositol and IP3 level), Verapamil (calcium channel blocker), Calpastatin (calpain inhibitor), trehalose, etc [60]. Genetic overexpression of autophagy genes such as Atg5 has been shown to upregulate autophagy [61]. Recently, TAT-Beclin1 peptide has been developed to specifically target autophagy, which unlike chemical inducers or genetic manipulation, can transiently upregulate autophagy both *in vitro* and *in vivo* [62].

Autophagy in disease pathogenesis

Autophagy is a multi-step process that starts with phagophore formation and ends with the degradation of cargo. Malfunction in any of the steps in this process, such as maturation of autophagosomes, damage to the autophagic vesicle, lysosomal degradation, or transport of autophagosomes to lysosomes [63, 64], may lead to

various cellular malfunctions. Lysosomal defective disorders are most certainly coupled with autophagy. Autophagy-lysosome pathway regulate cellular degradation, endosomal pathway and signal transduction, therefore impaired autophagy can downregulated lysosomal function through TFEB, alter endosomal pathway and signal transduction and finally cause cell death[33, 65]

Some of the pathologies associated with impaired autophagy are inflammatory diseases [66], lysosomal storage disease, cancer [67], cardiac diseases [68], myodegenerative disease [69], liver disease [70], and neurodegeneration [9].

Autophagy in neurodegenerative disease

Brain is a highly metabolically active organ consuming 60% of the energy requirement of the body, made primarily of neurons and glial cells. Both of these cell types have limited ability to store energy. Astrocytes are known to have some glycogen storage that can be used during hypoglycemia or high brain activation [71]. This underscores brain's dependence on other organs and systems for energy. However, brain does not see much fluctuation in energy levels and does not respond to starvation to induce autophagy [72]. This has been also shown by monitoring autophagy process during starvation in LC3-GFP transgenic mice [73].

Neurodegeneration can occur due to diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis [74], and disorders such as oxidative stress, protein aggregation [75], and neuroinflammation [76]. Physical insults to brain such as brain injury or traumatic brain injury (TBI) can also lead to neurodegeneration.

Autophagy has been reported to play an important role in neurodegeneration after TBI. Neurons are specialized polar cells with different cellular structures such as axon, dendrites, soma, and synapses. The long neurite structure of neurons function in cell-cell communication, vesicle transportation, and cargo degradation. Mature neurons are non-dividing cells, and basal autophagy is critical in avoiding accumulation of pathological proteins or damaged organelles. Autophagy is also important in neurogenesis, neurodevelopment, and synapses [77-79], and thus autophagy is constitutively active in neurons [80]. Autophagosome are formed in the distal end of neurons, which are then transported to soma by axonal retrograde transport. Neuronal autophagosomes are formed from endoplasmic reticulum membrane at the distal end of axon [38]. Neurons are highly polar structure with distinct cell body or soma, axon, and dendrites. Autophagy is compartmentalized, and there is a distinct pool of autophagosomes that are generated in axons and synapses and another pool of autophagosomes generated in the soma [81]. To further understand the role of autophagy in neurons, mice with neural specific Atg5 and Atg 7 knockout were made. These mice had retarded growth, defective motor function and behavior, and abnormal protein accumulation. Interestingly, the gross brain anatomy of these mice appeared normal, but cellular examination of brain showed neurodegeneration and apoptosis [82].

Traumatic Brain Injury (TBI)

Traumatic brain Injury (TBI) is a complex form of physical insult to the brain that can lead to severe disabilities and even death. TBI has also been linked to development of Alzheimer's disease and other neurodegenerative diseases. According to Center for Disease Control and Prevention, despite an increase in the rate of TBI over the last few years, number of deaths related to TBI has decreased owing to advances in medical intervention. However, chronic effects of posttraumatic injury syndrome such as seizures, motor neuron disease, abnormal Tau accumulation, etc., remains a significant challenge and warrants better molecular understanding for improved outcomes.

Recent research findings suggest that autophagy process is inhibited after TBI. Understanding the precise mechanism of changes in autophagy could open a new area of research for therapeutic drugs to manage TBI and improve post injury outcomes.

To study the role of autophagy in neurodegeneration, we used lateral percussion fluid injury as a TBI model in rats. Traumatic brain injury can be defined as a change in brain function or pathology caused by external force. TBI can be broadly classified as a penetrating or non-penetrating injury. Penetrating injury includes penetration of material through skull causing displacement or destruction of brain tissue at the site of trajectory, fracture and deposition of broken bones or penetrating material in the brain tissue, and introduction of foreign infectious material in the brain. An example of such injury is a bullet shot wound to the head. Such injuries are also described as focal injury. The impact of injury will vary

depending on the size and trajectory of the injuring material and the location of injury in the brain [3]. The second type is non-penetrating or closed injury, which is further distinguished into contact-based injury where brain inside the skull hits the inner surface of the skull causing injury to different outer regions of the brain or inertia-based injury which is caused due to the combination of linear or rotational force with angular acceleration or deceleration causing strain, shear or compression of the brain tissue. Such injures are also described as diffuse injury [3]. Most TBIs are a combination of both kinds of injury. Fluid percussion injury is a controlled and reproducible method that can replicate mechanical, physiological, neurological, and neuropathological process of closed head injury [90, 91]. Lateral fluid percussion injury involves parasaggital impact on the cortex causing more cortical damage with lesser compression of the brainstem, and this model is clinically more relevant to closed head injury [91].

TBI triggers diverse acute and long-term cellular changes (see figure. 1.2), which evolve over days and months after injury, and such chronic process is now linked to high risk of Alzheimer's disease in TBI patients [92]. One of the first cellular responses to injury in neurons is perturbation of calcium homeostasis. Mechanical injury, specifically of neurons, causes release of excitatory neurotransmitters such as glutamate and other amino acids causing calcium influx in the cell, and further release of calcium from intracellular reserve. Increase in intracellular calcium activates various calcium dependent pathways, which can cause cytotoxicity and cell death. Mechanical injury to the axons can also cause membrane poration and

influx of more calcium into the cell. Depending on the extent of injury and degree of these events, the cells may be repaired or progress to further cell damage and death [93].
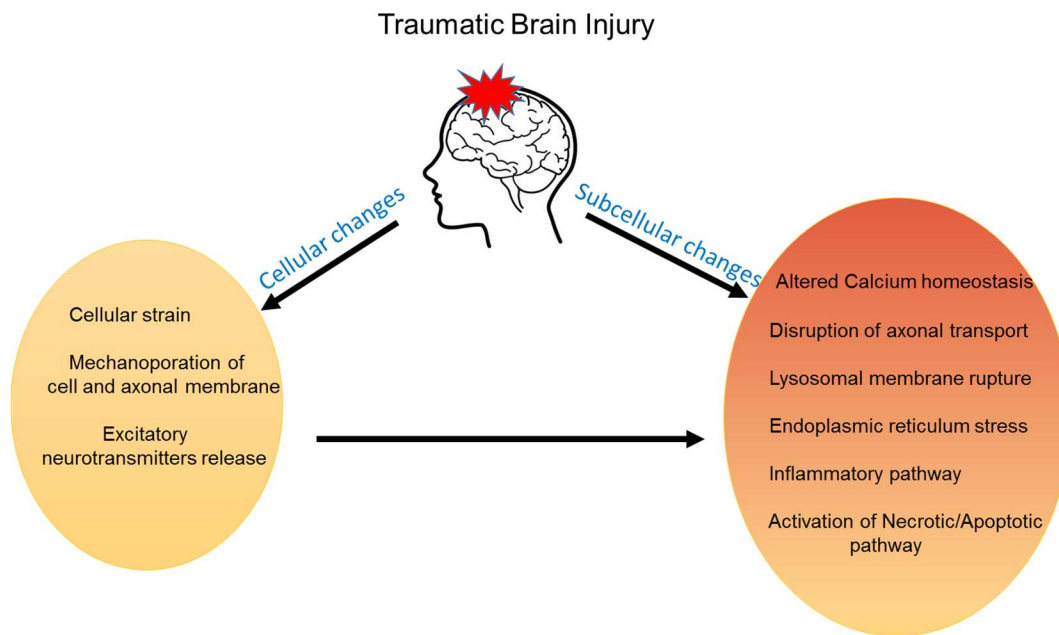
Figure 1.2: Cellular and subcellular events triggered by TBI that may progress to completion in days or months after Injury

Autophagy and Neurodegeneration

Neurodegeneration is generally associated with diseases described above and is characterized by loss of certain subset of neuronal population affecting anatomical structure of the brain. Neurodegeneration can be viewed as progressive neuronal cell death caused by neuropathy. Besides pathological neurodegenerative diseases, neurodegeneration can also be triggered by other factors such as oxidative stress [86], stroke [87], inflammation [88], and also by mechanical insults

such as traumatic brain injury (TBI). TBI has been shown to cause both acute and chronic neurodegeneration. Some of the common pathways that are activated in neurodegeneration, irrespective of its cause are excitotoxicity, dysfunctional mitochondria, protein misfolding, oxidative stress, dysregulation of protein translation, glial activation, and autophagy [87]. Neuronal cell death has been recently reported to be linked with inhibition of autophagy in TBI [89]. In this study, autophagy flux was reported to be inhibited after TBI and correlated with neurodegeneration in a mouse model. However, the mechanistic role of autophagy in neurodegeneration remains unclear. In our study, we sought to explore this question and determine how autophagy prevents neurodegeneration after TBI.
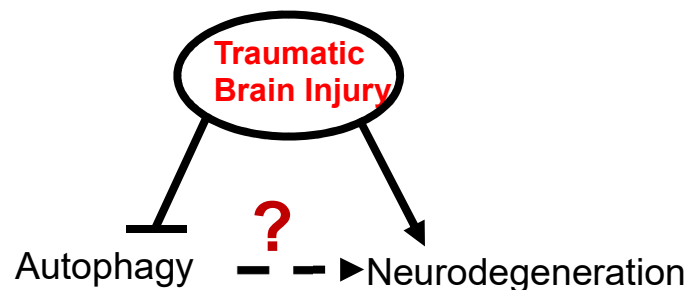
Figure 1.3: Working hypothesis: TBI causes impaired autophagy and Neurodegeneration. Inhibition of autophagy could lead to neurodegeneration by increasing ER stress.

Hypothesis:

We hypothesize that inhibition of autophagy after TBI would increase ER stress due to accumulation of aggregated proteins. We further postulate that chronic ER stress will lead to neuronal cell death.

To test our hypothesis we used lateral fluid percussion injury to model TBI in rats and TAT-Beclin1 peptide as an autophagy inducer in the brain after TBI.

Chapter 2: Scope of the work

We attempted to understand role of autophagy in neuronal cell death after TBI by altering the process using TAT-Beclin1 peptide and evaluating associated changes in signal transduction, cellular stress response, and cell death. Our findings suggest that TAT-Beclin1 peptide could serve as a novel therapeutic tool in management of TBI.

In chapter 3, we showed that autophagy is impaired in lateral fluid percussion injury model of TBI. We were able to restore autophagy flux using TAT-Beclin1 peptide. We hypothesized that TAT-Beclin1 dependent autophagy flux will decrease neuronal cell death by degradation of accumulated toxic protein post TBI. We showed that TBI induced impaired autophagy leads to buildup of pTau and TFEB mediated integrated stress response causing neuronal cell death. TAT-Beclin1 peptide could decrease pTau accumulation in neurons and attenuate neuronal cell death by inhibiting TFEB mediated integrated stress response.

In chapter 4 we extended our study of TBI induced impaired autophagy by determining its role in Wnt signaling. We hypothesized that impaired autophagy would sequester dishevelled in autophagosomes contributing to increased GSK3 activity. We showed that dishevelled sequestration *in vitro* and *in vivo* correlated with increase in pTau levels and increase in GSK3 activity post TBI and neuronal

stretch injury. Importantly, we found that TAT-Beclin1 peptide could decrease pTau in the rat brains and in primary cortical neurons and decrease neuronal cell death.

Chapter 3: Materials and Methods

Animal Model:

Sprague Dawley male rats were kept in 12 hr light and dark cycles with adequate supply of food and water. All experiments were carried out with animals approximately 3 months of age and weighing 300 – 350 g. Animals were handled according to NIH Guidelines for Care and Use of Laboratory Animals and approved by the IACUC of the Veterans Affairs Medical Centre at East Orange, New Jersey (V. A.). All animal procedures were carried out at V. A. and the tissue samples was provided for further studies by Dr. Kevin Pang.

Surgery and TBI:

The rats underwent craniectomy before fluid percussion injury, which was carried out as described previously [83]. Fluid percussion injury (FPI) is an established model for traumatic brain injury, representing both focal and diffuse injury. The brain injury and Acoustic Startle Response (ASR) was carried out following the established procedures described previously [83, 84]. ASR was assessed in animals before FPI, 1 day post injury, and before euthanizing the animals. TAT-Beclin1 peptide (#506048 Calbiochem) 0.5mg/kg was injected intraperitoneally or 500 µM of peptide was topically applied on the injury site above dura in 50 µl PBS solution on injured or sham rats immediately after the injury and continued for alternate days after injury. The rats were sacrificed 1, 3 and 7 days post injury for immunohistochemistry and western blotting.

Immunohistochemistry:

At day 3 post injury, rats were anesthetized using 50% urethane in saline. The animals were intracardially perfused with 0.1 M PBS followed by 4% paraformaldehyde (PFA) in PBS for 5 – 10 min. Brains were isolated and left in 4% PFA overnight. Brains were cryoprotected in 30% sucrose for 24 -48 hr for cryosectioning (25 µM thick) or directly used for vibratome sectioning (50 µM thick). The sections were permeabilized with 0.3% Triton X-100 (#T9284, Sigma) in PBS for 2 hours at 37°C, followed by three washes with 0.05% Triton X-100 in PBS. The sections were blocked with 10% normal goat serum in 0.05% Triton X-100 in PBS (#PBS01-03, Bioland Scientific) for 2 hr at room temperature. Sections were incubated with primary antibodies at 4°C overnight, followed by three washes with 0.05% Triton X-100 in PBS. Secondary antibody incubation was carried out at 37°C for 3 hours. Sections were washed in 0.05% Triton X-100 in PBS and mounted with anti-fading medium containing DAPI for nuclear staining (#30920, GeneTex).

Primary antibodies and dilutions used were: LC3 a/b 1:100 (#4108, Cell Signaling), TFEB 1:200 (#A303-673A, Bethyl), P62 1:100 (#GP62-C, Progen), βIII Tubulin 1:500 (#MMS-435P, Covance), GFAP 1:200 (#12389, Cell Signaling), Map2 1:200 (#ab5392, Abcam), PhosphoTau AT8 p-PHF-Tau p202/T205 1:200 (#MN1060), ATF4 1:100 (#10835-1-AP, Proteintech), CHOP 1:100 (#L63F7, Cell Signaling), and Cleaved caspase 3 (#D175, Cell Signaling).

Secondary antibodies and dilutions used were: Anti-mouse IgG-Cy3 1:500 (#715-165-151, Jackson IR), anti-rabbit IgG-Cy3 1:500 (#711-165-152, Jackson IR), anti-mouse IgG-Cy5 1:500 (#715-175-151, Jackson IR), anti-rabbit IgG-Cy5 1:500 (#715-175-152, Jackson IR), anti-guinea pig IgG-Alexa 488 1:500 (#706-545-148, Jackson IR), and anti-chicken IgY-cy2 1:500 (#703-225-155, Jackson IR).

Fluoro-Jade staining:

Brain sections were warmed to 50°C for 5 – 10 min on a slide. The slides were then immersed in 70% ethanol (#ET108, Spectrum) 2 minutes, followed by distilled water for 2 minutes. Sections were then incubated in 0.06% potassium permanganate (#7722-64-7, MCB) for 10 minutes, followed by distilled water for 2 minutes. The slides were then incubated in 0.0001% Fluoro-Jade C solution made in 0.1% acetic acid (# UN2789, Fisher Scientific) for 10 minutes in a dark chamber. Subsequently, the slides were incubated in 1:2000 DAPI (# S33025, Invitrogen) for 5 minutes in the dark chamber. Sections were washed in distilled water for 1 minute, followed by overnight drying protected from light, and finally rinsed in xylene (#UN1307, Fisher Scientific) and mounted with cytoseal-60 (#8310-4, Thermoscientific).

Assembly of PEEK rings on silicon membrane:

Silicone membrane with vulcanized gloss/gloss 0.005 inch thickness, 40 durometer hardness (Specialty Manufacturing, Inc), was washed in distilled water and assembled in PEEK rings (kindly provided by Dr. Bryan Pfister) with super-

resilient high-temperature silicone O-Ring, 1/16 fractional width (# 9396K65, McMaster-Carr) as described in [85]. The Peek rings were then washed in distilled water and autoclaved.

Neuronal culture:

Cortical neurons were isolated from E16.5 rat fetus. The neurons were dissociated with trituration and plated on poly-D-lysine-coated silicone membrane at a density of 200,000 cells /cm^2 area. The cells were cultured in Neurobasal medium (#21103-049, Gibco) supplemented with 2% B-27 (Gibco), 1% penicillin-streptomycin (Invitrogen), and 2 mM GlutaMAX (# 35050-061, Gibco). The cells were cultured for 7 days before subjecting to stretch injury.

Stretch injury:

Neurons were subjected to stretch injury in 6-well module as described elsewhere [85]. Uniaxial stretch injury was accomplished by controlled injury of neurons in a 2mm rectangular zone at the center of the silicone membrane. Rest of the area could be treated as uninjured control. The neurons were allowed to recover for 1, 3 and 7day post stretch injury.

Immunocytochemistry:

The injured and uninjured neurons were fixed onto silicone membrane with 4% paraformaldehyde for 15 mins at room temperature, followed by PBS wash. Cells

were blocked in 5% normal goat serum with 5% BSA in 0.1% Triton X-100 in PBS. The silicone membranes were cut from PEEK rings and placed in a humidity chamber for primary antibody incubation at 4°C overnight. The silicone membranes were washed with 0.1% Triton X-100 in PBS and incubated with secondary antibody at room temperature for 1 hour, washed in 0.1% Triton X-100 in PBS, and mounted with anti-fading medium containing DAPI.

Primary antibodies and dilutions used were: LC3 a/b 1:250 (#4108, Cell Signaling), P62 1:100 (#GP62-C, Progen), Dvl1 1:100 (# 3F12, Santa Cruz), and PhosphoTau, AT8 p-PHF-Tau p202/T205 1:500 (#MN1060).

Secondary antibodies and dilutions used were: Anti-guinea pig IgG-Alexa 488 1:1000 (#706-545-148, Jackson IR), anti-mouse IgG-Cy5 1:1000 (#715-175-151, Jackson IR), and anti-rabbit IgG-Cy3 1:1000 (#711-165-152, Jackson IR).

Imaging:

Immunohistochemistry and immunocytochemistry imaging was done in Zeiss spinning disc confocal microscope. Fluoro-Jade imaging was done in Zeiss, epi-florescent stereomicroscope.

Western Blots

Cortex from adult rats with or without lateral fluid percussion injury and with or without TAT-Beclin1 injected was collected at 1, 3 and 7 days after impact. Brain

lysates were prepared in lysis buffer [10% glycerol, 1% NP40, 20 mM Tris (pH7.4), 2.5 mM EDTA (pH 8.0), 2.5 mM EGTA (pH 8.0), and protease and phosphatase inhibitor cocktail (#88668, Thermoscientific)] by homogenizing tissue in the lysis buffer for 2 min, followed by sonication 3X for 5 sec/cycle. Lysates were centrifuged at 17,000 g for 15 mins at 4°C. Clear supernatant was collected and protein estimation was carried out using BCA method. The lysates were run on 12% SDS-PAGE gels, and the separated proteins were transferred to PVDF membrane (Immobilon) at constant 310 mA for 2 hours. The membrane was blocked in 5% non-fat milk in TBS containing 0.1 % Triton X-100 for 1 hour on shaker at room temperature. The membranes were incubated in primary antibody made in 5% BSA in TBST overnight at 4°C, followed by 1 hour incubation in secondary antibody made in 2.5% milk in TBST at room temperature. The membranes were then washed and imaged by LI-COR Odyssey system or developed using chemiluminescence substrate (ECL, Pierce).

Primary antibodies used for western blotting were: P-p70S6K (Thr389, #9205, Cell Signaling) 1:1000, LC3 a/b (#4108, Cell Signaling) 1:1000, TFEB (#A303-673A, Bethyl) 1:1000, Dvl1 (# 3F12, Santa Cruz) 1:70, p PHF Tau pS202/T205 (#MN1060, Thermo/Pierce), pGSK3β (# 5558D, Cell Signaling), β-Actin (#JLA20, DSHB). Secondary antibodies conjugated with Infrared Dyes (IRDye 680 or IRDye 800, LICOR), or to HRP (Jackson IR) were used for immunoblotting analysis.

RT-qPCR:

Total RNA was extracted using the RNeasy minikit (Qiagen, Hilden Germany). First-strand cDNA synthesis was performed using 2 µg of total RNA as the template and reverse transcribed using Superscript II (Invitrogen) primed with 50 pmol of random hexamers. Quantitative real-time PCR (RT-qPCR) was performed using the LightCycler 4.0 (Roche Diagnostics, Basel, Switzerland) and the QuantiTech SYBR green PCR kit (Qiagen) as per the manufacturer's protocol. Specific primers for each gene analyzed were designed using Primer3 software. The sense and antisense primers used were:

TFEB: GCAGCCACCTGAACGTGTA and TGTTAGCTCTCGCTTCTGAGT,

ATF4: CCCTTCACCTTCTTACAACCTC and GTCTGGCTTCCTATCTCCTTCA,

CHOP: GTCTAAGGCACTGAGCGTATCA and CACTTCCTTCTTGAACACTCTCTC,

BiP: AACGCCAAGCAACCAAAGAC and CTGCCGTAGGCTCGTTGAT,

GRP94: CGGTGTAGTGGGCGGA and GACCGACCCGAAGGTCA

EDEM1: ACAACTACATGGCTCACGCC and AGATTTGAAGGGTCCCCGC

HERPUD1: AGCCTGCTGGTTCTAATCGG and GCTTCAGGCCTTGAGATGTTT

B-ACTIN: GGTGTGATGGTGGGAATGG and GGTTGGCCTTAGGGTTCAGG.

The PCRs were performed in 20-µl volumes with the following parameters: 95°C for 15 min, followed by 40 cycles of 94°C for 20 s, 59°C for 20 s, and 72°C for 20 s. The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. The data were analyzed using the Lightcycler software 4.0, with all samples normalized to β-actin.

Quantification and Statistics:

The data presented in the thesis is a representation from three animals per cohort. The experiments were carried altogether with 3-7 animals per condition with 2-3 or more of technical repeats.

Western blots: Densitometric analyses, accounting for intensity and size of bands for specific proteins were measured using either ImageJ or LI-COR software. The intensity was normalized with the loading control (Actin or Tubulin), and fold change was determined by normalizing the mean with the sham. Statistical significance in experiments consisting of 3 experimental groups was determined using one-way ANOVA while significance in experiments consisting of 4 groups was determined using two-way ANOVA followed by Tukey's test using Prism 7 software, and p-value < 0.05 (95% confidence level) was considered statistically significant. The data presented in the thesis show statistical comparison between Sham, TBI and TBI with TAT-Beclin1. Sham and TAT-Beclin1 alone did not show significant difference in most experiments.

Immunofluorescence Microscopy: Images were quantified by selecting regions of interest around βIII-tubulin/MAP2 positive cells (neuron) and quantifying the fluorescent intensity of the protein of interest using ImageJ software. The numbers were normalized to sham levels and relative fold increase was determined.

Fluoro-Jade analysis was carried out by counting the number of bright florescent dots in at least 4 different regions from the same section. The means were

normalized to sham to determine the fold change between the experimental groups.

Colocalization analysis was carried out for the region of interest in neurons and astrocytes by splitting the two channels for which the colocalization is to be determined using ImageJ. Mander's overlap coefficient (MOC) was calculated using Coloc 2 plugin in ImageJ. Mean MOC coefficient from 50-100 cells was used in each group

For ATF4/CHOP imaging, analysis was done by counting ATF4 alone or ATF4 and CHOP positive cells in all three experimental groups. Mean values of the group for ATF4 alone and ATF4+CHOP positive cells were determined,

Cellular localization of TFEB in MAP2-negative glia, MAP2-positive neurons was determined by scoring TFEB signal in the nucleus (DAPI-positive areas) or in the cytosol (DAPI-negative areas). For this, 50-100 cells were counted, and percentage of cells having TFEB in the cytoplasm, cytoplasm and nucleus, and nucleus alone was determined.

N = number of technical repeats, n = number of animals, ns = not significant.

Chapter 4: TAT-Beclin1 mediated restoration of autophagy decreases pTau buildup and rescues neuronal cell death after TBI

Introduction

Autophagy is a multistep catabolic process that is conserved amongst all eukaryotes. Despite intense and rigorous scientific interest in understanding the process of autophagy over several decades, new studies continue to unravel the complicated molecular mechanisms and varied context-dependent roles autophagy plays in vastly diverse cellular and physiological processes, during development, homeostasis, repair, and aging. Autophagy is critical in brain tissue and especially in post-mitotic cells such as neurons. In mouse models, brain-specific knockout of autophagy genes such as Atg5 and Atg7 causes severe neurodegeneration. Moreover, human neurodegenerative diseases, such as Parkinson's, Alzheimer, Huntington, Multiple sclerosis and Amylotrophic lateral sclerosis [9, 74], are reported to have dysfunctional autophagy. Thus, while autophagy has been implicated to play an important role in regulation of neurodegeneration, the molecular mechanisms and signaling pathways that link autophagy and neurodegeneration remain unclear.

Endoplasmic Reticulum stress after TBI

TBI triggers a myriad of cellular response and intracellular changes. Increase in intracellular calcium and rupture of lysosomes are amongst these changes which impact on homeostatic autophagy pathway and cause endoplasmic reticulum stress (ER stress). ER stress is reported in neurons after ischemic stroke [94]. TBI causes perturbation of calcium homeostasis, buildup of aggregated proteins such as phosphorylated Tau, and inhibition of protein degradation pathway, leading to increase in ER stress [95]. Imbalance between increase in misfolded protein,

protein aggregation, and reduced degradation upregulates unfolded protein response (UPR) [96]. The UPR response is mediated by three membrane bound receptors on ER – PERK (PKR-like ER kinase), IRE1 (inositol requiring kinase 1), and ATF6 (activating transcription factor 6) [97]. These transmembrane receptors are regulated in the ER by chaperon protein GRP78 or BIP. Under homeostatic conditions, GRP78 binds to these three proteins and maintains them in an inactive state. However, when misfolded proteins get accumulated, the unfolded proteins bind to and sequester GRP78, thereby dissociating the three receptors and activating them for stress response [98]. PERK receptor is activated by autophosphorylation and dimerization. Activated PERK triggers inhibition of protein translation by phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), thus decreasing the protein load on ER [97].

It also causes an increase in ATF4 levels, a transcription factor that mediates UPR response and regulates transcription of ER chaperones, autophagy genes, amino acid metabolism genes, and pro-apoptotic transcription factor CHOP [99, 100]. ATF4 signals for cell survival under stress, however persistent stress triggers ATF4 and CHOP-mediated cell death. Chronic ATF4 signals GADD34 along with CHOP GADD34 along with phosphatase PP1 dephosphorylates eIF2α initiating protein synthesis this further increases cellular stress which is also called as integrated stress response (ISR) as shown in figure 4.1 (103) Upon activation, IRE receptor dimerizes and mediates unconventional splicing of X-box binding protein1 (XBP1) mRNA to generate another transcription factor sXBP1, causing increased

expression of ER chaperone proteins that can decrease the ER stress [101]. Additionally, ATF6 upon dissociation from GPR78 translocates to the golgi where it is cleaved to generate transcription factor, causing an increase in ER chaperone protein expression [101].
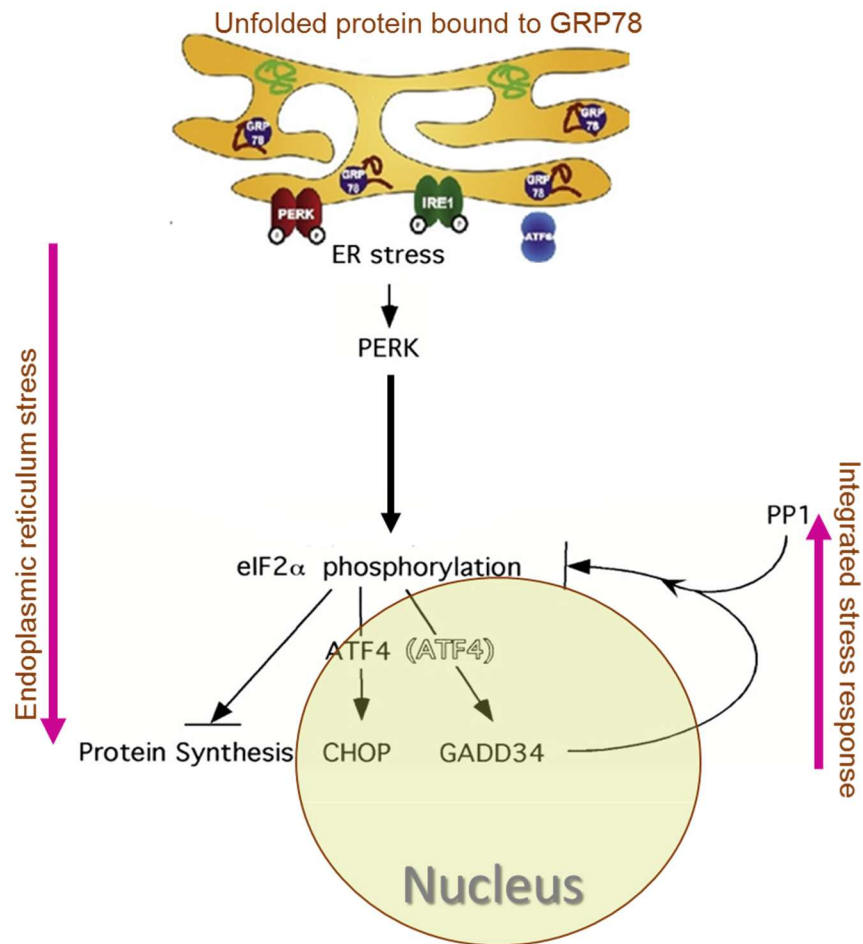
Figure 4.1: ER stress in response to misfolded/unfolded protein buildup. PERK, IRE1 and ATF6 are three sensors on ER. PERK activation leads to CHOP mediated apoptosis and GADD34 with PP1 dephosphorylates eIF2α restarting protein synthesis and integrated stress response

ER stress has been found to be activated in different models of TBI *in vivo*. PERK pathway is activated after blast-induced TBI in rats [102], and ATF6 pathway is activated in mild-concussive TBI mouse models [103]. Accumulation of CHOP or Growth Arrest and DNA Damage-inducible 153 (GADD153) in cells after TBI indicates ER stress. Increase in CHOP levels has been shown in control cortical injury model in rats [104]. Other biochemical markers that have been used to study ER stress are ATF4, pPERK, and Caspase 12 [105].

Altered autophagy after TBI

Autophagy pathway is altered after TBI, however its role in TBI is equivocal. Autophagy in relation to TBI has been studied using autophagy markers such as LC3, Beclin1, and P62. TBI has been shown to increase the level of autophagy markers LC3, Beclin1, and P62, indicating induction of autophagy. The increase in autophagy after TBI has also been shown to correlate with cell death [106, 107]. Autophagy has also been described as a cytoprotective process after TBI in studies where autophagy is induced using pharmacological drugs such as rapamycin, causing decreased cell death [108]. Autophagy has thus been described as a double-edged sword. Such conflicting outcomes confound the role of autophagy in TBI. A major limitation of these studies was that the level of autophagy was determined using autophagy marker LC3 alone which only indicates increase in autophagy induction or accumulation of autophagosomes. None of these studies assessed autophagy flux, which completes the autophagy

pathway. In a recent study determining the autophagy flux after TBI, it was reported that the increase in autophagy marker is due to accumulation of autophagosomes and inhibition of autophagy flux, and not due to increase in autophagy [89]. Moreover, neurons, microglia, and astrocytes tend to accumulate autophagosomes at different time points after CCI-based TBI. Neurons are first affected by inhibition of autophagy, followed by microglia and oligodendrocytes [89]. Neuronal accumulation of autophagosomes is cytotoxic leading to neuronal cell death after TBI. Inhibition of autophagy has been attributed to lysosomal malfunction as determined by lysosomal hydrolase enzyme Cathepsin D levels and its activity. Early decrease in Cathepsin D level and activity leads to prolonged inhibition of autophagy [89]. How TBI causes this initial decrease in Cathepsin D level and activity is not yet understood. However, TBI induced inhibition of autophagy flux is a reversible process since autophagy normalizes to sham condition one week after TBI [89]. Our study corroborated similar findings indicating inhibition of autophagy after TBI. The toxic effect of accumulation of autophagosomes and inhibition of autophagy flux can be overcome by both autophagy inhibitor and autophagy inducer. Autophagy is likely beneficial when the flux is active but could be cytotoxic when flux is hindered. The study from wild type and heterozygous Beclin1 knockout mouse model (impaired autophagy flux) showed greater neuronal survivability in wild type than Beclin1 knockout mice after TBI [109].

Autophagy is linked to ER stress, which is further supported by *in vitro* studies where treatment of cells with ER stress inducer tunicamycin and thapsigargin induced autophagy through IRE pathway [110]. Conversely, inhibition of autophagy by deletion of ATG5 gene or addition of 3-methyladenine has been shown to cause ER stress-induced apoptotic cell death in MEFs. Transcription factor EB (TFEB) was recently described to link autophagy and ER stress that is consolidated as integrated stress response (ISR).

TFEB links ER stress and autophagy

TFEB, the master regulator of autophagy, is known to regulate lysosomal biogenesis and genes that belong to the network of Coordinated Lysosomal Expression And Regulation (CLEAR genes) and autophagy. TFEB overexpression in different cell types such as HeLa, COS7, and MEFs increases autophagosome formation as determined by LC3II levels, and knockdown of TFEB causes decreased LC3II levels [65]. TFEB localizes to cytoplasm under homeostatic condition. However, upon amino acid starvation or serum deprivation, TFEB translocates to the nucleus which can be reversed by refeeding the cells with serum or amino acids. Thus, TFEB localization is regulated by growth factor and nutrient availability. TFEB undergoes phosphorylation by several kinases under nutrient replete conditions to localize in the cytoplasm; MAPK, mTOR, PKCβ, and AKT are kinases known to phosphorylate TFEB [65, 111-113]. TFEB localizes to lysosomes in the cytoplasm where it gets phosphorylated by mTOR. Upon phosphorylation, it binds to 14-3-3 scaffold protein in the cytoplasm as shown in

figure 4.2 [114]. Nuclear translocation of TFEB requires dephosphorylation in response to nutrient deprivation. Recently, calcineurin was identified as the phosphatase dephosphorylates TFEB in a calcium-dependent manner, which is effluxed from lysosomes in response to nutrient starvation through mucolipin 1 channel [15]. In AD models *in vivo*, TFEB has been shown to induce clearance of neurofibrillary Tau tangles formed by phosphorylated Tau, and rescue synaptic defects and neurodegeneration [115]. TFEB is recently being linked to contribute to ER stress acutely for survival and chronically in meditating cell death [116].

TFEB translocates to the nucleus in the presence of ER stress through PERK sensor on the membrane. TFEB upregulates lysosomal and autophagy genes to
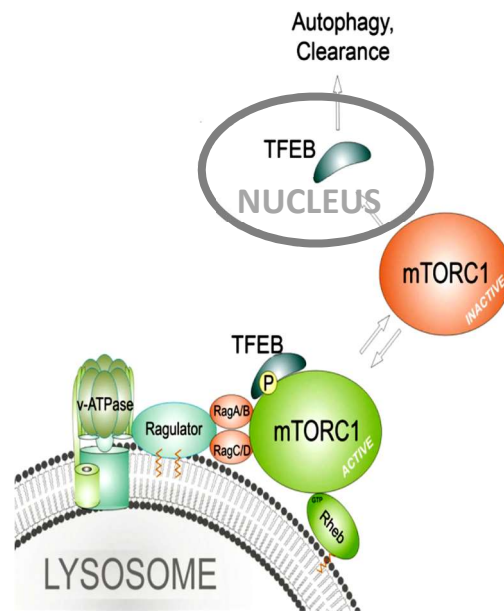

Figure 4.2: TFEB transcribes CLEAR network genes and regulate autophagy. TFEB is phosphorylated on lysosomes by mTORC1. Dephosphorylation translocates TFEB into the nucleus [1].

adapt to cellular stress. However, chronic ER stress induces TFEB mediated upregulation of ATF4 and CHOP leading to apoptotic cell death [116].

Neuronal cell death after TBI

Traumatic brain injury leads to neuronal and glial cell death. The trauma also cause axonal injury and leads to overall pathology of the brain and neurodegeneration. The dying cells exhibit both apoptotic and necrotic cell death. Neuronal cell death occurs near acute injury and glial cell death is more prominent in diffused injury. Post TBI neurons are lost in cortex, hippocampus, cerebellum and thalamus [117-119]. Calcium increase after TBI leads to activation of calcium dependent, non-lysosomal cysteine proteases; calpain. Calpain can lead to apoptosis and necrosis however *in vitro* study modeling neuronal injury associate calpain with necrosis [120]. Apoptotic cell death are regulated by cascade of cysteine proteases caspase. Caspases are divided into two groups the activator group and executor group. Caspase 8 and 9 belongs to initiator group and caspase 7, 6, 3 and 2 [121]. The caspases are regulated by different intrinsic and extrinsic signaling pathway converging into Bcl-2 family proteins. Some of them are pro-apoptotic such as Bax, Bad, Bid, and Bcl-xS and some are anti-apoptotic like Bcl-2 and Bcl-XL [122]. Apoptosis and autophagy converges at Bcl-2 family proteins. Autophagy mediator Beclin1 binds to Bcl-2 or Bcl-XL. Dissociation of Bcl-2 from Beclin1 and association to pro apoptotic BH3 only proteins leads to apoptosis [123]. Increase in Bcl-2 levels is found in cells surviving traumatic brain injury in rats and human brains conversely decrease in Bcl-2 correlated to increase cell death [124-126]. ER stress regulates Bcl-2 family proteins and can mediate ER stress induced cell death [127].

To understand the role of autophagy in neurodegeneration after TBI, we need tools to induce autophagy during TBI and monitor neurodegeneration. One can use genetic or pharmacological approach to alter autophagy, such as conditional knockout of Atg or other autophagy genes to inhibit autophagy, or drugs such as 3-Methyladenine, Wortmannin, rapamycin, trehalose, etc., for inducing autophagy. TAT-Beclin1 peptide a novel candidate that can induce autophagy without affecting any other pathway was synthesized in Levin lab [62]. TAT-Beclin1 peptide is derived from an important autophagy mediator Beclin1, and is especially useful in inducing autophagy *in vivo* for animal studies.

Role of Beclin1 in autophagy

Beclin1, also called as Atg6 or Vsp30, is a part of a lipid kinase complex that is required in the initiation of autophagosome formation. It is a BH3-domain-containing protein which was discovered in a yeast two-hybrid screening using Bcl2 anti-apoptotic protein for binding [128, 129]. Beclin1 knockout is embryonically lethal and is found to be associated with endoplasmic reticulum, mitochondria, perinuclear membrane, and golgi apparatus [62, 130]. Molecular structure of Beclin1 can be divided into 3 domains: BH3 domain, central coil-coiled domain (CCD), and an evolutionary conserved domain (ECD). The ECD domain is important in mediating autophagy since it binds to PI3KC3/vps34, whereas BH3 domain binds to Bcl2 protein that helps in regulating its activity, and CCD is needed for binding to other proteins of the PI3KC3 complex such as Ambra, UVRAG, and Atg14L. The BH3 domain of Beclin1 oligomerizes to form a protein complex

platform for the nucleation of phagophore formation [130, 131]. Beclin1 is transcriptionally regulated by NFκB, E2F, and miR30 [132-134]. Beclin1 is post-translationally modified to regulate its role in autophagy and endocytosis through phosphorylation mediated by death associated protein kinase (DAPK) and Akt. Additionally, K63 linked ubiquitination by tumor necrosis factor receptor associated factor 6 (TRAF6) and deubiquitination enzyme A20 regulate ubiquitination for oligomerization of Beclin1 to induce autophagy [135-137]. Regulation of autophagy by post-translational modification of Beclin1. Autophagy is induced when Beclin1 binds to PI3KC3 complex proteins, and autophagy is inhibited when Beclin1 binds to Bcl2 or BclXL proteins [138]. Beclin1 has also been reported to play a role in protein sorting during endocytosis for degradative pathway [137]. Beclin1 is a tumor suppressor and deletion of one allele causes malignancy in lung cancer, lymphoma, breast cancer, and liver cancer [139, 140]. Amongst other diseases, loss of Beclin1 allele could increase the risk of Alzheimer's like phenotype, cardiomyopathy, etc. [141, 142]

Beclin1 also regulates anti-viral defense mechanism. Many viruses have evolved with some protein to bind Beclin and inhibit its function, thereby inhibiting autophagy and facilitating viral replication and release from the host. Some of the viral proteins that bind to Beclin1 are Bcl2-like protein in ɤ Herpesvirus [143], ICP34.5 in α Herpesvirus [144], Nef in HIV-1, and M2 protein in influenza virus.[145, 146].

TAT-Beclin1 peptide: An autophagy inducer

Beclin1 is an important regulator of autophagy during homeostasis and disease. It is also highly conserved in mammals, which makes it a candidate protein to be used ectopically to alter autophagy. Levine lab has synthesized an 18 amino acid long peptide derived from Beclin1 that can induce autophagy. This peptide domain is a part of Beclin1 that binds to Nef protein in HIV-1 and inhibits autophagy. The peptide does not include BH3 domain and thus is independent of regulation by Bcl-2 or Bcl-XL. The peptide binds to Golgi-associated plant pathogenesis-related protein 1 (GAPR-1), a golgi protein localized on the cytosolic part of golgi membrane. GAPR-1 is an endogenous negative regulator of autophagy as it binds to Beclin1 inhibiting it's binding to PI3KC3 protein complex.

The peptide was fused to TAT protein transduction domain (PTD) of HIV-1 to increase the cell permeability and two glycine linkers to 18 amino acid peptide. Thus, the peptide induces autophagy by binding to the negative regulator GAPR-1 and causing endogenous Beclin1 free for autophagy and endocytosis. The peptide was shown to induce autophagosome formation in HeLa cells expressing LC3-GFP and in MEF cells. The peptide induced canonical autophagy pathway, as determined by knockdown of *BECN1* and *Atg7* genes that decreased LC3II levels. In mouse models, TAT-Beclin1 peptide reduced the viral titer after infection with West Nile virus, chikungunya virus, and HIV-1 virus. It was also shown to induce autophagy in mice as monitored by LC3-GFP [62]. TAT-Beclin1 peptide has also been shown to promote regeneration of neurons after spinal cord injury by degradation of SCG10, a microtubule disassembly protein, and stabilization of

microtubules [147]. In our study to regulate autophagy after TBI and determine neurodegeneration we used TAT-Beclin1 peptide. TAT-Beclin1 peptide regulates autophagy both *in vitro* and *in vivo,* the peptide targets specifically autophagy and endocytosis. One can regulate autophagy transiently at different time points without any genetic manipulation.
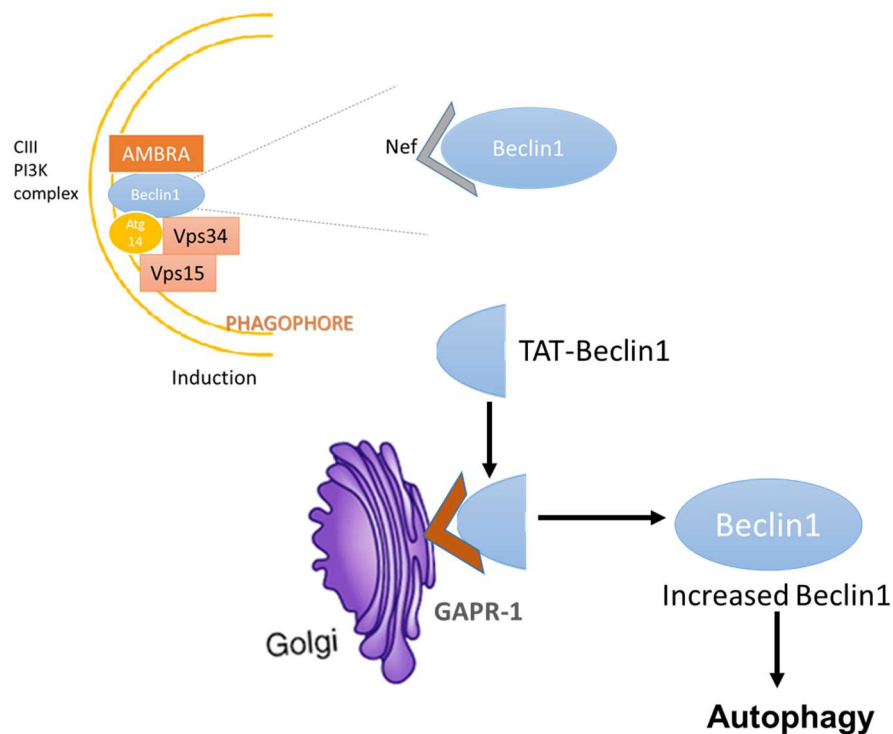

Figure 4.3: TAT-Beclin1 peptide is derived from Nef-binding domain of Beclin1. TAT-Beclin1 binds to and sequesters GAPR-1, a negative regulator of Beclin1, on golgi membrane, freeing endogenous Beclin1 to induce autophagy.

Results

TAT-Beclin1 dependent autophagy induction prevents lysosomal inhibition after injury

<u>Biochemical analysis of LC3 and P62 in cerebral cortex lysate 1 day post TBI</u>

Autophagy is a multiple step process which involves autophagosome formation that are identified as LC3II positive double membrane vesicle and fusion of autophagosomes with lysosomes. This can also be detected by degradation of lysosomal substrate such as P62. Physical insult to the brain such as TBI alters the homeostatic state of cell, including autophagy. For instance, TBI leads to accumulation of aggregated proteins in the cells, indicating that the degradation pathway is inhibited. Research from Sarkar *et al.* showed that TBI causes accumulation of autophagosomes and inhibition of autophagy [89]. Based on these observations, we intended to determine whether inhibition of autophagy in TBI could be prevented using TAT-Beclin1 peptide. To study autophagy flux in TBI with or without TAT-Beclin1 treatment, we measured LC3I, LC3II and P62 levels in the cortex. LC3II is a marker for autophagosome formation. We found that LC3II levels increased in PID1 (Post-injury day 1) in our TBI model, indicating increased autophagosome formation. Since the increased LC3II level does not give provide much information on the flux of the autophagy, we determined P62 levels which is a substrate of lysosome. P62 levels were significantly higher in TBI animals indicating inhibition of lysosomal degradation as seen in figure 4.4 Taken together, increased LC3II and P62 levels in TBI indicates accumulation of autophagosomes and inhibition of lysosomal degradation. This confirmed the findings of Sarkar *et*

al. in our concussion-based TBI model. TAT-beclin1 treatment significantly decreased LC3II and P62 levels indicating attenuation of accumulation of autophagosomes and functional lysosomal degradation. Thus, using TAT-Beclin1 peptide on TBI animals likely causes prevention of lysosomal inhibition. TAT-Beclin1 alone causes increase in LC3I and LC3II levels with a concomitant decrease in P62 levels suggesting that TAT-Beclin1 peptide is able to pass blood-brain barrier and reach neural cells in case of peritoneal delivery, increasing the basal autophagy flux but not significantly. In case of topical applications, TAT-Beclin1 directly reached the injured brain cells. Sarkar *et al.* reported that the autophagosome accumulation was high from day 1 to day 3 and eventually normalized to sham levels by day 7 in CCI injury model. We next wanted to determine inhibition of autophagy flux on day 3 and rescue with TAT-Beclin1 peptide.

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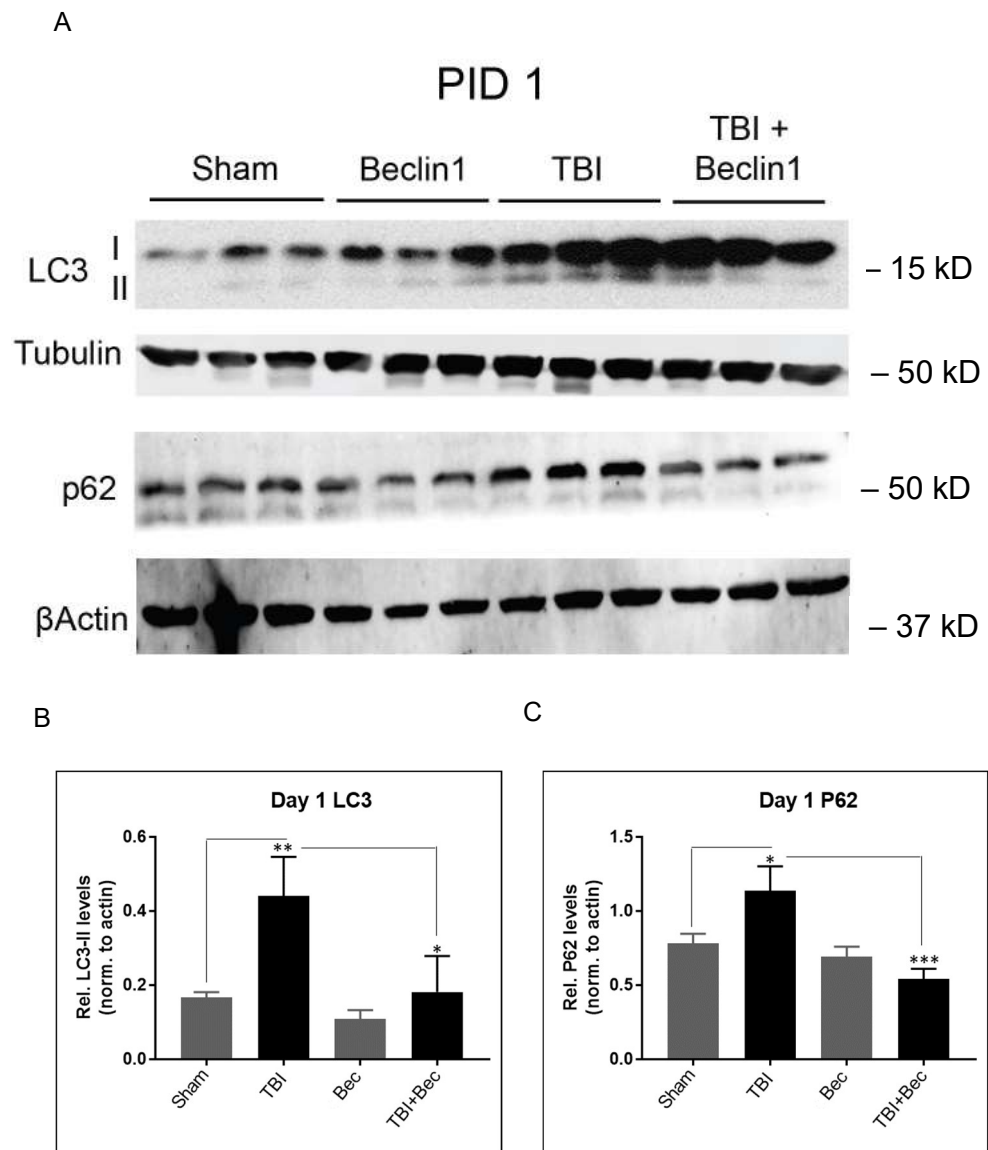**Figure 4.4:** TAT-Beclin1 prevents autophagy inhibition 1 day after TBI. **A)** Western blots of autophagy marker LC3 and P62, for determining autophagy flux. **B,C)** Densitometric analysis of LC3-II and P62 which is normalized to the loading control Tubulin and Actin respectively, this is further normalized to sham to represent the fold change. Data is shown as mean ± SE, n (number of animals) =3 *P<0.05, **P< 0.01, ***P<0.001

Biochemical analysis of LC3 and P62 in cerebral cortex lysate 3 and 7 days post TBI

To determine the autophagy flux on day 3 post injury, LC3I, LC3II and P62 levels were determined in the cerebral cortex after TBI with or without TAT-Beclin1 treatment. LC3 levels were significantly elevated in TBI brains with or without TAT-Beclin1 treatment, and P62 levels were high in TBI indicating inhibition of autophagy flux and lysosomal inhibition similar to day 1 post injury. Moreover, as shown in figure 4.5, the inhibition of autophagy is prevented using TAT-Beclin1 peptide as evidenced by decrease in P62 levels in TBI with TAT-Beclin1 treatment. In our LFP TBI model, autophagy inhibition is stronger on day 1 than day 3 post injury based on the fold increase in LC3II and P62 levels. TAT-beclin1 peptide alone increases the basal autophagy flux based on LC3II and P62 levels. In LFP TBI, TAT-Beclin1 is preventing lysosomal inhibition both on day 1 and day 3 after injury.

It has been reported that 7 days post injury, autophagy flux eventually normalizes to sham conditions (Sarkar 2014). To determine normalization of autophagy in our TBI model by day 7 and role of TAT-Beclin1 peptide at this time point after injury, we determined the levels of LC3II and P62 similar to day 1 and day 3 in lysates of cerebral cortex. As shown in figure 4.6, LC3II levels were significantly high in 7 days post TBI, and P62 level was slightly higher than sham but not significantly high, indicating inhibition of autophagy flux. Thus, by day 7, autophagy flux is normalizing to sham conditions. Interestingly, brains in TBI with TAT-Beclin1 treatment had significantly low levels of LC3 II and P62 as shown in figure 4.6. By

day 7 post TBI, TAT-Beclin1 increases autophagy flux compared to TBI alone brains.

A
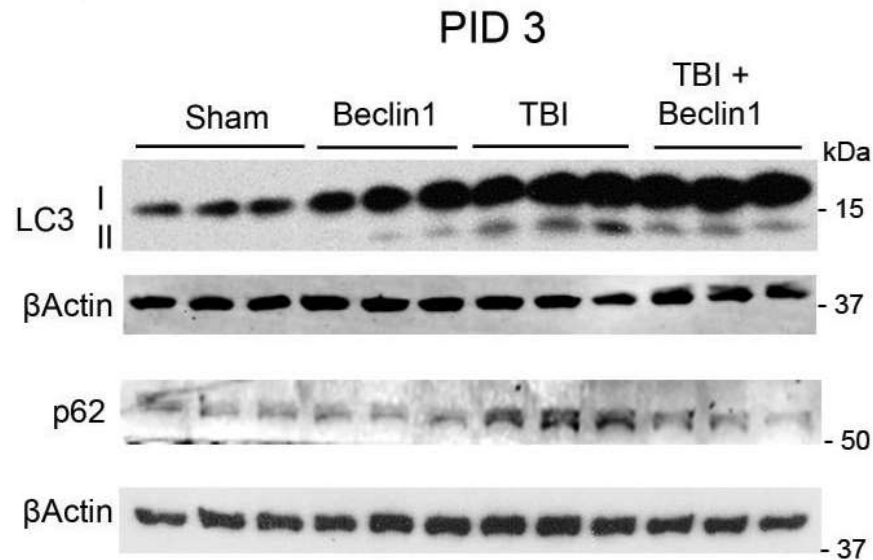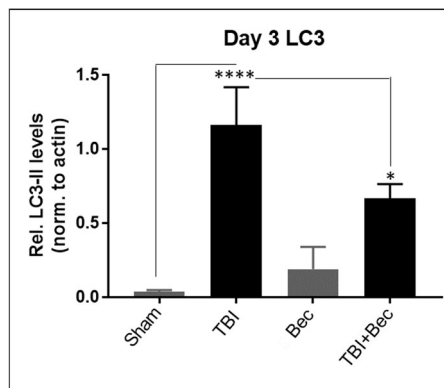
Figure 4.5: TAT-Beclin1 prevents autophagy inhibition 3 day after TBI. **A)** Western blots of autophagy marker LC3 and P62, LC3II levels have significantly increased in TBI and TBI with TAT-Beclin1 treatment. P62 levels are significantly low in TAT-Beclin1 treated TBI animals compared to high levels in TBI animals. **B,C)** Densitometric analysis of LC3-II and P62 which is normalized to the loading control Tubulin and Actin respectively, this is further normalized to sham to represent the fold change. Data is shown as mean ± SE, n=3 *P<0.05, ****P<0.0001.
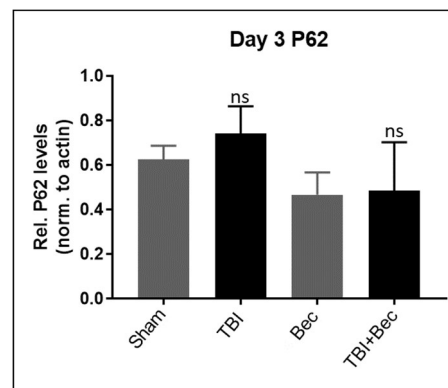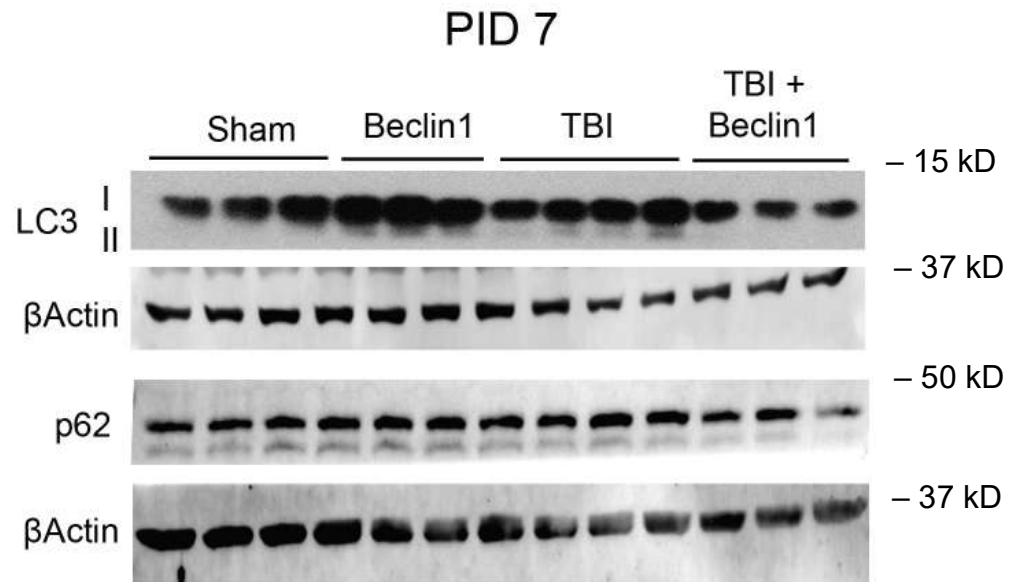
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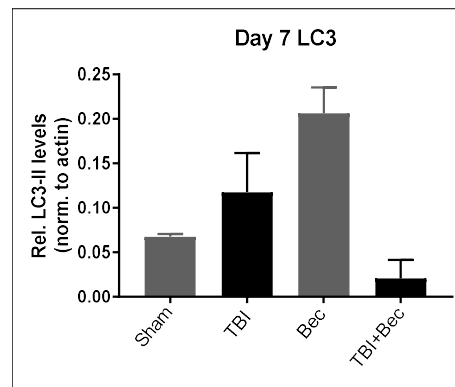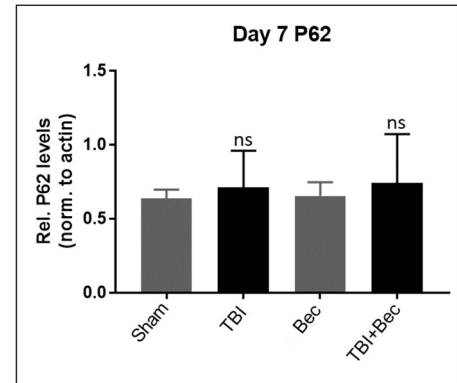
Figure 4.6: TAT-Beclin1 increases autophagy flux by day 7 after TBI. **A)** Western blots of autophagy marker LC3 and P62, LC3II levels were high in TBI and significantly low in TBI with TAT-Beclin1 treatment, indicating accumulation of autophagosomes in TBI, TAT-Beclin1 decreases LC3II level. P62 levels did not change significantly on day 7 after TBI, TAT-Beclin1 significantly decreased the P62 level indicating increase in autophagy flux. **B,C)** Densitometric analysis of LC3-II and P62 which is normalized to the loading control Tubulin and Actin respectively, this is further normalized to sham to represent the fold change. Data is shown as mean ± SE, n=3

TAT-Beclin1 prevents accumulation of P62 and LC3 in neurons indicating an induction of autophagy

Three days after injury, there was accumulation of autophagosomes shown by increased levels of LC3 and P62 suggesting inhibition of lysosomal degradation (figure 4.5). TAT-Beclin1 treatment on TBI brain decreased LC3II and P62 levels indicating normalization of autophagy flux. In order to confirm whether the inhibition of autophagy is happening in neurons, we stained rat brain sections acquired from day 3 after injury with antibodies against autophagosome markers LC3 and lysosomal substrate P62, as well as neuronal marker Tuj1 to identify neurons. The neurons were identified as Tuj1 positive cells and corresponding levels of LC3 and P62 was determined. Under sham condition, neurons had very low levels of LC3 puncta indicating reduced autophagosomes. The data agreed to previously described levels of autophagy under normal condition [72], where basal level of autophagy in neurons was described to be constitutively active, making it difficult to visualize LC3 puncta under normal conditions.

We found that in LFP TBI condition, there was increased LC3 positive puncta and P62 levels in neurons, indicating inhibition of autophagy flux after TBI. Our result was similar to what was described by Sarkar *et al.* [89]. In our study, this outcome is replicated in rat using LFP TBI model as opposed to CCI TBI model used by Sarkar *et al.* Thus, these data underscore the commonality in autophagic changes after brain injuries, irrespective of different kinds of TBI or animal model.

Importantly, we find that TAT-Beclin1 peptide prevents inhibition of autophagy flux in neurons after TBI, indicated by presence of LC3II puncta and decreased P62 accumulation.
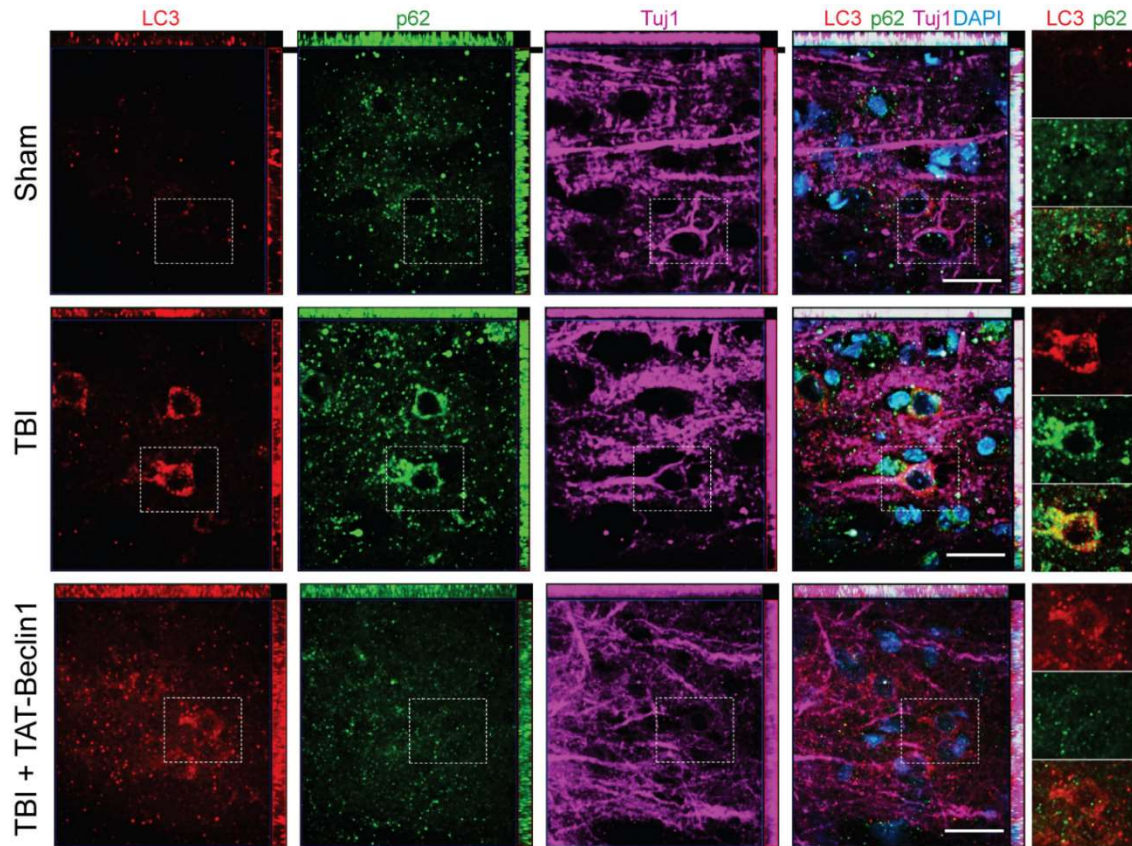
Figure 4.7: Autophagosomes and P62 accumulate in neurons after TBI on 3 day post injury, P62 level decrease with TAT-Beclin1 peptide. Images (63X) of Rat cortical brain sections stained with antibodies against autophagy marker LC3, lysosomal substrate P62, and neuronal marker Tuj1. Scale bar = 50 µm. The boxed region are neurons that are zoomed further. The quantification of the Data is shown in the next figure. Microscopic images confirm the outcome from western blot that TAT-Beclin1 peptide prevents inhibition of autophagy flux after TBI.

A

rel. LC3 signal intensity

B

rel. p62 signal intensity

LC3/p62 Colocalization

C
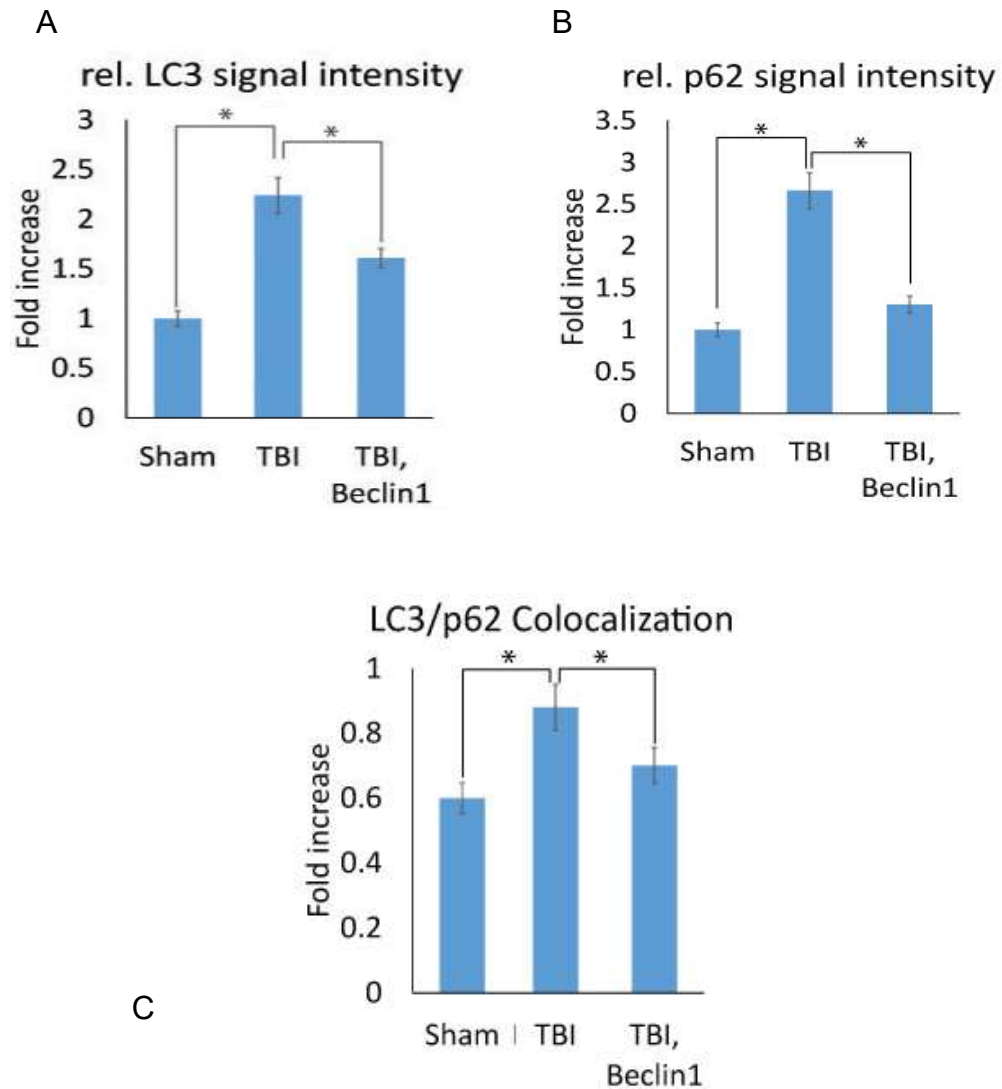
Figure 4.8: Autophagosomes and P62 accumulate in neurons after TBI on 3 day post injury, P62 level decrease with TAT-Beclin1 peptide. **A)** The bars represent intensity reading of LC3 signals in Tuj1 positive cells normalized to sham **B)** The bars represent intensity reading of P62 levels in Tuj1 positive cells normalized to sham. **C)** The bars represent percentage of cells having colocalization of LC3 and P62 in Tuj1 positive cells. Data are represented as mean ± SE, n=3. *P< 0.05

TAT-Beclin1 prevents autophagy flux inhibition and increases clearance of pTau buildup

Our earlier data indicated that LFP TBI causes inhibition of autophagy flux and TAT-Beclin1 peptide restores the autophagy flux after TBI. Tauopathy is one of the biomarker for mild-to-moderate TBI [148]. We therefore wanted to investigate whether pTau buildup occurs upon LFP TBI and, importantly, whether the pTau accumulation is cleared upon restoration of autophagy flux by TAT-Beclin1. Microscopic analysis on TBI brain and TBI + TAT-Beclin1 brain cortex indicated that there was accumulation of PHF form of pTau around the injury zone. To identify the cell type in which there is pTau buildup, we stained cells with antibodies against neuronal marker Tuj1 and astrocyte marker GFAP. The data indicated that most neurons were positive for pTau accumulation in the soma (figure 4.9). The number of astrocytes were more in TBI brains but with TAT-Beclin1 peptide the number of astrocytes were less, indicating neuroinflammation after TBI which appeared to be attenuated with TAT-Beclin1 peptide. Quantification of relative pTau fluorescent intensity in Tuj1-positive cells indicated approximately 14-15 fold increase in pTau levels in neurons, which was significantly attenuated with TAT-Beclin1 peptide after TBI to 4-5 fold compared to sham. We further estimated total pTau level in the lysates from cortex which is shown in figure 4.10.

A
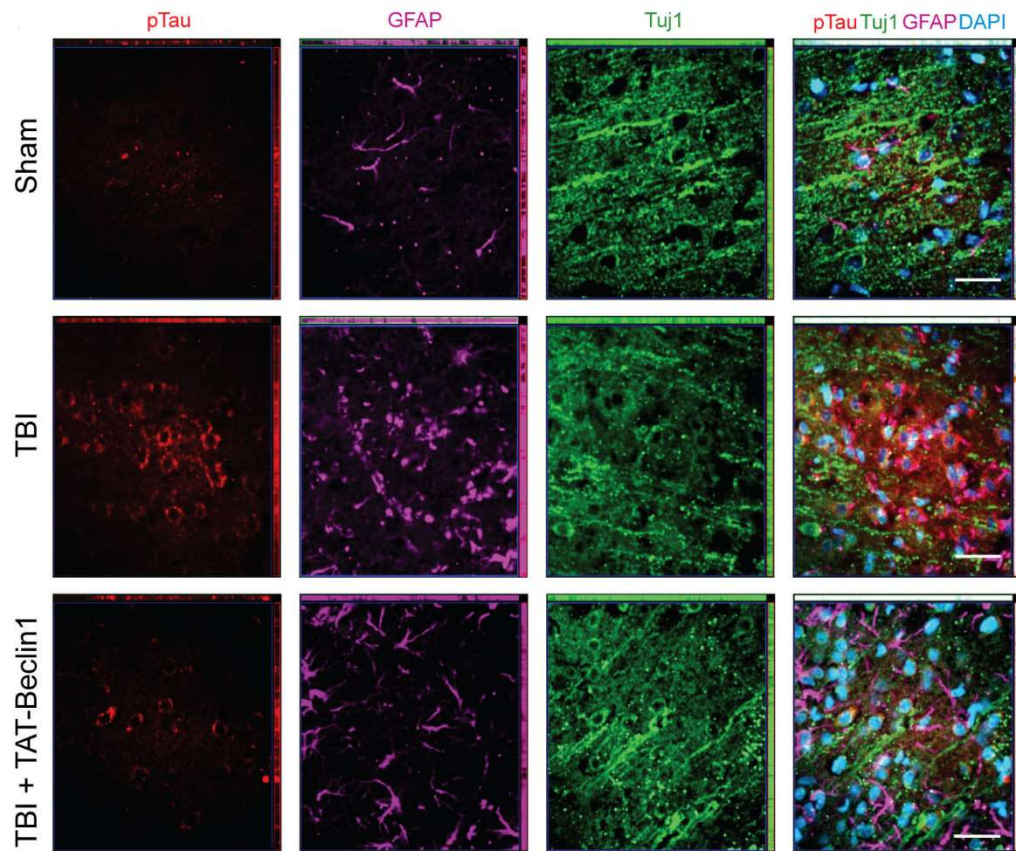
B

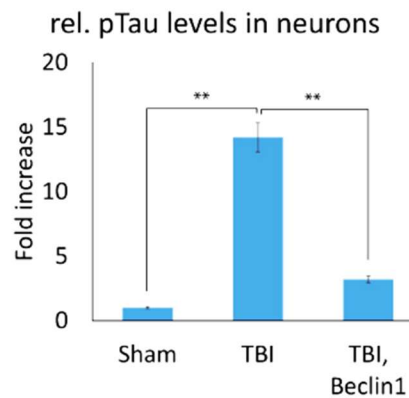**Figure 4.9:** Paired helical form (PHF) of pTau is buildup after TBI and is attenuated by the TAT-Beclin1 peptide **A)** Images (40X) of Rat cortical brain sections stained with antibodies against PHF pTau AT8, neuronal marker Tuj1 and astrocyte marker GFAP. Scale bar = 50 μm **B)** The bars represent relative intensity of pTau signal in Tuj1 positive cells normalized to sham. Data are represented as mean ± SE, n=3 ** P< 0.01.

pTau levels increase in cerebral cortex after TBI which is cleared by TAT-Beclin1 peptide

Biochemical analysis of pTau accumulation in cortex of TBI with or without TAT-Beclin1 replicates the observation from microscopic analysis. Total pTau level in the TBI brain significantly increases (4-5 fold) compared to sham, and TAT-Beclin1 peptide attenuated the pTau build up in the cortex indicating that the restoration of autophagy flux prevents/clears pTau buildup after TBI. The Western blot was difficult to replicate with other groups of 3 animals each, due antibodies. Therefore, no statistical significance could be calculated. But the western blot could replicate the pTau accumulation that was observed in 6 animals under each experimental group in microscopic analysis, giving us confidence in the results. This experiment also confirms the restoration of functional autophagy after TBI using TAT-Beclin1 peptide. Comparing the microscopic and biochemical data, pTau which is a neurotoxin appears to accumulate in neurons more than astrocytes, although other cell types were not investigated. Next, we wanted to study the impact of pTau clearance and restoration of autophagy on neuronal degeneration caused due to TBI.
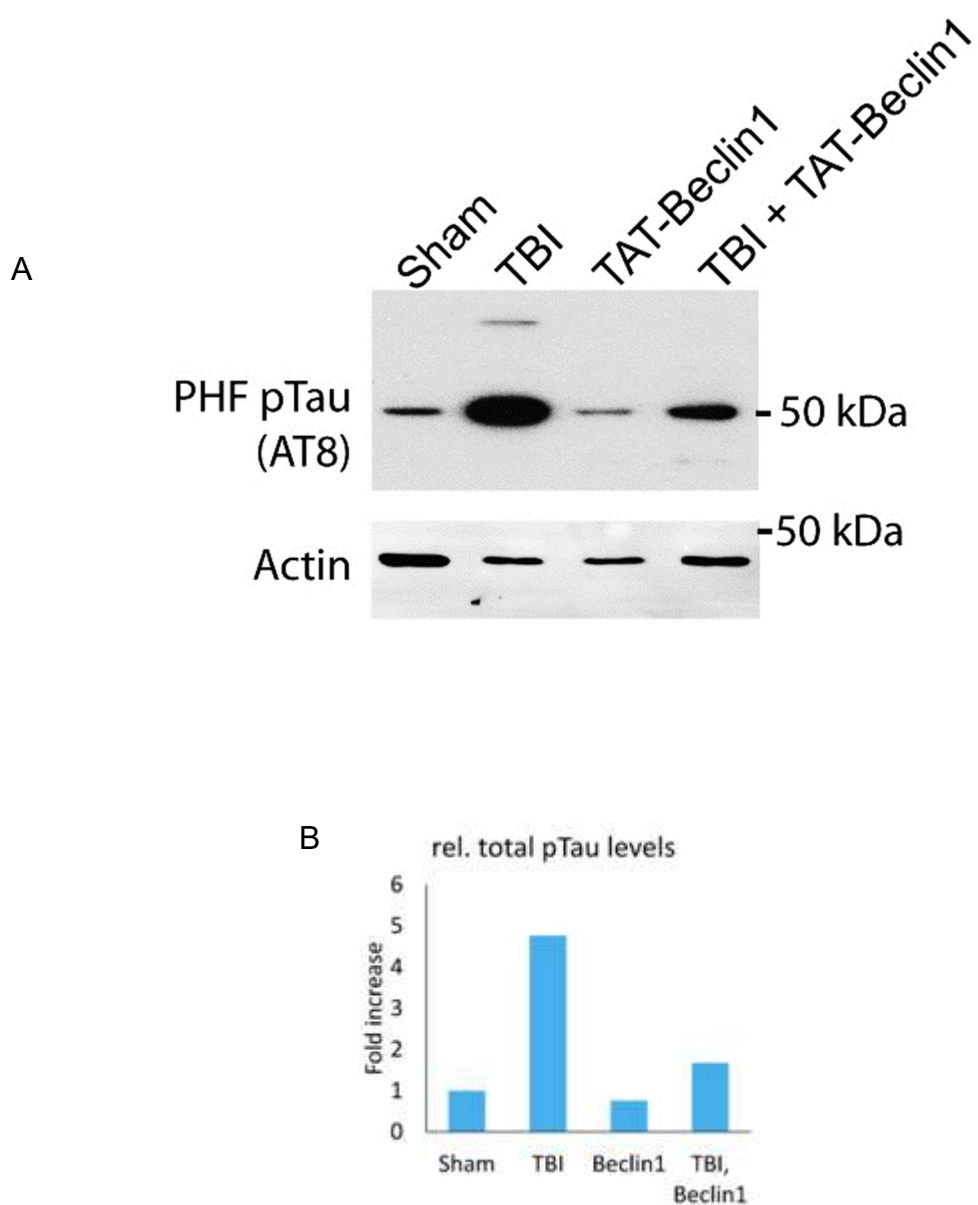
Figure 4.10: TAT-Beclin1 peptide attenuates pTau accumulation after TBI **A)** Western blot of PHF pTau (AT8) in cortex in TBI with or without TAT-Beclin1 treatment. **B)** Densitometric analysis of pTau, normalized to the loading control actin, this is further normalized to sham to represent the fold change. Data is represented is from n=1.

TAT-Beclin1 attenuates neurodegeneration in brain after TBI

TAT-Beclin1 prevents/clears neurotoxic pTau accumulation in TBI brains, indicating that it may play a role in neurodegeneration caused by TBI. Fluoro-Jade staining determines neurodegeneration caused by neurotoxins or mechanical insults specifically in neurons [149]. We therefore used this method to determine the extent of neurodegeneration in TBI brains with or without TAT-Beclin1 peptide. The images of the brain sections showed clear difference in terms of neurodegeneration, as indicated in figure 4.11. The bright fluorescent positive cells as seen in TBI brains were scored as neurodegenerative cells. As expected, TBI brains had significantly higher levels of neurodegeneration. Interestingly, TAT-Beclin1 treated rats with TBI had significantly less neurodegeneration. Our earlier experiments indicated TAT-Beclin1 restores autophagy, clears pTau, and attenuates neurodegeneration. However, we do not know which autophagy mediated signaling pathway plays a role in neurodegeneration after TBI and how is TAT-Beclin1 able to regulate the pathway to prevent neurodegeneration? To further investigate the signaling pathway involved, we explored if the observed neurodegeneration leads to apoptotic cell death.

<u>Fluoro-Jade staining on brain sections to determine neurodegeneration in TBI with or without TAT-Beclin1 peptide.</u>

A
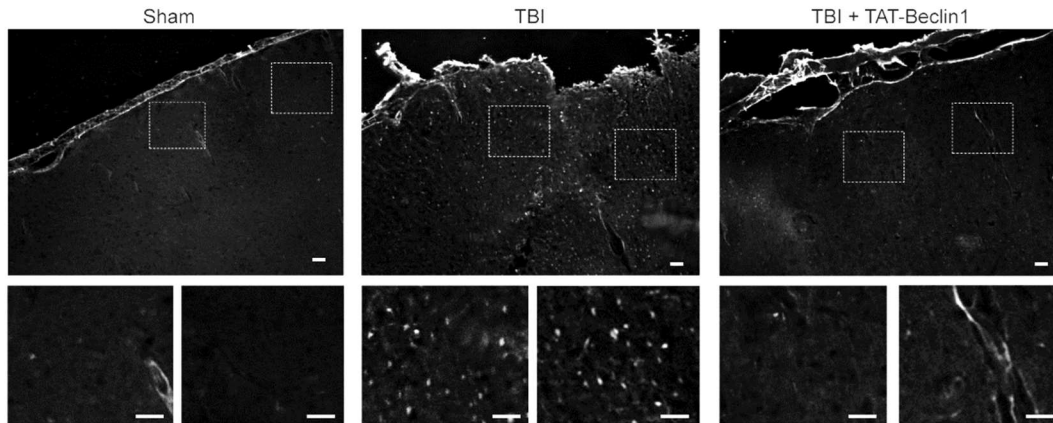
B

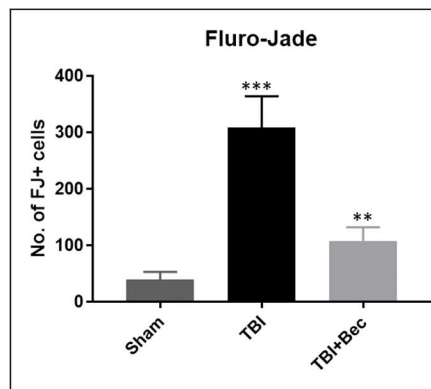**Figure 4.11:** TAT-Beclin1 peptide attenuates neurodegeneration. **A)** Images (100X) brain sections are stained with Fluoro-Jade stain to detect neuronal degeneration. Scale bar = 100 μm **B)** The bars represent number of bright florescence normalized to sham thus showing relative levels of neurodegeneration. Data are represented as mean ± SE, n=6. **P< 0.01 *** P< 0.001

TAT-Beclin1 decreases apoptotic cell death in neurons after TBI

Neurodegeneration is defined as progressive loss of structure and function of neurons leading to neuronal cell death. To determine if neurodegeneration leads to apoptotic cell death, we stained the brain sections with antibody for cleaved caspase 3 which is the activated form of caspase. The activated cleaved caspase 3 is involved in activation of other caspases, DNA fragmentation, and cytoskeleton disassembly in the apoptotic pathway. Late stage apoptotic cells have nuclear cleaved caspase 3, whereas early stages will have cytoplasmic cleaved caspase 3. In our staining, TBI brain sections had more nuclear and cytoplasmic cleaved caspase 3 indicating both early and late stage apoptotic neurons. In TAT-Beclin1 treated TBI rats, the number of apoptotic neurons were significantly reduced. This observation supports our study that TAT-Beclin1 rescues neurodegeneration after TBI and specifically apoptotic cell death is regulated by the peptide in neurons. The staining could not be replicated in other set of 3 animals per experimental conditions due to poor primary antibody binding. However, we made one attempt to carry out In Situ End-labelling (ISEL) staining for detecting apoptosis in the cortex region of brain sections giving us similar results. Thus giving confidence in the outcome of the experiment. Apoptosis is regulated by Bcl-2; inhibition of Bcl-2 protein will activate apoptotic pathway. CHOP is a transcription factor that inhibits Bcl-2, activating apoptosis. CHOP in turn is regulated by transcription factor ATF4 that is expressed during endoplasmic reticulum stress. ATF4 generally signals for survival response but when is chronically active will switch to apoptotic cell death through CHOP. We therefore wanted to investigate if these two transcription

factors are involved in neuronal cell death. This study will also unveil the underlying

signaling pathway involved in autophagy mediated neuronal cell death.

A
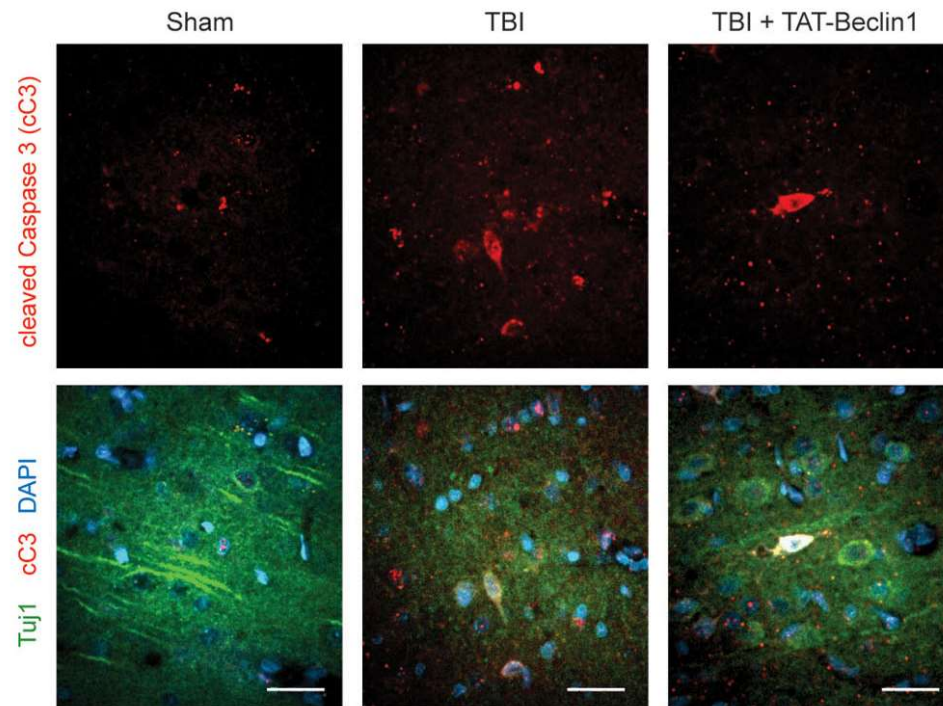
B

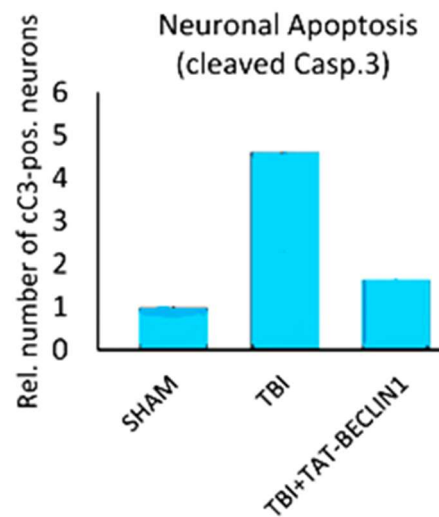**Figure 4.12:** TAT-Beclin1 peptide decreases apoptotic cell death in neurons. **A)** Images (63X) brain sections is stained with antibodies against cleaved caspase3 and Tuj1 to detect apoptosis in neurons. The white arrows point the nuclear cleaved caspase 3 Scale bar = 50 µm **B)** The bars represent relative number cleaved caspase 3 positive in neurons normalized to sham. Data are represented from, n=1

TAT-Beclin1 attenuates neurodegeneration by inhibiting CHOP/ATF4 signalling

Brain sections were stained with antibodies against ATF4 and CHOP proteins to evaluate their role in neurodegeneration after TBI. ATF4 is expressed under ER stress via PERK signaling. Increase in cytoplasmic ATF4 along with nuclear translocation indicate activation of ATF4 signaling. ATF4 activates survival signals upon ER stress, however increased ATF4 signaling chronically will induce apoptotic pathway by activating CHOP signaling. As shown in figure 4.14, 40x images of the cortex shows significant increase in ATF4 levels and ATF4 positive neurons in TBI cortex. Some of these neurons showed distinct nuclear localization of transcription factor ATF4 and corresponding increase in CHOP levels in those cells indicating active ATF4/CHOP signaling in neurons after TBI which would eventually activate apoptotic pathway through inhibition of Bcl-2. TAT- Beclin1 peptide in TBI animals significantly reduced ATF4 positive cells and overall levels along with a corresponding decrease in CHOP levels in the cortex near the injury zone. This important observation indicated that TAT-Beclin1 peptide is inhibiting ATF4/CHOP signaling after TBI, thereby reducing apoptotic neuronal cell death. Thus TAT-Beclin1 peptide appears to reduce ER stress caused due to TBI by restoring autophagy flux and clearing accumulation of neurotoxic proteins such as pTau. To confirm this, mRNA levels for integrated stress response genes (ISR) that also include ER stress response genes was determined by qPCR. Data from the lab shows that TBI increased ISR gene transcripts significantly including TFEB, master regulator of autophagy. Interestingly, TAT-Beclin1 peptide significantly

decreased TFEB, ATF4, CHOP, and HERPUD1 transcript levels, but other ER stress markers such as Bip, GRP94, and EDEM1 did not show significant decrease in the transcript abundance. qPCR data indicates that TAT-Beclin1 peptide does not attenuate ER stress completely, but impacts on only specific markers. Interestingly, qPCR data indicated high TFEB transcript levels after TBI as shown in figure 4.13. In a recent study, it was shown that chronic nuclear translocation of TFEB along with ER stress activates ATF4/CHOP pathway to induce apoptotic cell death *in vitro* [116]. We therefore wanted to determine the TFEB protein levels and its cellular localization in neurons of the injured cortex with or without TAT-Beclin1 peptide.
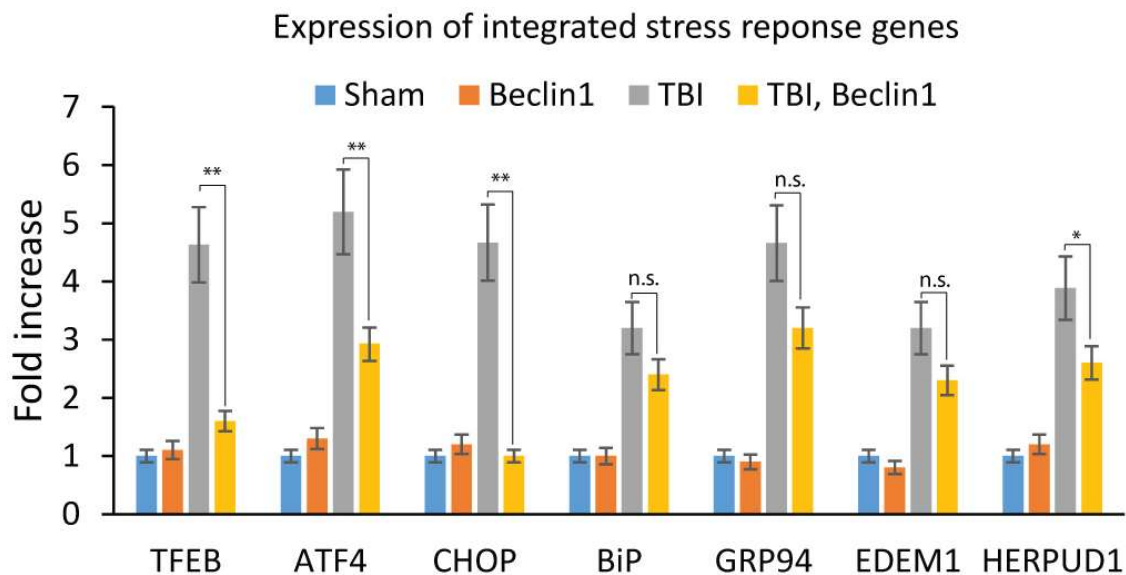
Figure 4.13: TAT-Beclin1 peptide decreases transcript levels of TFEB, ATF4 and CHOP in injured brain on PID3. The other ER stress marker such as Bip, GRP94 etc does not significantly decrease with TAT-Beclin1 treatment after TBI. The bars represent fold increase in transcript level on PID3 Data are represented as mean ± SE, n=4. *P< 0.05 **P< 0.01

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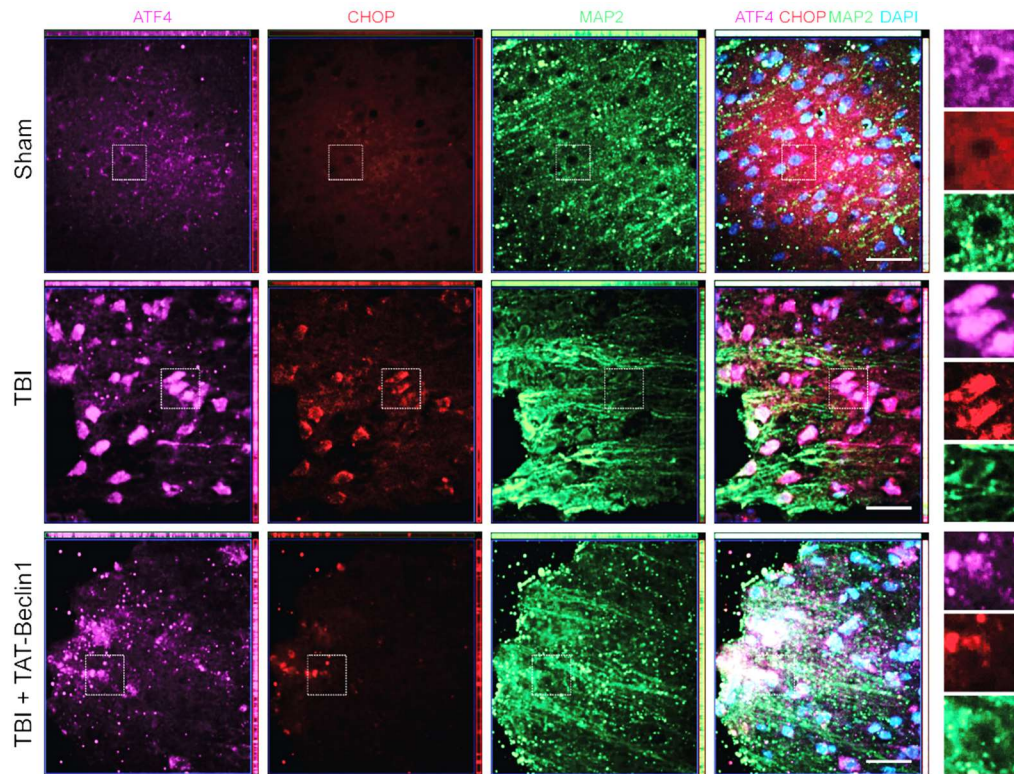
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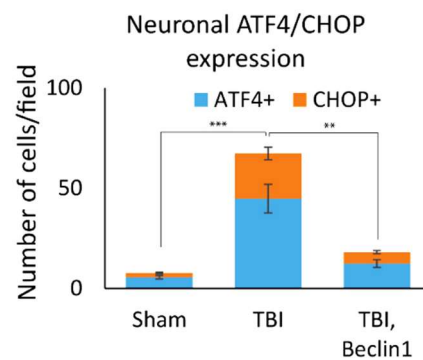
Figure 4.14: ATF4/CHOP signal is inhibited in TAT-Beclin1 treated TBI brains. **A)** Images (40X) brain sections is stained with antibodies against ATF4, CHOP and Map2 as neuronal marker. Scale bar = 50 µm **B)** The bars represent number of Map2 positive cells that are also positive for ATF4/CHOP per field. Data are represented as mean ± SE, n=6. **P< 0.01 *** P< 0.005

TAT-Beclin1 dependent autophagy inhibits TFEB signaling in cortex of TBI brains

We next performed immunohistochemistry on brains sections and western blot on cortex lysates to determine the TFEB levels and distribution in the cortex. As shown in figure 4.15, immunohistochemistry results indicate significant increase in TFEB levels in the cortex after TBI, whereas TAT-Beclin1 peptide reduced TFEB levels in TBI animals. This observation was confirmed by western blotting. As seen in figure 4.16, there was a 3-fold increase in TFEB levels in TBI animals and TAT-Beclin1 peptide treatment caused a significant decrease in TFEB levels. The result suggests that TFEB may play a role in increasing ATF4 and CHOP levels in TBI. Next, we investigated the cellular localization of TFEB in neurons after TBI. Being a transcription factor, TFEB should enter the nucleus for its activity.

Microscopic analysis of TFEB levels and localization in neurons after TBI
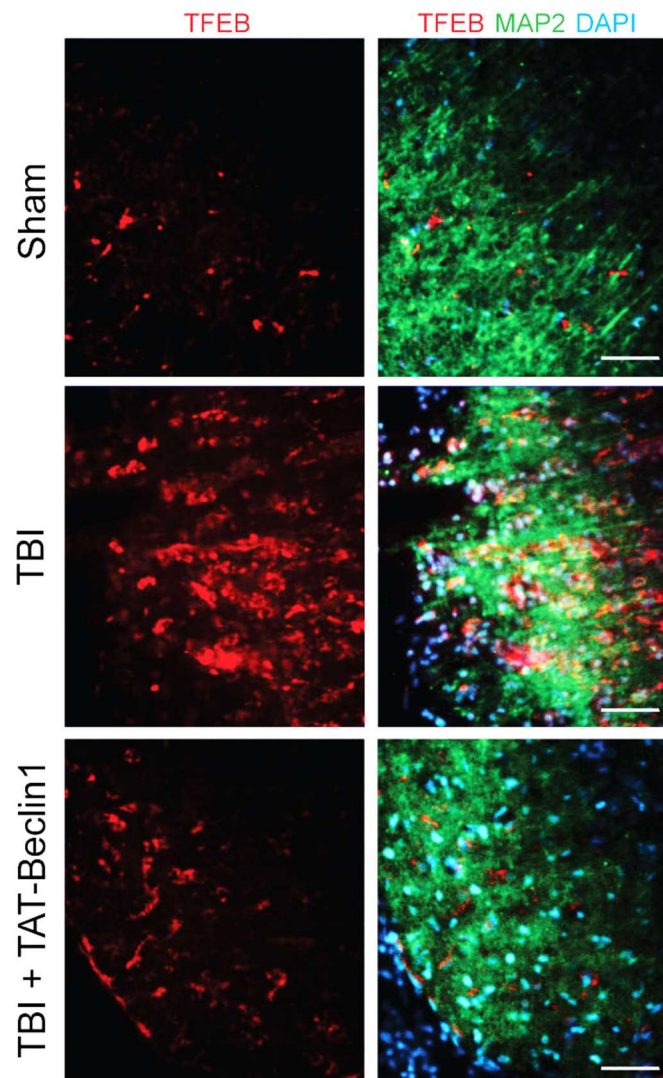
Figure 4.15: TAT-Beclin1 peptide decreases TFEB levels after TBI. Images (20X) brain section is stained with antibodies against master regulator of autophagy; TFEB. TBI has increased TFEB levels at the injury zone. Scale bar = 100 μm

Biochemical analysis of TFEB levels in cortex of TBI brain with or without TAT-
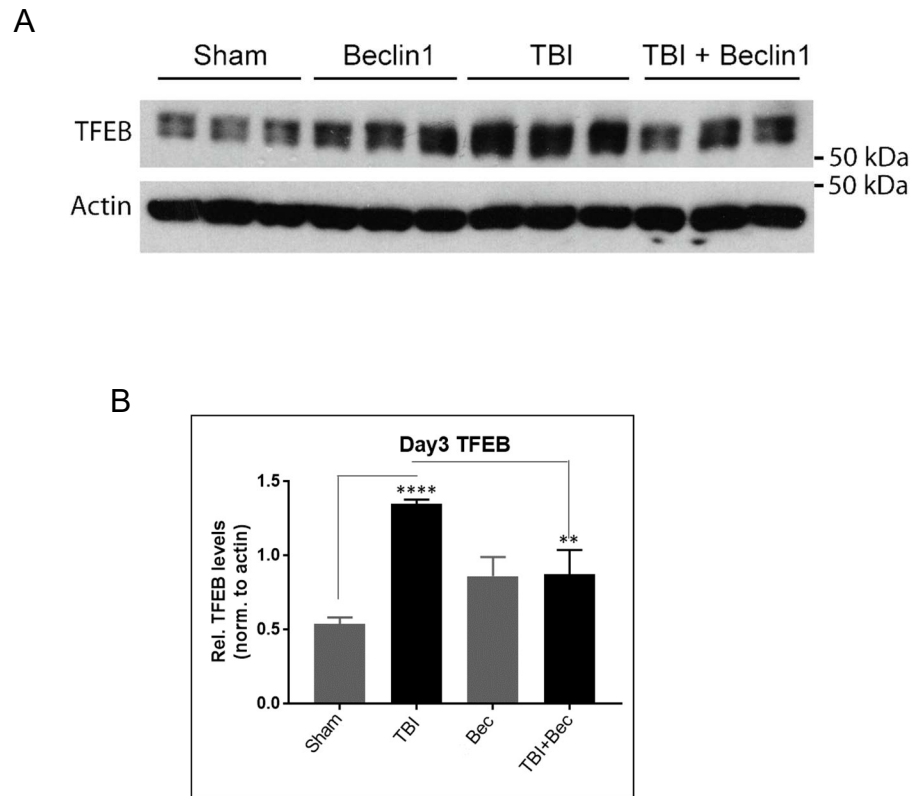
Beclin1 treatment

Figure 4.16: TAT-Beclin1 peptide decreases TFEB levels after TBI. **A)** Western blot for TFEB in cortex region of the brain in TBI with or without TAT-Beclin1 treatment. **B)** Densitometric analysis of TFEB normalized to the loading control actin. This is further normalized to sham to represent the fold change. Data is shown as mean ± SE, n=3 **P< 0.01, ****P<0.0001

TAT-Beclin1 peptide inhibits TBI-induced nuclear localization of TFEB in neurons

TFEB is described as a master regulator of autophagy [65]. It is a transcription factor that regulates autophagy through CLEAR network. Recent studies indicate that TFEB is involved in regulating lysosomal biogenesis, endocytosis, and integrated stress response [65]. In figure 4.17, immunohistochemistry results show perinuclear distribution of TFEB in the neurons as indicated in the magnified areas under sham condition; here TFEB appears to be membrane bound and not dispersed in the cytoplasm. TFEB has been reported to localize to lysosomes where mTOR phosphorylates TFEB and maintains it in the cytoplasm. Interestingly, in the same field, MAP2 negative cells also show TFEB distribution in the cytoplasm, indicating that TFEB is expressed in both neurons and glia cells. As shown before, TFEB level increases significantly in TBI brains (figure. 4.15, 4.16). Under higher magnification, upon TBI, neurons which are identified as MAP2 positive cells show increased levels of TFEB and clear nuclear translocation as shown in the magnified neuron. TFEB is distributed both in the cytoplasm and in the nucleus. Other non-neuronal cells, identified as MAP2-negative cells, also showed increased TFEB levels and nuclear translocation. Morphologically these cells resembled astrocytes. The number of non-neuronal cells with TFEB staining also increased in TBI. TAT-Beclin1 treatment reduced TFEB levels, corroborating our previous results (figure. 4.15, 4.16). Interestingly, TAT-Beclin1 treatment inhibited nuclear translocation of TFEB in neurons, as indicated in boxed region, and in non-neuronal cells. Thus, TAT-Beclin1 peptide appears to decrease cell

death through inhibiting ATF4/CHOP pathway in neurons by decreasing TFEB signaling. TFEB can localize to nucleus in its dephosphorylated form, but is retained in the cytoplasm upon phosphorylation by mTOR serine/threonine kinase. We therefore determined the activity of mTOR in TBI brains with and without TAT-Beclin1 peptide.
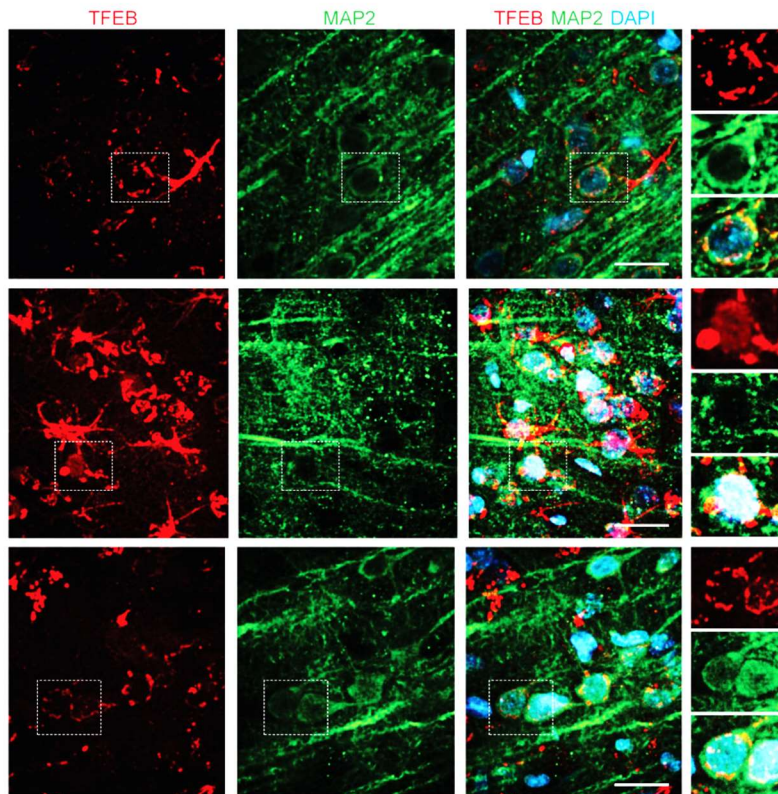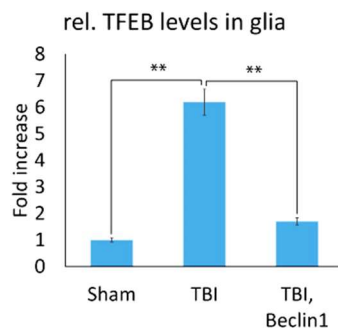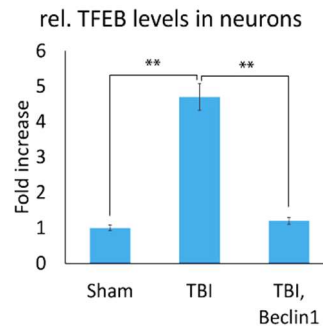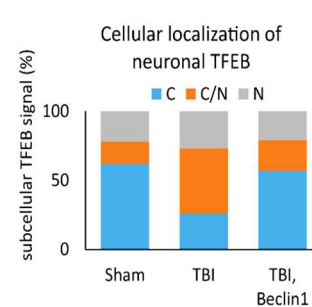
Figure 4.17: TAT-Beclin1 peptide decreases TFEB levels after TBI and inhibits nuclear translocation. **A)** Images (63X) brain section is stained with antibodies against master regulator of autophagy; TFEB. TBI has increased TFEB levels and nuclear translocation in Map2 positive neurons. Boxed area shows neurons with TFEB signal. Scale bar = 20µm **B)** The bars represent fold change of florescent signal in Map2 negative cells relative to Sham. **C)** The bars represent fold change of florescent signal in Map2 positive cells. **D)** The bar represents percentage distribution of TFEB signals in cytoplasm and nucleus in Map2 positive cells. Data is shown as mean ± SE, n=3 **P< 0.01.

mTOR activity increases in cerebral cortex that is prevented by, TAT-Beclin1

mTOR is a serine/threonine kinase and plays a central role in metabolism, growth, and survival. It regulates transcription factor TFEB by phosphorylating it and inhibiting its nuclear translocation. Activated mTOR inhibits nuclear translocation of TFEB, transcription of CLEAR network genes, and eventually autophagy. Conversely, mTOR inhibition will facilitate nuclear translocation of TFEB. To evaluate mTOR signaling pathway, phosphorylation of mTOR downstream substrates such as pP70S6K, p4EBP, or pULK is used. In figure 4.17, we observed nuclear translocation of TFEB after TBI. We therefore wanted to determine mTOR activity in TBI with and without TAT-Beclin1 peptide. As shown in figure 4.18, mTOR activity was high in TBI brain cortex at 3 days post injury. Our results are in agreement with observations made earlier by other groups working on different TBI models [150, 151]. Increased mTOR signaling should inhibit nuclear translocation after TBI, which was not observed in our experiment. Additionally, mTOR level was similar to sham condition with TAT-Beclin1 treatment indicating inhibition of mTOR activity and inhibition of nuclear translocation of TFEB. The results indicate that nuclear translocation of TFEB is not regulated by mTOR in TBI. In addition to mTOR, calcineurin – a calcium dependent phosphatase – is known to play a role in dephosphorylation of TFEB. However, western blot analysis of TFEB in figure 4.16 shows that there is no reduction in phosphorylated TFEB, likely indicating that calcineurin may not regulate TFEB translocation in our context. In a recent study, mTOR-independent regulation of TFEB by another

serine/threonine kinase AKT has been reported [112]. Therefore, we next determined activated AKT (pAKT) levels in the brain cortex lysate.

A
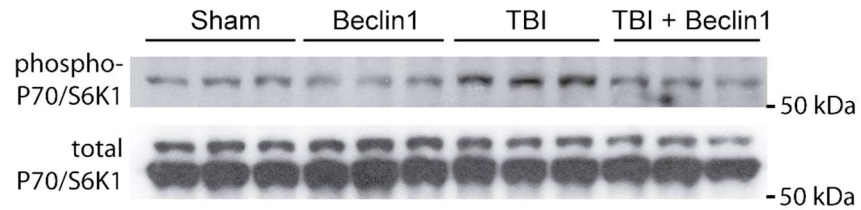
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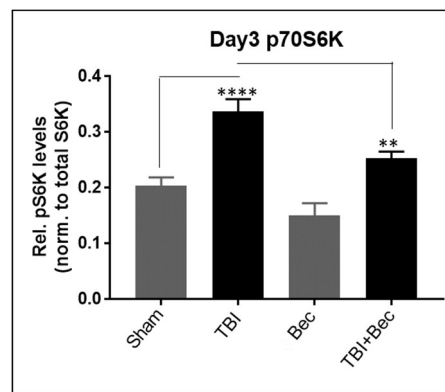
Figure 4.18: mTOR activity is high in TBI brains and is inhibited by TAT- Beclin1 peptide. **A)** Western blot for pP70/S6K as a marker for mTOR activity in brain cortex in TBI with or without TAT-Beclin1 treatment **B)** Densitometric analysis of pP70/S6K normalized to the loading control total P70/S6K, this is further normalized to sham to represent the fold change. Data is shown as mean ± SE, n=3 **P< 0.01, ****P< 0.0001

AKT activity is attenuated in TBI, TAT-Beclin1 peptide rescues Akt activity after TBI.

AKT, also called as protein kinase B, is a serine/threonine kinase that plays an important role in signaling pathway involved in survival and growth in response to external stimulus. Akt when phosphorylated at T308 and S473 by PDK1 and mTORC2, respectively, becomes activated. In order to determine the level of activated Akt in brain cortex lysates, western blot analysis was performed, which showed significant reduction in pAkt levels post TBI compared to sham condition. The reduction in activated Akt levels upon TBI might explain the nuclear translocation of TFEB. Akt has been reported to phosphorylate TFEB and inhibit its nuclear translocation [112]. Moreover, inhibition of Akt is sufficient to cause nuclear translocation of TFEB independent of mTOR. Thus, Akt may regulate TFEB and integrated stress response to cause neurodegeneration after TBI which is alleviated by TAT-Beclin1 peptide.

A
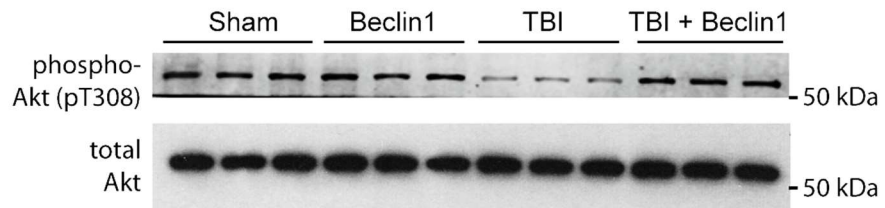
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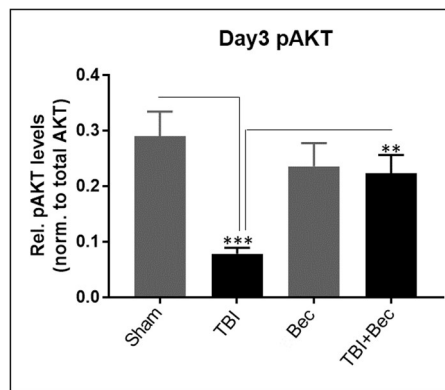
Figure 4.19: AKT activity is low in TBI brains and is normalized by TAT- Beclin1 peptide. **A)** Western blot for pAKT (T308) as a marker for AKT activity in brain cortex in TBI with or without TAT-Beclin1 treatment **B)** Densitometric analysis of pAKT normalized to the loading control total AKT, this is further normalized to sham to represent the fold change. Data is shown as mean ± SE, n=3 **P< 0.01, ***P<0.001

Discussion

In the present study, we demonstrated that induction of autophagy at early stage after TBI reduces neurodegeneration and apoptotic cell death. Importantly, we show that TFEB plays a role in mediating TBI-induced neurodegeneration.

Sarkar *et al.* reported that autophagy is impaired after TBI in controlled cortical injury model in mice [89]. Similarly, in rats using mild fluid percussion injury model, our findings suggest that autophagy flux is significantly reduced on days 1 through 7day post injury. Unlike earlier studies that relied on autophagy marker LC3 alone to understand the role of autophagy after TBI, in Sarkar *et al.* as well as in our study, autophagy flux was measured after TBI using lysosomal substrate P62. Another study in spinal cord injury (SCI) model has also demonstrated inhibition of autophagy post-injury [152]. Taken together, our data in LFP-based TBI as well as other studies suggest that traumatic injury to nervous system causes impaired autophagy which is restored to normal levels in 7 days.

TAT-Beclin1, a small 18 amino acid long peptide, was described to induce autophagy by binding with endogenous inhibitor of Beclin1, GAPR-1 [62]. In this study, it was demonstrated that the peptide is effective in inducing autophagy in cell culture as well as in neonatal mice, where it was shown to reach the brain. Additionally, in adult mice, the peptide was observed to be effective in peripheral nervous system. We have demonstrated that TAT-Beclin1 peptide is effective *in vitro* in cortical neurons isolated from E16.5 rat embryos. In our *in vivo* study with

adult rats, we observed that TAT-Beclin1 is able to induce autophagy in the brain, both when injected peritoneally or upon direct topical application at the injury site. Thus, TAT-Beclin1 peptide could serve as an important tool in modulating autophagy in adult rat brains.

We showed in rat brain sections that pTau accumulates in neurons and astrocytes upon TBI. Similar outcomes have previously described in other models of traumatic brain injury [153, 154]. Many studies have contributed in understanding the molecular processes regulating pTau buildup in brain after TBI [155]. Recently impaired intracellular clearance system such as autophagy and intercellular glymphatic pathway have been implicated in pTau buildup in Alzheimer's disease mouse model as well as in TBI [115, 153].In tauopathy mouse model exogenous expression of TFEB was found to clear specifically hyperphosphorylated aggregated prone tau (174). However, role of TFEB in neurotoxic pTau clearance in Rats during injury is not known. In our study, we found that TAT-Beclin1 induced autophagy reduced pTau accumulation in cortical site of injury. We further discovered that when TAT-Beclin1 was applied immediately after the TBI, it was able to induce autophagy as well as significantly reduce pTau buildup however at this time point TFEB levels in injured brains treated with TAT-Beclin1 was low and showed less nuclear translocation. Such contrasting results in mouse and rat model indicates differential role of TFEB due to injury treatment or different animal model.

Impaired autophagy is implicated in neuronal cell death in controlled cortical injury and SCI. Although the autophagy normalizes by day 7 post injury, cell death occurs acutely at the injury site as well as chronically at the adjacent site. Our data showed neuronal cell death at the cortical injury site 3 days post TBI, and at this time point autophagy continues to be impaired. Of note, we found that TAT-Beclin1 peptide restores autophagy 3 days post TBI and significantly decreases neuronal cell death. Similar observation was made when we monitored apoptotic cell death in TBI brains with or without TAT-Beclin1 peptide. This indicates that functional autophagy at an early stage of TBI increases survivability. Similar observation was made when mTOR inhibitor rapamycin was used at an early stage of TBI [108]. Rapamycin treatment at early stage of TBI inhibits mTOR activity and induces autophagy. How TAT-Beclin1 is able to restore impaired autophagy after TBI is still a puzzle. It is reported that lysosomal cathepsin D protein level and enzymatic activity is reduced after TBI, causing impaired autophagy through lysosomal inhibition [89]. It was further found that cathepsin D levels increased on days 3 and 7 post-TBI, correlating with recovery of autophagy by day 7. Normal recovery of autophagy by day 7 after injury could be the result of increase TFEB/ATF4 signaling as we observed at day 3, high TFEB and ATF4 signaling at day 3 could be one of the cellular survival and repair signal. Mechanistically, TAT-Beclin1 increases endogenous Beclin1 level, which can participate in autophagy, endocytosis, or bind to Bcl-2 inhibiting apoptosis. We do not understand how any of these processes rescue lysosomal activity and restore autophagy flux. Recently autophagy flux in CA neurons of hippocampus in Alzheimer's patient was

determined to me inhibited. The inhibition of the flux is coupled with increased TFEB levels and other autophagy markers. Further investigation indicated that inhibition of flux is caused by increase lysosomal fusion, increased cargo for lysosomal digestion (243). We think similar process could be taking place in the TBI brains as indicated by high levels of autophagy markers and TFEB. TAT-Beclin1 peptide decrease the lysosomal inhibition by formation of phagophore and sequestration of cargo thereby decreasing overloading of lysosomes.

TBI induced impaired autophagy and increased mTOR activity along with perturbed calcium homeostasis can contribute to ER stress. We found that ER stress markers ATF4 and CHOP significantly increase in neurons at the cortical injury site. ATF4 signaling is mediated by PERK and ATF6 sensor on ER. ATF4 is implicated in cell survival; however, chronic ER stress activates ATF4/CHOP pathway for apoptotic cell death. In this study, we found that after TBI, neurons had increased levels of ATF4 that translocated to the nucleus, concomitantly increasing CHOP to drive apoptotic cell death. CHOP induces apoptosis by inhibiting anti-apoptotic BCL-2 and upregulating pro-apoptotic Bim. In addition, CHOP also increases GADD34 level that dephosphorylates eIF2α and resumes protein synthesis, activating integrated stress response (ISR) and apoptotic cell death [156]. We found that TAT-Beclin1 peptide reduced ATF4/CHOP level in neurons after TBI correlating with decreased apoptotic cell death. This likely suggests that TAT-Beclin1 peptide is reducing ER stress and ISR by inducing autophagy and activating degradation pathway for misfolded or aggregated

proteins after TBI. Recently, transcription factor TFEB, which is known to regulate autophagy and lysosomal genes, was reported to play a role in ISR during ER stress *in vitro* [116]. Our qPCR data suggest that expression levels of ER stress and ISR markers such as ATF4, CHOP, BiP, GRP94, EDEM1, HERPUD1 and TFEB were elevated in cerebral cortex after injury. Surprisingly, TAT-Beclin1 peptide decreased specifically ISR genes and TFEB but not all ER stress markers, suggesting that TAT-Beclin1 is inhibiting ISR response and apoptotic cell death.

TFEB protein level was elevated in cortical lysates in neuronal and glial cells after TBI. We were able to demonstrate TBI induced nuclear translocation of TFEB in rat brains, and TAT-Beclin1 peptide reduced TFEB protein level in neurons and glial cells and also inhibited nuclear translocation. mTOR kinase negatively regulates nuclear translocation of TFEB by phosphorylation, however we found high mTOR activity after TBI and nuclear translocation of TFEB. High mTOR activity after TBI could be the result of increased inflammation Activated mTOR pathway may contribute to high TFEB protein level through upregulation of protein translation, but it is likely that nuclear translocation of TFEB is independent of mTOR activity in TBI. We also observed no obvious decrease in the phosphorylated TFEB in western blot analysis. Moreover, TAT-Beclin1 decreased mTORC1 activity in the whole tissue lysate and inhibited TFEB translocation in the nucleus. Thus, this likely points to a different kinase involved in regulating nuclear translocation of TFEB.

It is possible that nuclear translocation of TFEB is regulated by ISR in TBI. ISR mediated nuclear transport of TFEB requires activated PERK. We have not monitored PERK activity in our study; however, determining PERK activity in cortical lysate will confirm the role of TFEB in ISR mediated cell death after TBI. Other kinases that can phosphorylate TFEB are MAPK, PKCβ, and AKT. Recently AKT was shown to phosphorylate TFEB and induce nuclear translocation in mTOR-independent manner [112]. However, we found that AKT activity was low in cortical lysates after TBI. AKT activity is expected to increase in TBI in response to growth factor stimulation; conversely ER stress can negatively regulate AKT when GADD34 binds to AKT. The binding of GADD34 inhibits ubiquitination of AKT by TRAF6 and subsequent transport to plasma membrane, which is required for its activation [157]. Decrease in active AKT could be attributed to ATF4/GADD34 pathway after TBI. TAT-Beclin1 peptide increased AKT by attenuating ATF4/GADD34 pathway as indicated from our data post injury. It is possible that increased mTOR activity after TBI is contributed by neuroinflammation. This was evident when we observed increased astrocytes near the cortical injury site. Interestingly, TAT-Beclin1 decreased astrocyte activation at the injury site (as shown in figure 4.9 of results section). This suggests that decrease in mTOR activity with TAT-Beclin1 peptide after TBI is mediated at least in part by reduced inflammatory response. The paradoxical increase in AKT activity with TAT-Beclin1 after TBI could be due to increased receptor mediated endocytosis due to increase in endogenous Beclin1 since Beclin1 also participates in endocytosis. However, I have not shown a direct phosphorylation activity on TFEB. The identification of

AKT specific phosphorylation on TFEB will establish the direct regulation of TFEB by AKT.

Statistical Analysis:

The data presented in the thesis is a representation from three animals per cohort. The experiments were carried altogether with 3-7 animals per condition with 2-3 or more of technical repeats. The drawback of having small animal numbers per cohort is the possibility of having type I error in the results using Analysis of variance (ANOVA). With 3 animals per cohort it is difficult to detect any variation or normality between animals using ANOVA. Type I error occurs when we reject the null hypothesis or accept false positive. There are different ways to correct for the error and one of the way is to decrease the confidence level from 5% to 1% in the outcome or increase the number of animals per cohort and repeating independent experiments. However, approaching same question with two different methods of experiments and getting similar result could indicate the likelihood of the outcome. The scientific questions in the thesis are approached by using western blot and microscopic analysis giving us very similar outcome. This increases the probability of the outcome.

Conclusions and Future Directions

Conclusions

1. Functional autophagy can increase survivability after TBI and inhibit accumulation of toxic proteins.

2. TFEB mediates ISR and cell death in neurons after TBI which is probably regulated by AKT.

3. Increasing AKT activity and suppressing mTOR activity after TBI may improve cell survival, reduce neuronal loss and may decrease the risk factor for developing neurodegenerative disease after TBI.

4. TAT-Beclin1 peptide can be used to regulate autophagy in adult rat brain

Future Directions:

1. To determine expression of CLEAR network genes in terms of protein and mRNA level in cerebral cortical lysate with and without TAT-Beclin1 treatment.

2. Determine phospho PERK level in cerebral cortical lysate to confirm its role in TFEB translocation.

3. To determine role of AKT in nuclear translocation of TFEB in primary cortical neurons after stretch injury *in vitro* using chemical activator and inhibitor of AKT as well as in the presence of TAT-Beclin1 peptide.

4. Monitor receptor mediated endocytosis in cerebral cortical lysate with and without TAT-Beclin1 peptide to determine its role in endocytic pathway

5. Determine neuronal cell death in TFEBKO mice with TBI to understand the role of TFEB in neuronal cell death through integrated stress response.

.**Chapter 5: Preliminary study indicates TAT-Beclin induced autophagy attenuates Dishevelled-1 sequestration in autophagosomes after TBI**

Introduction

Dishevelled is an evolutionarily conserved mediator of canonical and non-canonical Wnt pathway. It is required for closure of neural tube during development, and is also known to regulate cell polarity, cell fate, and cardiac and neuronal development [158]. Three isoforms of Dishevelled (Dvl) protein have been described in humans Dvl-1, Dvl-2 and Dvl-3.

Dvl is activated by phosphorylation which is mediated by different kinases such as casein kinase1 (CK1) [159], casein kinase 2 (CK2) [160], PAR1 [161], and Metastasis-associated kinase [162]. Phosphorylation of Dvl facilitates subcellular localization to microtubules and creates binding site for many effector proteins to participate in both canonical and non-canonial Wnt signaling [158].

Canonical and non-canonical Wnt signaling

In canonical Wnt signaling, overexpression of Dvl activates β-catenin signaling in LRP receptor mutant drosophila, whereas overexpression of LRP receptors in Dvl-mutant background inhibits Wnt signaling [163, 164]. Thus, Dvl is an important mediator of Wnt signaling and functions downstream of Wnt receptor complex, Frizzled and LRP5/6. Dvl inhibits GSK3 activity, and also inhibits β-catenin destruction complex PC/Axin/GSK3 responsible for phosphorylation and degradation of β-catenin [165]. Stable β-catenin translocate into nucleus, where it binds to LEF/TCF family of transcription factors and upregulates Wnt regulated genes.

In non-canonical Wnt signaling, Dvl regulates planar cell polarity (PCP) signaling by competitively binding to Diego and Prickle [166]. The pathway activates Rho and Rac GTPase for remodeling actin cytoskeleton and activating JNK pathway.

Dishevelled is generally considered as a mediator collecting signals from the plasma membrane and relaying it to specific effector proteins to activate specific signaling pathway [167]. Dvl contains three main structural domain: DIX, PDZ, and DEP. DIX domain is involved in canonical Wnt signaling and for interacting with Axin [168]. PDZ domain orchestrates protein-protein interactions in both canonical and non-canonical Wnt signaling. PDZ domain binds to PCP effector proteins such as PAR1, Diego, Strabismus, Prickle and DAAM1. It can also bind to canonical Wnt effectors such as GSK3 CK1/2, GBP/FRAT, Dapper, protein phosphatase 2, etc. [158]. Lastly, the DEP domain functions in non-canonical signaling where it binds to Rac GTPase and activates it [169].

Dvl-2 is expressed ubiquitously in all developmental stages and tissues [170]. Dvl-3 is also expressed in all stages of development [171]. Dvl-1 is expressed ubiquitously in all stages of development, particularly in neuron dense areas of the central nervous system in the embryonic and postnatal stages [172].

Role of Dvl-1 in neurodegeneration
Dvl-1 regulates activity of GSK3, and localizes to neurons in the cortex, hippocampus, pons and cerebellum. GSK3 activity is required for the polar structure of the neuron. In cerebellar neurons, Wnt signaling regulates axonal

morphology, increase in growth cone, axonal branching, and decrease in axon length. All these processes require remodeling of microtubules, which is regulated by GSK3 activity [173]. GSK3 facilitates remodeling of microtubule by phosphorylation of microtubule binding protein MAP-1A [174]. GSK3 also phosphorylates Tau, which is an axonal microtubule binding protein, and alters stability of microtubules. Thus, GSK3 plays an important role in microtubule organization in neurons by phosphorylation of MAPs.

Dvl-1 localizes to axonal microtubule, similar to Tau, and protects it from depolymerization in the presence of nocodazole. Dvl-1 thus stabilizes microtubules in the axons, and the microtubule stabilization is mediated by inhibition of GSK3β [175].

Dvl-2 and Dvl-3 is negatively regulated by ubiquitination and degradation [176] [177]. Dvl-2 is ubiquitinated and degraded by autophagy pathway through ubiquitin recognition molecule P62 [176]. Additionally, TBI has been shown to inhibit Wnt signaling pathway and increase GSK3 activity [178]. It was reported that inhibition of GSK3 activity by lithium in rats decreased neuronal loss and improved cognition. Therefore, in our study we attempted to understand role of Dvl-1 in regulation of GSK3β after TBI induced autophagy impairment.

Results

TAT-Beclin induced autophagy attenuates Dishevelled-1 sequestration in autophagosomes after TBI

Wnt pathway is involved in synaptic plasticity, cognition, and neuronal regeneration during homeostasis and injury [179]. Dishevelled is a common mediator of canonical and non-canonical Wnt pathway, and Dishevelled-1 (Dvl-1) is expressed in all stages of development in the central nervous system [172].

In our study, we investigated the effect of TBI-induced impaired autophagy flux on Dvl-1 mediates Wnt signaling. Immunohistochemistry for Dvl-1 co-stained with autophagy marker LC3 shows that the autophagosomes that accumulate due to inhibition of autophagy are positive for Dvl-1signal, indicating sequestration of Dvl-1 in autophagosomes. Dvl-1 sequestration increases the overall level of Dvl-1 in the cells as seen in figures 5.1 and 5.2, but likely renders them non-functional inside the autophagosomes. After TBI, Wnt signaling is reduced, impacting repair and neuronal regeneration, and sequestration of Dvl-1 into autophagosomes could contribute to the attenuation of Wnt signaling after TBI. Interestingly, restoration of autophagy flux by TAT-Beclin1 peptide after TBI decreases sequestered Dvl-1 evident by reduced colocalization of Dvl-1 with LC3. It is possible TAT-Beclin1 induced clearance of sequestered Dvl-1 after TBI restores homeostasis and inhibits neuronal cell death.

Immunohistochemistry of Dishevelled-1 in brains sections after TBI with or without

TAT-Beclin1 peptide.
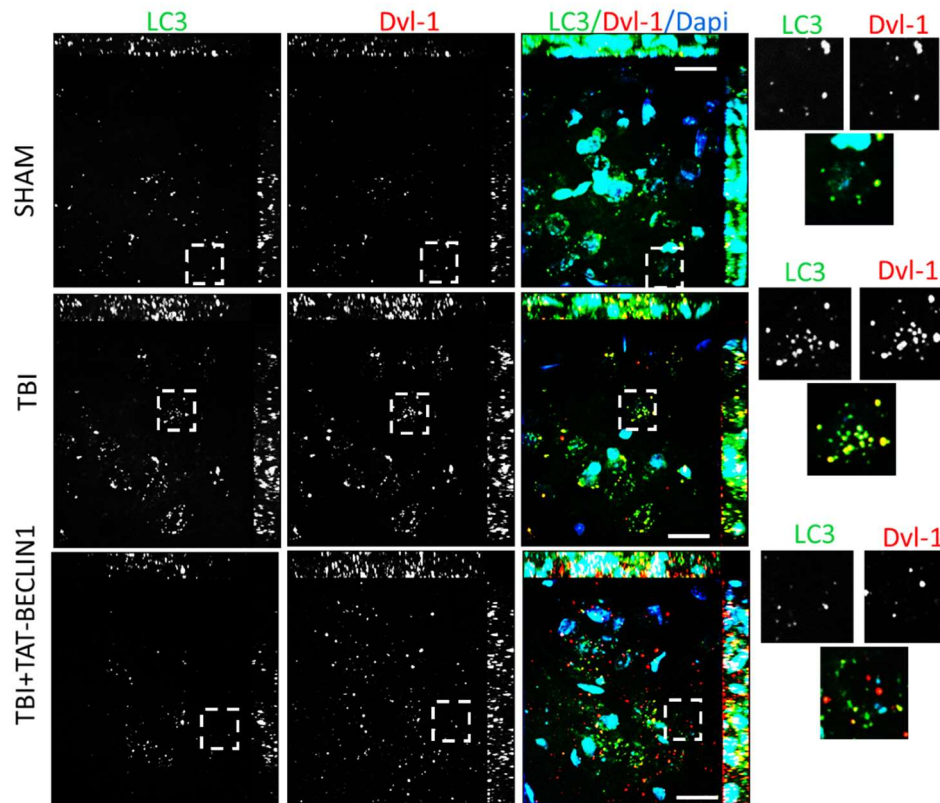
Figure 5.1: TAT-Beclin1 peptide decreases Dvl-1 sequestration after TBI **A)** Images (63X) Brain section is stained with antibodies against autophagy marker LC3 and Dvl-1, Dvl-1 is sequestered in accumulated autophagosomes after TBI Boxed area shows cell with LC3 and Dvl-1 colocalization. Scale bar = 20 μm. Data is shown from n=3

Next, we wanted to investigate Dvl-1 levels in cerebral cortex after TBI with or without TAT-Beclin1 peptide. Western blot indicates robust increase in Dvl-1 levels after TBI which significantly decreases by 3-fold when treated with TAT-Beclin1 peptide in the cerebral cortex. Similar results were observed in the hippocampus indicating that TBI impacted both the cortex and hippocampus. Increase in Dvl-1 level after TBI along with immunohistochemistry observation from figure 5.1 indicated sequestration of Dvl-1 in autophagosomes after injury, which is prevented by treatment with TAT-Beclin1 treatment. Next, we wanted to determine whether cortical neurons *in vitro* when stretched on silicone membrane would replicate fluid percussion injury in mice and whether it will similarly impact on Dvl-1 sequestration and autophagy inhibition.

Dvl-1 level is increased in in the cerebral cortex after TBI.
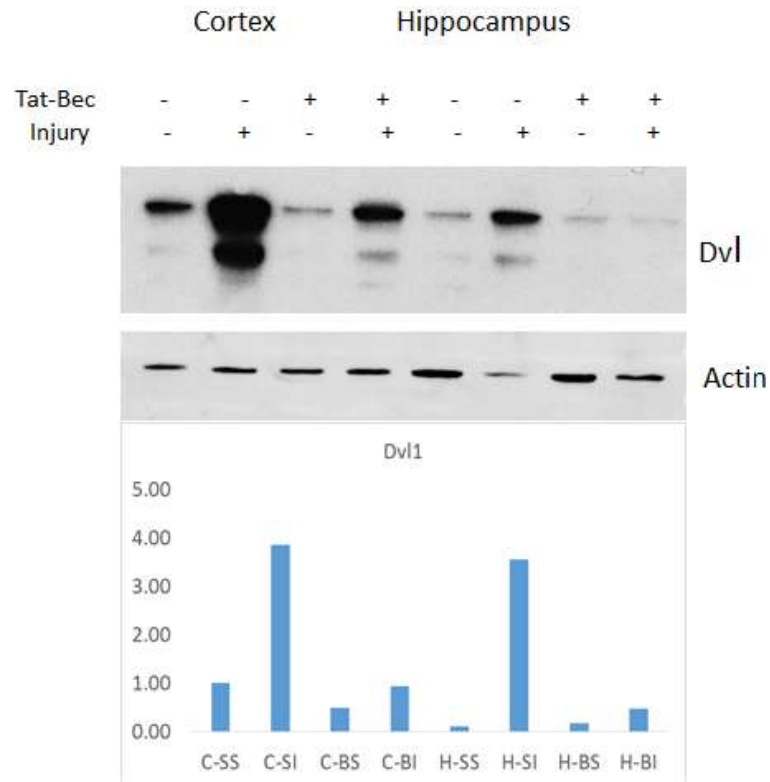
Figure 5.2: TAT-Beclin1 decreases Dvl-1 level 7 days after TBI. **A)** Western blots of autophagy marker LC3 and Dvl-1 Dvl-1 levels were high in TBI and significantly low in TBI with TAT-Beclin1 treatment, **B)** Densitometric analysis of Dvl-1 which is normalized to the loading control actin this is further normalized to sham to represent the fold change. n = 1

Dvl-1 is sequestered into autophagosomes after stretch injury

To establish an *in vitro* injury model and evaluate the impact of Dvl-1 sequestration on neuronal death after TBI, we cultured primary cortical neurons on silicone membrane isolated form E16.5 embryos from rats. After 7 d̲ays of i̲n *v̲itro* culture (DIV), cortical neurons were subjected to 50% stretch representing moderate TBI. Immunostaining of these cells at different time points post stretch injury showed increase in autophagy marker LC3 and lysosomal substrate P62 day 3 post stretch injury. This indicates accumulation of autophagosomes and inhibition or decrease in autophagy flux. Dvl-1 colocalized with the LC3 and P62 puncta indicating sequestration into autophagosomes. However, Dvl-1 was not sequestered on days 4 and 5 post stretch injury, suggesting recovery from inhibition of autophagy. Quantification of number of cells with Dvl-1 sequestration indicated that nearly 40% of these cells responded to stretch injury with sequestration of Dvl-1 in LC3 and P62 positive autophagosomes. Of note, lateral fluid percussion injury in rat models' mild percussion injury, whereas 50% stretch of neurons *in vitro* models moderate TBI. Therefore, we reduced the stretch force to 30% and monitored Dvl-1 sequestration on day 3 post stretch injury.

Immunostaining of 50% stretched cortical neurons to determine localization of Dvl-1 and P62 as autophagy marker at different time point post stretch.
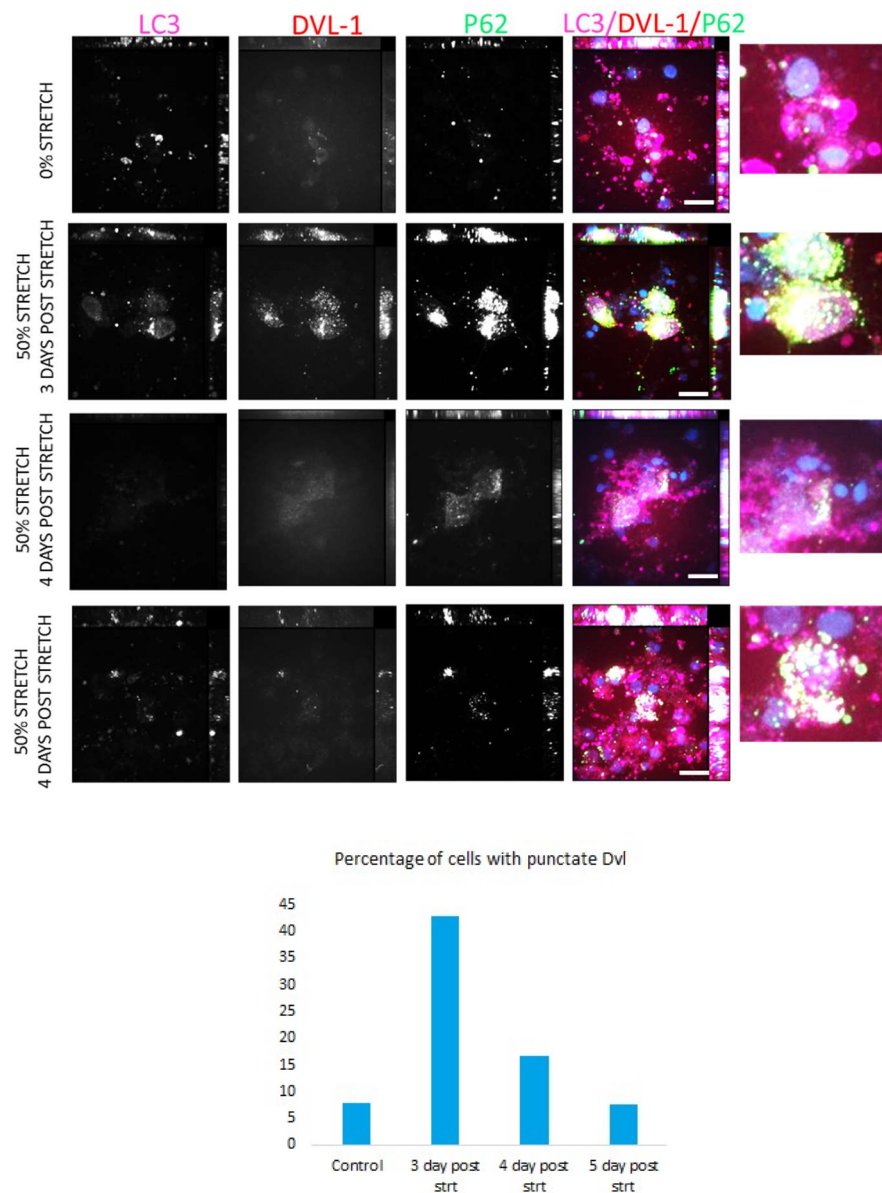
Figure 5.3: Dvl-1 is sequestered in autophagosomes through P62 adaptor protein in cortical neurons after 50% stretch on silicon membrane **A)** Images (63X) cortical neurons are stained with antibodies against autophagy marker LC3, P62 and Dvl-1, Dvl-1 is sequestered in accumulated autophagosomes through P62 adaptor on day 3 post stretch. Scale bar = 20 μm **B)** Bars represent percentage of cells with punctate Dvl-1 signal colocalizing with LC3 and P62 post 50% stretch. N = 2.

TAT-Beclin1 decreases Dvl-1 sequestration after stretch injury

Our previous observation *in vivo* indicated sequestration of Dvl-1 in LC3 positive autophagosomes after TBI. This sequestration of Dvl-1 was inhibited with TAT-Beclin1 treatment. The *in vitro* experiment with cortical neurons using 50% stretch injury showed similar Dvl-1 sequestration of Dvl-1 into autophagosomes. We next determined whether 30% stretch injury, which mimics mild TBI would also induce Dvl-1 sequestration, and whether this could also be rescued by TAT-Belcin1 treatment *in vitro*. As shown in figure 5.4, 30% stretch injury neurons showed high levels of autophagy marker P62 which colocalized with Dvl-1. This was similar to 50% stretch injury as well as *in vivo* brain sections. Of note, treatment with 5 µM TAT-Beclin1 peptide immediately after the stretch injury decreased Dvl-1 level and reduced its colocalization with P62. It has been reported that injury decreases Wnt signaling and increases GSK3β activity [180]. Therefore, we next determined GSK3β activity in cerebral cortex of rats after TBI with or without TAT-Beclin1 peptide.

Immunostaining to determine the level and localization of Dvl-1 and P62 3 day post stretch with or without TAT-Beclin1 peptide in 30% stretched cortical neurons
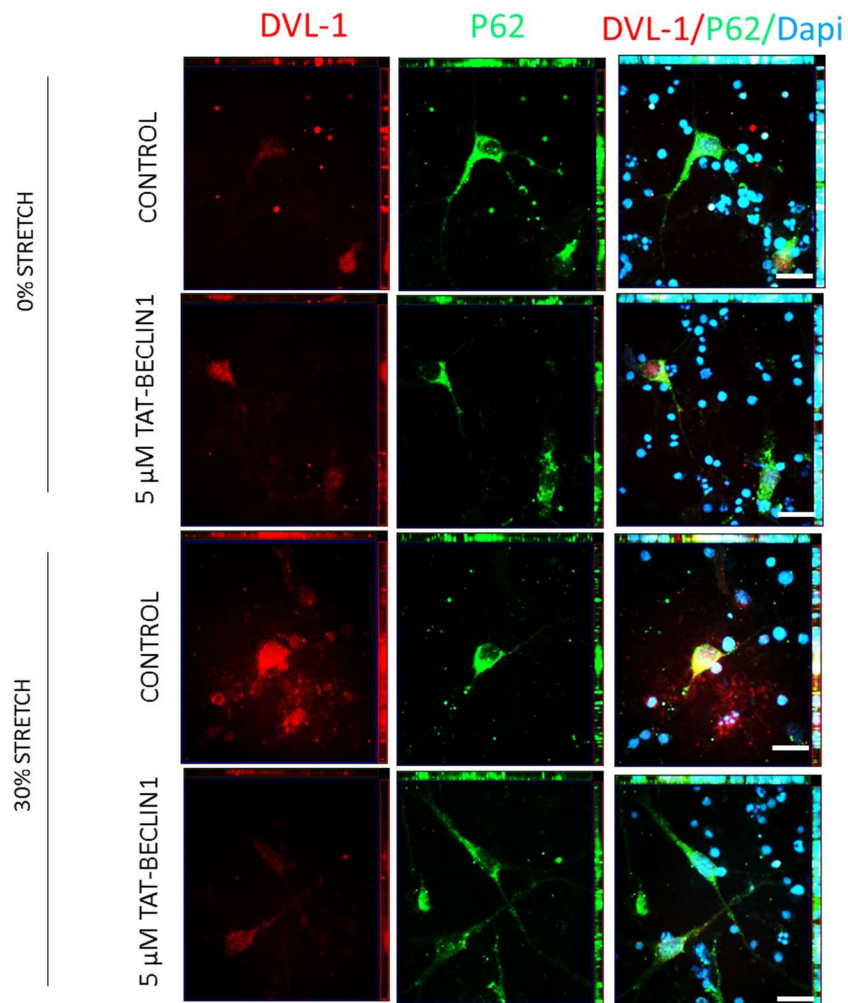
Figure 5.4: TAT-Beclin decreases Dvl-1 accumulation in 3 day post stretch injured cells **A)** Images (63X) cortical neurons 30% stretched are stained with antibodies against autophagy marker, P62 and Dvl-1, Dvl-1 and P62 level increases in stretched neurons and colocalizes. Scale bar = 20 µm N = 2

TBI increases GSK3β activity

Glycogen synthase kinase3 (GSK3) play an important role as a regulator of cell proliferation, protein synthesis, cell differentiation, and cell death. In TBI, increased GSK3 activity promotes neuronal cell death. Conversely, inhibition of GSK3β using small molecule inhibitor has been shown to protect from neuronal cell death in TBI [178]. GSK3β activity is regulated by both AKT and Wnt pathway. Akt phosphorylates serine 9 residue of GSK3β and inhibits its activity. Our western blot shows decrease in pGSKβ levels in the cerebral cortex after TBI, consistent with previous findings. TAT-Beclin1 peptide further decreased pGSK3β level after TBI suggesting an increase in GSK3β activity. Tau is a microtubule binding protein, abundantly found in neurons, and a direct substrate of GSKβ activity. Thus, increase in GSK3β activity can lead to phosphorylation of Tau and pTau buildup in neurons. We therefore determined pTau buildup in cortical neurons after stretch injury with or without TAT-Beclin1 peptide as a marker for GSK3β activity.

Western blot analysis to determine GSK3β activity in cerebral cortex after TBI with or without TAT-Beclin1 peptide.
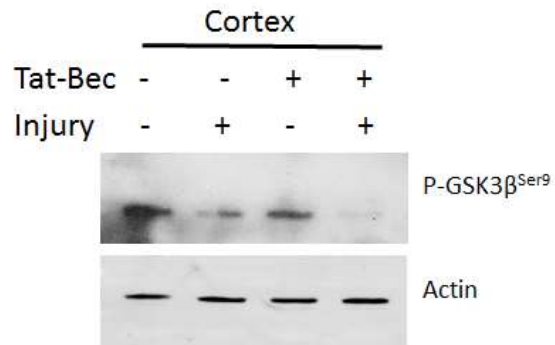
Figure 5.5: Both TBI and TBI +TAT-Beclin1 increases GSK3β activity in the cerebral cortex 3 days post injury. Western blots of phospho-GSK3β marker for GSK3β activity. represents n = 1

TAT-Beclin1 decreases pTau buildup after stretch injury

Tau regulates assembly and stability of microtubules in neurons and is predominantly found in neuronal axons. GSK3β is one of many kinases that phosphorylates Tau. Hyperphosphorylation of Tau alter its binding to microtubule and destabilizes it. TBI is described to induce pTau accumulation in neurons, and was also observed in our TBI model *in vivo*. Data from figures 5.4 and 5.5 indicates attenuated Wnt signaling and increased GSK3 activity after stretch injury due to Dvl-1 sequestration. As seen in figure 5.6, immunostaining for pTau and LC3 in cortical neurons injured with 30% stretch showed increase in pTau in the neurites of the cells. Surprisingly, TAT-Beclin1 peptide decreased pTau formation in cortical cells despite increased GSK3 activity from figure 5.5. LC3 levels were increased 3 days post stretch injury as observed in our previous figures, and TAT-Beclin1 decreased LC3 levels indicating induction of autophagy. TAT-Beclin1 induced autophagy may increase clearance of pTau after stretch injury. figure 5.7 shows LC3 and pTau levels in cortical neurons post injury with 30% stretch with or without TAT-Beclin1. The staining clearly shows decrease in LC3 and pTau levels with TAT-Beclin1 treatment after stretch injury indicating activation of degradation pathway and clearance of pTau.

Immunostaining to determine the pTau and LC3 level in 30% stretched cortical neurons with or without TAT-Beclin1 peptide
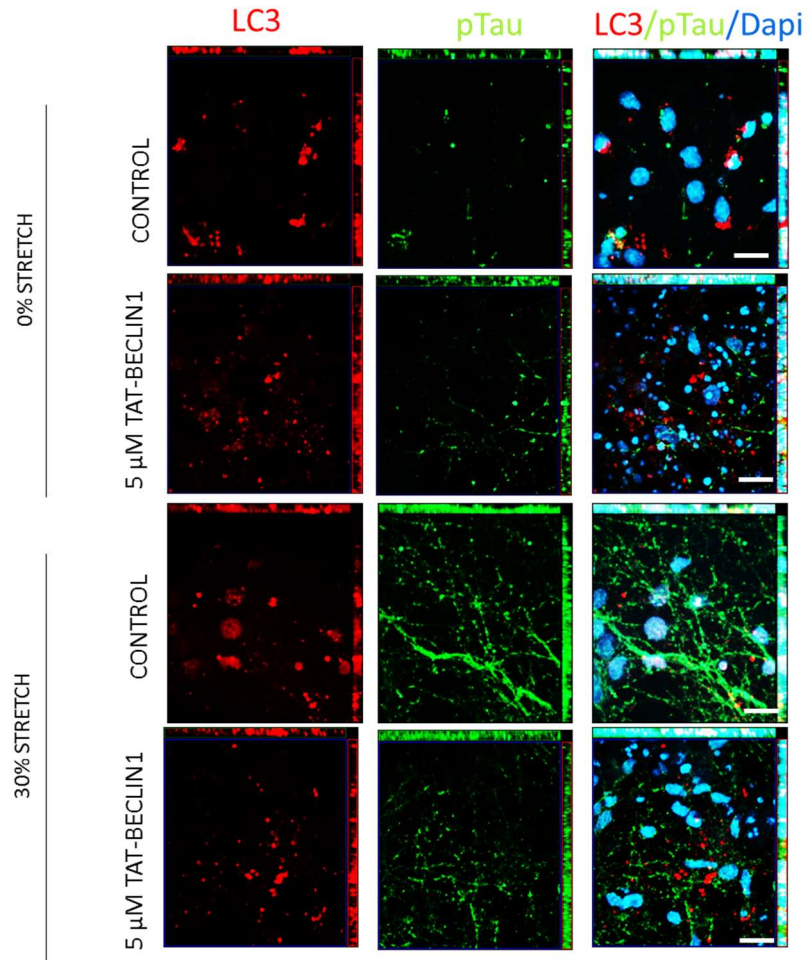
Figure 5.6: TAT-Beclin1 decreases pTau buildup in cortical neurons 3 day post stretch injury. Images (63X) cortical neurons 30% stretched are stained with antibodies against autophagy marker LC3 and pTau, LC3 and pTau levels increases after stretch injury and both protein level decreases with TAT-Beclin1 treatment. Scale bar = 20 μm. N = 2
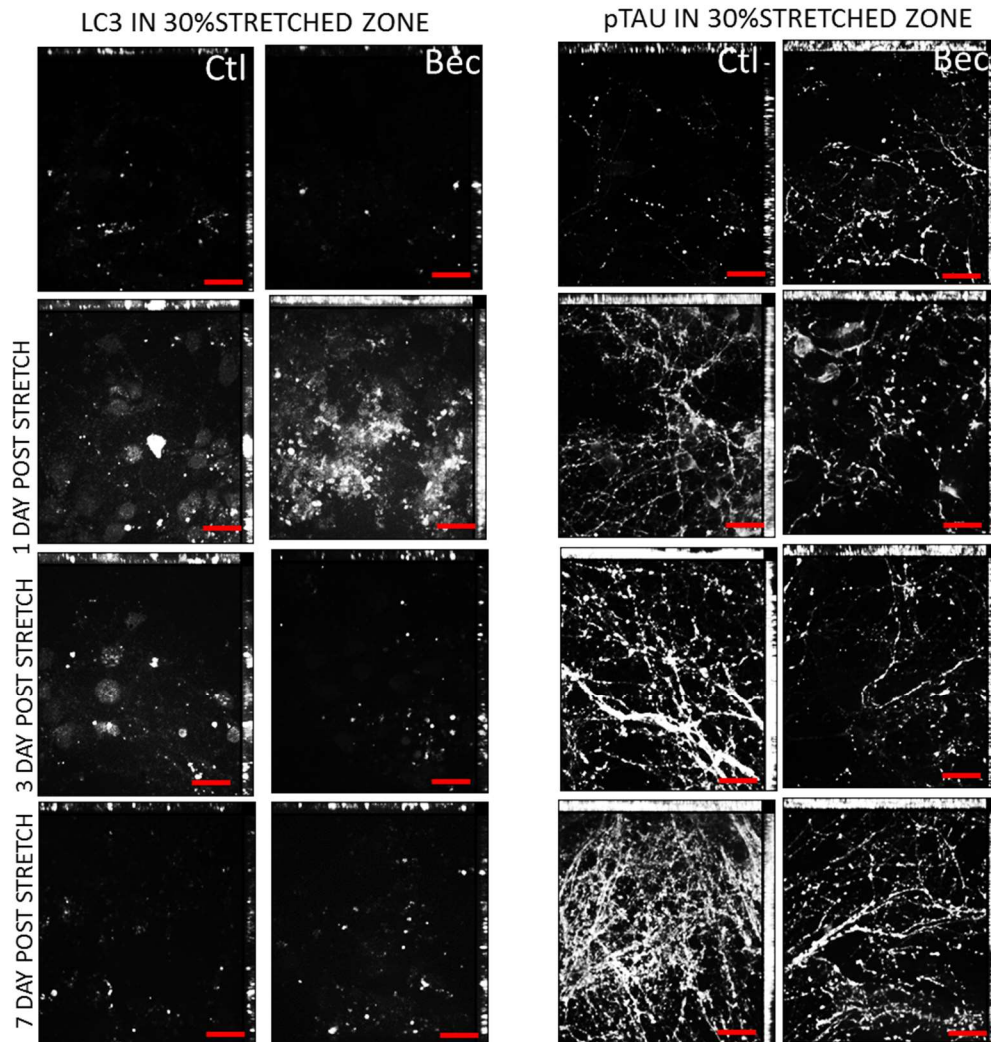
Figure 5.7: TAT-Beclin1 decreases pTau buildup in cortical neurons 1,3 and 7 day post stretch injury. Images (63X) cortical neurons 30% stretched are stained with antibodies against autophagy marker LC3 and pTau, LC3 and pTau level increases after stretch injury. Scale bar = 20 µm. 5 µM TAT-Beclin1 treatment decreases both pTau and LC3 levels post stretch injury N= 2

Discussion

In our study to understand role of autophagy in Wnt signaling, we found that impaired autophagy caused by TBI lead to sequestration of Dvl-1 in autophagosomes 3 days post injury. We used the term "sequestration" since the autophagy flux is inhibited and Dvl-1 is engulfed inside the autophagosomes rendering it dysfunctional. Biochemical analysis shows that sequestration of Dvl-1 in autophagosomes after TBI lead to significant increase in total Dvl-1 level in the cortex. TAT-Beclin1 peptide decreased Dvl-1 level, normalizing to sham condition, suggesting active degradation of sequestered Dvl-1 post TBI. Of note, increase in Dvl-1 level after TBI did not correlate with a decrease in p-GSK3β levels, indicating high GSK3 activity. Increase in Dvl-1 levels would suggest increase in Dvl-1 mediated Wnt signaling, which negatively regulates GSK3 activity. This was not observed in our studies. Moreover, pTau level was high in cerebral cortex post injury consistent with a high GSK3β activity. Increased GSK3β activity is responsible for neuronal death and TBI pathology [178].

We were able to demonstrate Dvl-1 sequestration and pTau buildup in primary cortical neurons after different intensities of stretch injury. Wnt signaling is negatively regulated by autophagy mediated degradation of Dvl-1 through P62 in homeostatic condition [176]. The presence of Dvl-1 in autophagosomes after TBI suggests that injury induces inhibition of autophagy by increasing Dvl-1 degradation though increase in ubiquitination process [181], however due to inhibition of autophagy flux, Dvl-1 remain trapped in autophagosomes.

We observed in our immunohistochemistry data that TAT-Beclin1 peptide restores autophagy after TBI and inhibited sequestration of Dvl-1. Similar observation was made in primary cortical neurons subjected to stretch injury. TAT-Beclin1 peptide reduced Dvl-1 level close to normal condition through active degradation. Decrease in Dvl-1 level and sequestration in autophagy in cortex after TBI correlated with decrease in pTau (Paired helical filament Tau) accumulation in cerebral cortex after TBI and in primary cortical neurons after stretch injury. Dvl-1 degradation to sham levels and decrease in pTau after TAT-Beclin1 peptide treatment would indicate inhibition of GSK3β activity. However, GSK3β activity continued to stay remain elevated after TAT-Beclin1 peptide in TBI animals. It is likely that the TAT-Beclin1 mediated decrease of pTau in brain and primary cortical neurons is a result of clearance of buildup pTau rather than GSK3β activity.

Sequestration of Dvl-1 and pTau in autophagosomes may implicate that GSK3β may be associated with Dvl-1, phosphorylating Tau after TBI. Dvl-1 has a DEP domain for binding of GSK3β [158]. The sequestration of Dvl-1 may potentiate phosphorylation of Tau and protect GSK3β from Akt mediated phosphorylation and degradation. Thus sequestration may contribute in activation of GSK3β. In the presence of TAT-Beclin1, autophagy is activated mediating active degradation of Dvl-1 and GSK3β and inhibit phosphorylation due to AKT. Sequestration of Dvl-1 during injury may decrease functional Dvl-1 in the neurons which may affect synapse formation as Dvl-1 and Wnt signaling together plays an important role in presynaptic clustering, recycling of synaptic vesicles and neurotransmitter release (244). Decrease in functional Dvl-1 may contribute to destabilization of

microtubules after injury through Wnt signaling. Study shows that Dvl stabilizes microtubules through Axin in primary neurons independent of transcription and translation process (245). TAT-Beclin1 is reducing sequestration by increasing autophagy flux contributing to more synapse formation, neural communication through neurotransmitter release and neuronal regeneration. Preliminary data from our collaborator suggested that there is an increase in synaptic bouton when TAT-Beclin1 was treated after injury indicating increased synapse formation. Wnt signaling in TBI is altered leading to increase GSK signaling (226), sequestration of Dvl-1 could contribute to this alteration of Wnt leading to neuronal cell death, inhibition of the sequestration by TAT-beclin1 could increase Wnt signaling and decrease neuronal cell death.

In this study we demonstrated that 5 μM TAT-Beclin1 peptide was effective on primary cortical neurons in inducing autophagy and clearance. This was represented by increase in LC3 and P62 level in stretch injured neurons from day 1 to day 7 indicating inhibition of autophagy flux and TAT-Beclin1 peptide treatment decrease the level of LC3 and P62 from day 3 to day 7 indicating functional autophagy.

Statistical Analysis:

The data presented in this part of the thesis were carried altogether with 3-1 animals per condition with 1-2 or more of technical repeats. Due to small number of animals and technical repeats the results could not be evaluated for statistical significance. The work can be viewed as preliminary study towards understanding

the effect of autophagy on Dvl-1 which functions both in canonical and non-canonical Wnt signaling and in stabilization of microtubules.

Conclusions and Future Directions

Conclusions:

1. TBI induced impaired autophagy sequesters functional signaling mediators such as Dvl-1 inhibiting signaling process.

2. TAT-Beclin1 can rescue sequestration of functional proteins by inhibiting accumulation of autophagosomes. This would decrease cellular stress

3. Decrease in pTau level due to TAT-Beclin1 in TBI brains and stretch injured cortical neurons is due to autophagy mediated clearance.

Future Directions:

1. To determine role of sequestered Dvl-1 on neuronal death after stretch injury in primary cortical neurons by overexpression of exogenous Dvl-1 in injured primary cortical neurons.

2. Immunohistochemistry and biochemical assay to determine subcellular localization and level of $GSK3\beta$ in neurons after TBI with or without TAT-Beclin1 peptide.

Chapter 6: Concluding Remarks

TBI is a leading cause of death and disability in young adults and children. While there is a decrease in rate of TBI-related death from 21.9 to 11.4 %, management of chronic effects and long-term disability after TBI that affects the quality of life continues to remain a health and socioeconomic challenge [182]. Large population of TBI survivors suffer from cognition deficit and/or psychological disorders. Most of these changes can be attributed to chronic physiological and molecular changes that occur after TBI. Changes such as loss of white matter, neuronal death, protein misfolding such as Tauopathy, neuroinflammation, and changes in neurotransmitter system can together cause disability. These changes are collectively called as polypathology. TBI is associated with polypathology [183]. Often these multiple pathologies are interconnected and may get regulated by common cellular pathways.

In this study, we focused on accumulation of misfolded proteins such as Tau that can lead to neuroinflammation and neurodegeneration, as well as alter autophagy pathway in TBI. We attempted to understand the role of autophagy in TBI-related misfolded proteins, neuroinflammation, cell signaling, and cell death. We further studied whether TAT-Beclin1 peptide can induce autophagy in our TBI model, and whether this could lead of clearance of aggregated proteins accumulated in the autophagosomes.

We showed in our study that autophagy is impaired in lateral fluid percussion injury that represents concussion based TBI. TBI-related autophagy impairment is reversible but takes a week to normalize. However, it is this window period from day 1 to day 7 post TBI that both acute and chronic changes in cellular pathway occurs. We found that TAT-Beclin1 peptide was able to rescue TBI-related impaired autophagy on day 1 post injury. We further demonstrated that TBI-related autophagy impairment leads to increase in neurotoxic pTau level in neurons and glia. pTau was particularly accumulated in autophagosomes. Similarly, we also found that Wnt signaling mediator Dvl-1 is sequestered in autophagosomes. This suggests that accumulation of autophagosomes due to inhibition of autophagy flux has a potential to sequester both functional as well as misfolded, toxic proteins without degradation. This could increase cellular stress and induce ER stress pathway. Initial inhibition of autophagy pathway may contribute to protein accumulation, particularly the toxic ones such as pTau fibrillary forms, amyloid precursor protein and its cleaved product, and Synuclein [184]. Sequestration of functional protein such as Dvl-1 can affect Wnt signaling after TBI.

We detected ER stress and ISR markers such as ATF4 and CHOP were elevated in the neurons of cerebral cortex after TBI. Interestingly, we also found increase in TFEB level with nuclear translocation, indicating upregulation of TFEB regulated genes even when autophagy was inhibited after TBI. TFEB has been implicated in increasing ATF4/CHOP mediated cell death during integrated stress response [116]. In our study, we found that TFEB mediated ISR, and not ER stress alone,

regulates neuronal cell death. TAT-Beclin1 peptide was able to decrease TFEB level and inhibit nuclear translocation, which correlated with decrease in ATF4/CHOP levels and significant decrease in neuronal cell death. Our qPCR data after TBI with and without TAT-Beclin1 peptide showed decrease in ISR proteins with TAT-Beclin1, but no significant change in ER stress markers. Thus, we demonstrate that inhibited autophagy flux and sequestration of toxic or misfolded proteins can induce TFEB dependent ISR and neuronal cell death in TBI.

We also found both *in vivo* and *in vitro* sequestration of functional proteins such as Dvl-1 in autophagosomes, which lead to decreased Wnt signaling and upregulated GSK3 activity, causing pTau formation. Inhibition of autophagy flux in TBI facilitates buildup of neurotoxic pTau, which is known to destabilize microtubules of the axons and cause neurodegeneration. pTau accumulation is a risk factor for Tauopathy and Alzheimer's disease after TBI [184]. Thus, TBI-induced impaired autophagy can impact signaling pathway by sequestration of signaling molecule in autophagosomes.

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