5. Chapter II: Trib3 regulates autophagic neuron death in response to Amyloid-β

5.1 Introduction

Autophagy or self cleaning is a well conserved mechanism, required for the maintenance of the physiological process of the cells. Studies have shown that autophagy plays a pivotal role in the removal of misfolded proteins that accumulate within the cell. While autophagy is needed for physiological conditioning, non-dividing cells, like neurons, are notably sensitive to changes in autophagic degradation [1-3].

Autophagy is considered as an important physiological process required for the clearance of aggregate-prone proteins. The possibility of failure of autophagy could act as a mechanism predisposing cells to death. This has relevance to several pathological processes in a variety of diseases [4, 5]. Defects in autophagy would lead to accumulation of broken organelles and degradation of proteins and aggregates leading to their accumulation within the cell. Formation of intracytoplasmic aggregates can be seen in AD, where accumulation of tau occurs within the protoplasm while Aβ accumulates outside the cell. In Parkinson's disease, the major part of the aggregates is α-synuclein while in Huntington's disease, mutant huntingtin is the primary constituent of the aggregates [6].

Under normal conditions, autophagy is a gift, as it maintains proper physiological condition at basal levels. There occurs a high demand for cellular internal control through autophagy in some cells, especially in post-mitotic cells, like neurons and myocytes [7-10] and dysfunctional autophagy has been related to cell death in several neurodegenerative disorders. Thus, inducing the autophagic process to eliminate aggregates of degraded proteins and organelles would therefore be considered a logical therapeutic approach in neurodegenerative diseases.

There is therefore enough evidence that autophagy exerts a protective role against neurodegeneration, however how it forestalls neurodegeneration still remains to be elucidated. One probable answer to this could be the ability of autophagy to selectively eliminate macromolecule aggregates or inclusion bodies, via the selective degradation ability of the autophagic proteins p62 and hsc 70 [11, 12].

Stimulation of autophagy attenuated the illness severity in models of Huntington's disease [13, 14] or amyotrophic lateral sclerosis [15] and promoted clearance of α-synuclein in PC12 cells [16]. Huntingtin inclusions induce autophagy and sequester Beclin 1 [17, 18]. Mice lacking either Atg5 or Atg7, key elements of the autophagy

pathway, were shown to develop ubiquitin positive inclusions, behavior abnormalities and undergo neuronal loss [7, 19, 20]. These reports indicate the protective role of autophagy against neurodegeneration. On the contrary ultrastructural analysis have shown the presence of double membrane autophagic vesicles in dystrophic neurites in AD brains, and APP/PS1 transgenic mice [21, 22] and additionally recently, autophagy of mitochondria in AD brains [23-25] have also been reported. Autophagosomes are reported to be the focal point for Aβ production [26]. Continuous autophagy could act as a cause for cell death [27], or inexplicably needed for cell death [28]. However alterations occurring at the molecular level during autophagic cell death still stay complicated. Together, the information recommends that autophagy plays a crucial role within neurons. The key question on whether or not autophagy protects neurons or executes their death in neurodegeneration remains unexplained. So far, no genetic or causative defects within the autophagy pathway are linked to any of the neurodegenerative disorders and therefore the role of autophagy in AD remains unclear. What makes autophagy a very fascinating pathway to review in neurodegeneration, is its postulated role in Aβ production, degradation of macromolecules and protein aggregates, and its role within the cellular stress response.

Autophagy can therefore either promote cell survival, or can lead to cell death. The molecular mechanism underlying this dual role still remains obscure. It was reported that Tetrahydrocannabinol (THC), the active component of marijuana, induced human glioma cell death by stimulating autophagy [29, 30]. They showed that THC treatment resulted in active ER stress response that promoted autophagy. This occurred due to inhibition of mammalian target of rapamycin complex 1 (mTORC1) via Trib3. They further went on to show that autophagy preceeded apoptosis in cannabinoid induced cancer cell death. These findings intrigued us to check the role of Trib3 in regulating autophagy upon Aβ induced cell death.

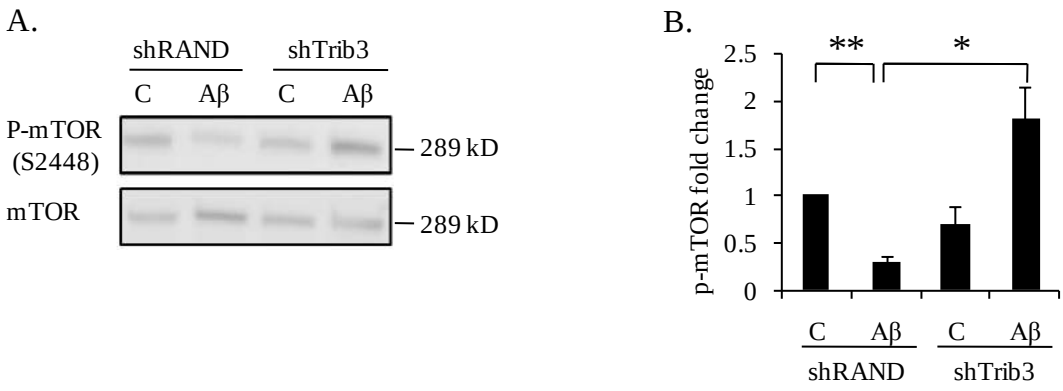
Further, there were reports which stated Trib3 directly bound to the autophagic receptor p62 and disrupted autophagy flux. They went onto explain that Trib3 interrupted the interaction between p62 and LC3 resulting in anomalous autophagy flux. The disruption of this interaction causes defective clearance of ubiquitinated proteins which goes on to hinder the ubiquitin-proteasome-dependant degradation. They further showed that inhibiting the interaction between Trib3 and p62 could restore the normal degradation of ubiquitinated proteins and promote animal survival. [31]. We therefore sought to

understand the mechanism via which Trib3 regulated autophagy in Aβ induced neuronal cell death.

5.2 Results

5.2.1 Trib3 induces autophagy in neurons evoked by Aβ via the Akt-mTOR pathway

It has been reported recently that Trib3 inhibits Akt/mTORC1 axis in cancer cells in response to cannabinoid and thus induces autophagy [30, 32]. Several reports suggest the role of impaired autophagy in the pathogenesis of AD [33-37]. Therefore we wanted to investigate whether Trib3 could also induce and regulate autophagy in Aβ treated neuronal cells. We transfected differentiated PC12 cells, with shTrib3 or shRand and followed it with Aβ treatment. Western blot analysis was performed to check the level of phospho-mTOR at S2448 (p-mTOR) and total mTOR. We observed that upon treatment, p-mTOR levels decrease and this decrease in phosphorylation level can be blocked by downregulating Trib3 (Fig. 1A & B). A direct target of p-mTOR is Ulk1 [38]. mTOR when phosphorylated leads to the inactivating phosphorylation of Ulk1 at Serine-757. This results in reduced autophagy. We therefore analysed the phosphorylation status of Ulk1 protein. We found that Ulk1 phosphorylation at Serine-757 reduced upon Aβ treatment which was rescued by downregulating Trib3 (Fig. 1C & D). Analysis of pULK1 levels in transgenic mice revealed that there is decreased expression of pULK1(S757) in the transgenic mice brain as compared to the brain of wild type (Figure 1E & F).Taken together these results indicate that Trib3 when induced upon Aβ treatment leads to enhanced levels of autophagy by inhibiting mTOR and activating Ulk1 activity.



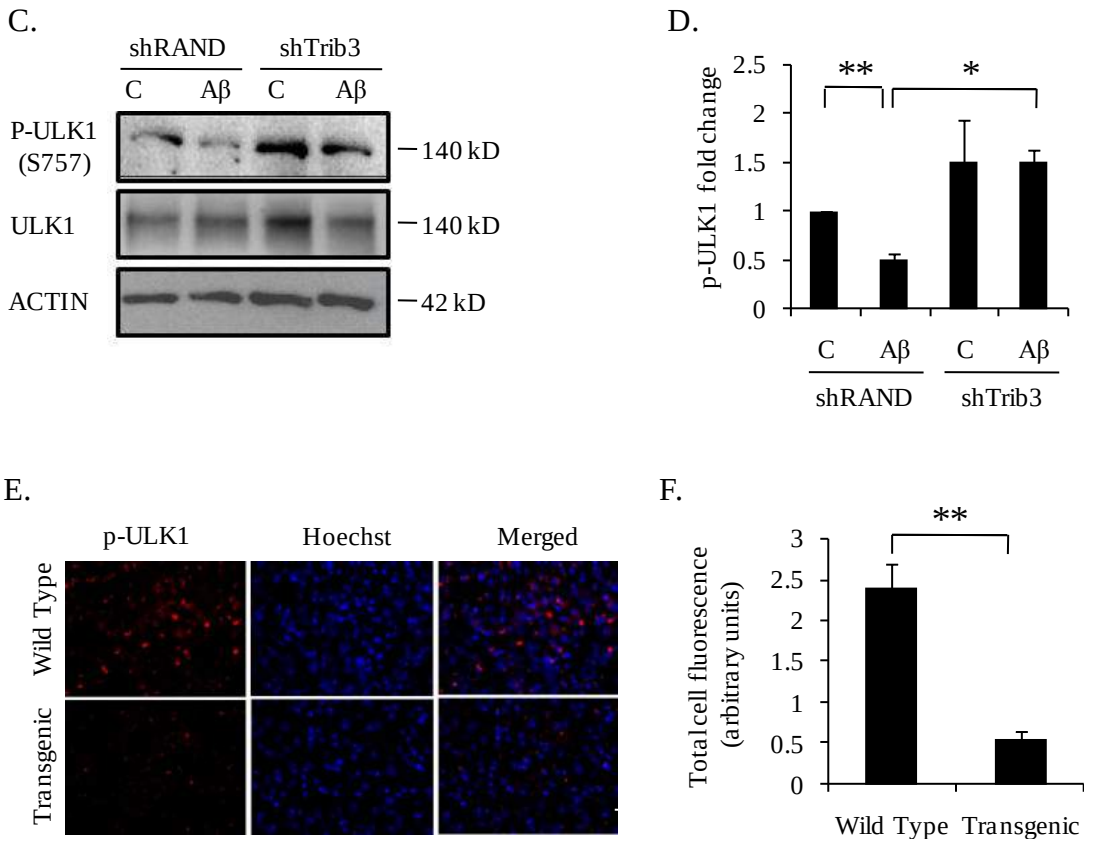


Figure C.2.1: Trib3 induces autophagy in neurons. **(A)** *PC12 cells were transfected with shTrib3 or shRand, primed and then treated with 5 μm Aβ. Phosphorylation status of mTOR at S-2448 was analysed by western blotting of cell lysate using p-mTOR antibody, total level of mTOR was also checked.* ***(B)*** *Graphical representation of fold change of p-mTOR level by densitometric analysis upon transfection with shTrib3 or shRand in presence or absence of Aβ 5 μM. Data represents mean ± SEM of three independent experiments.*p < 0.05.* ***(C)*** *PC12 cells were transfected with shTrib3 or shRand, primed and then treated with 5μM Aβ. Phosphorylation status of Ulk1 was analysed by western blotting using P-Ulk1 antibody, total level of Ulk1 was also checked.* ***(D)*** *Graphical representation of fold change of P-Ulk1 level by densitometric analysis upon transfection with shTrib3 or shRand in presence or absence of Aβ 5μM. Data represents mean ± SEM of three independent experiments.*p < 0.05.* ***(E)*** *Brain sections obtained from AβPPswe-PS1de9 transgenic mice and control littermates were stained with p-ULK1 (S757) antibody. Nuclei were stained with Hoechst. Representative image of one of the brain sections with similar results in each case is shown.* ***(F)*** *Graphical representation of corrected total cell fluorescence of pULK1 in transgenic and wild type brain sections. Difference in intensity of pULK1 staining is quantified by NIH imageJ as described in experimental procedures. Data represent mean ± SEM of thirty different cells from three independent experiments. *p < 0.05.*

5.2.2 Trib3 induces formation of autophagosomes in neuronal cells in response to Aβ

Having checked that Trib3 leads to activation of p-ULK1 and hence autophagy, we next went on to check the various markers for autophagy. LC3 is a well known autophagy marker. LC3 remains in the soluble form in cells. After the induction of the first gene in autophagy i.e. ULK1, there occurs a schematic upregulation of a number of autophagic genes which help in the double membrane structure, an autophagosome formation. The structure can be identified as an autophagosome only after the addition of LC3 on its membrane. When there is an induction of autophagy then LC3 is cleaved into LC3-I and LC3-II. The cleaved form is further conjugated to a phosphatidyl ethanolamine group. This lipidated form of LC3 binds to the membrane of the autophagosomes and is therefore used as a marker for autophagy. The LC3-II remains on the outer wall of the autophagosomes. The presence of LC3-II marked membranes indicate the occurrence of autophagosomes which go onto indicate an induction in the process of autophagy. We checked whether there was an induction in autophagy after Aβ treatment. We performed immunocytochemical staining of Aβ treated differentiated PC12 cells. We found an increased punctated staining in the treated condition (Fig. 2A &B). We next wanted to check if Trib3 played a role in induction of autophagy in this scenario. For this we transfected differentiated PC12 cells, with shTrib3 or shRand and followed by Aβ treatment. Western blot analysis was performed to check the level of cleaved LC3. We observed that upon treatment, LC3 was cleaved to LC3-I and LC3-II, while cleavage of LC3 can be blocked by downregulating Trib3 (Fig. 2C & D). We checked the levels of LC3 in APP/PS1 transgenic mice by performing immunohistochemical staining of the brain sections from APP/PS1 transgenic mice and wild type mice. We observed that there was an increased punctated staining of LC3 in transgenic mice as compared to wild type mice (Fig 2E & F).We also checked the role of Trib3 in induction of autophagy in cortical neurons. For this we downregulated Trib3 using shRNA against it in the primary cortical neurons and performed immunocytochemical staining for LC3.We observed that downregulating Trib3 reduced the formation of autophagosomes which were induced in the presence of Aβ treatment (Fig 2G & H).

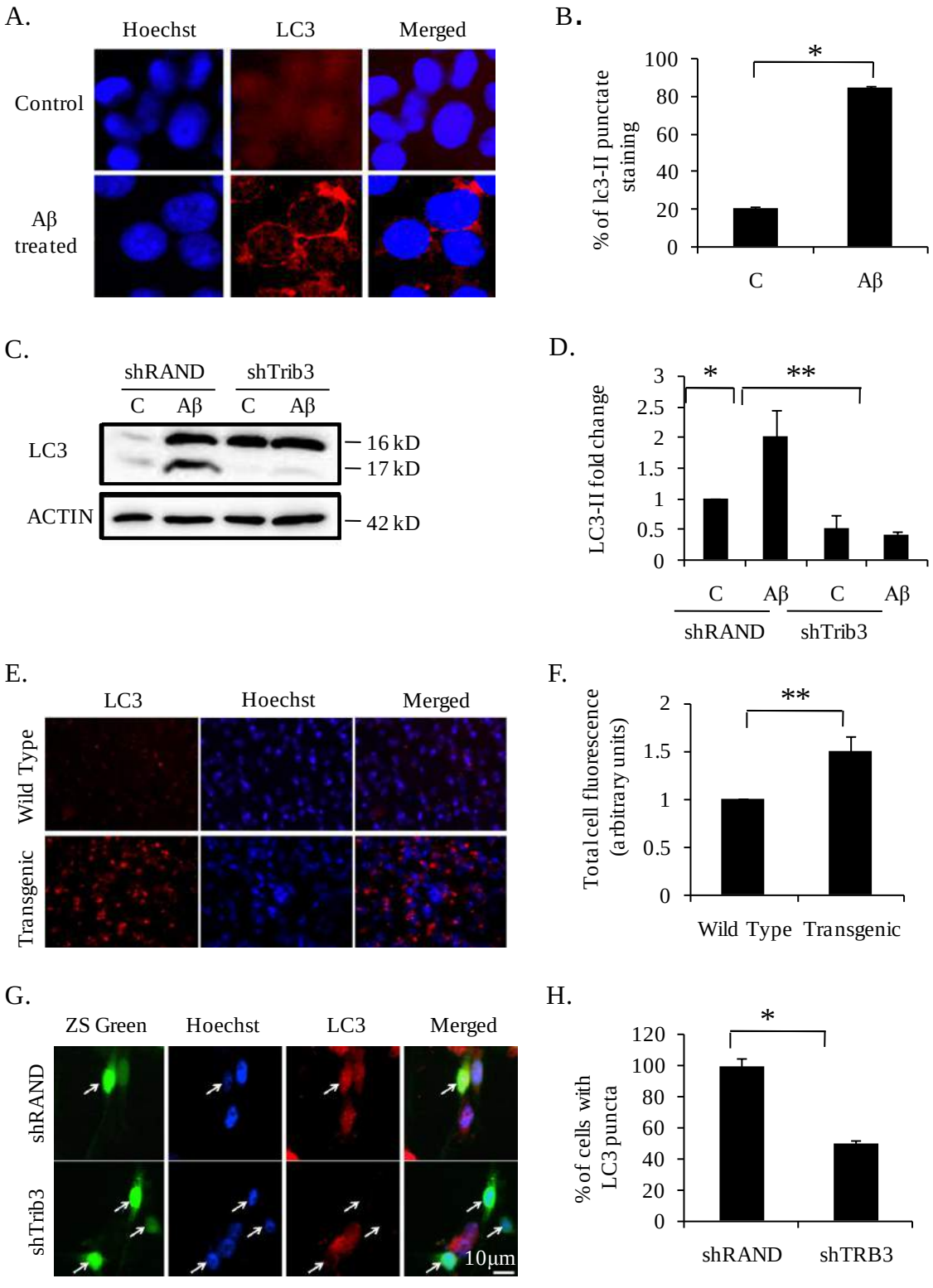


Figure C.2.2. Trib3 induces formation of autophagosomes in neuronal cells in response to* Aβ:** ***(A) *Differentiated PC12 cells were treated with 5µM Aβ for 24 h. immunocytochemical staining was performed with LC3 antibody.* ***(B)*** *Graphical representation of % of cells positive for LC3 punctated staining. Data represents mean ± SEM of three independent experiments.*p < 0.05.* ***(C)*** *PC12 cells were transfected with shTrib3 or shRand, cells were primed and treated*

with 5 μM Aβ for 24 h. Cleaved levels of LC3 was analysed by western blotting using LC3 antibody. ***(D)*** *Graphical representation of fold change of LC3-II levels by densitometric analysis upon transfection with shTrib3 or shRand in presence or absence of Aβ (5 μM). Data represents mean ± SEM of three independent experiments.*p < 0.05.* ***(E)*** *Brain sections obtained from AβPPswe-PS1de9 transgenic mice and control littermates were stained with LC3 antibody. Nuclei were stained with Hoechst. Representative image of one of the brain sections with similar results in each case is shown.* ***(F)*** *Graphical representation of corrected total cell fluorescence of LC3 in transgenic and wild type brain sections. Difference in intensity of LC3 staining is quantified by NIH imageJ as described in experimental procedures. Data represent mean ± SEM of thirty different cells from three independent experiments. *p < 0.05.* ***(G)*** *Cultured cortical neurons (5DIV) were transfected with shTrib3 and shRand, the cells were maintained for the next 48 h and then treated with Aβ 1.5 μM for 16 h, after which they were immunostained with LC3 antibody (Red).* ***(H)*** *Graphical representation of percentage of cells expressing LC3 puncta. Data represent mean ± SEM of thirty different cells from three independent experiments. *p < 0.05.*

5.2.3 Trib3 causes impaired autophagic flux in neurons exposed to Aβ

Protein degradation and organelle turn over require a basal level of autophagy. However reports indicate that increased autophagy promotes survival of neurons by facilitating enhanced clearance of aggregated proteins [35, 36]. Yet, if autophagic activity is continuously induced it can lead to cell death [39]. Dysregulation of autophagic flux mainly leads to autophagic death. The later stages of autophagy, mainly the fusion of the autophagosome with the lysosome is referred to as autophagy flux. Any hindrance occurring at this stage is referred to as frustrated autophagy. A protein which plays a pivotal role at this stage is p62. p62 is a ubiquitin binding scaffold protein. It colocalises with ubiquitinated protein aggregates. p62 contains an N-terminal PB1 domain which helps in the essential formation of polymers of p62. The formation of these polymers occurs when the acidic surface of one p62 PB1 domain binds to the basic surface in the next p62 PB1 domain [40, 41]. The PB1 domain is reported to have a role in the autophagic degradation of p62. It bears a C-terminal UBA (Ubiquitin associated domain) via which it binds to the ubiquitinated proteins. p62 also known as Sequestosome-1 is a factor that targets cargo for autophagy [42]. p62 works as a carrier of unwanted proteins to the autophagosome to enable their degradation. The generalized function of p62 therefore lies in ensuring aggregation and subsequent turnover of the unwanted ubiquitinated proteins. The protein is itself degraded by autophagy, but, if at any point of time there occurs an accumulation of p62 within the cells, it points out to a

different condition altogether. It has been reported that p62 is accumulated when autophagic degradation is inhibited, hence, considered as marker of autophagy flux [43],[44]. We checked the autophagy flux in Aβ treated cells by monitoring p62 levels. Interestingly, we found that there was an increased accumulation of p62 in the cells treated with Aβ (Fig. 3A & B).

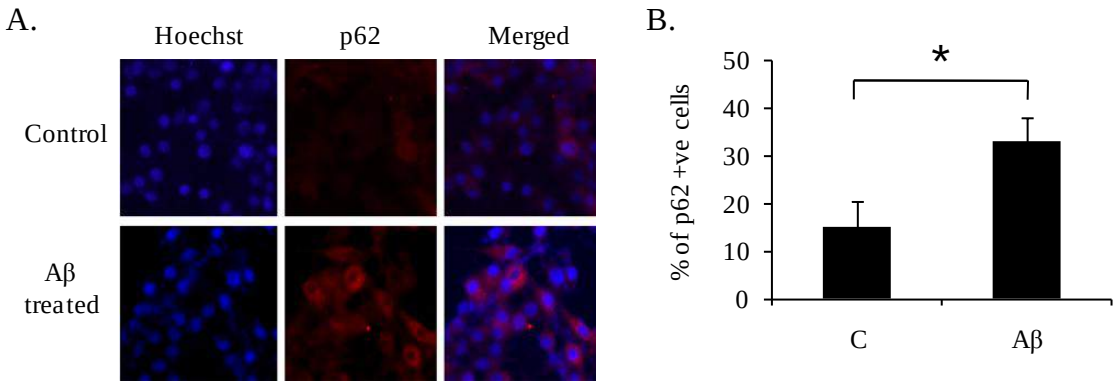
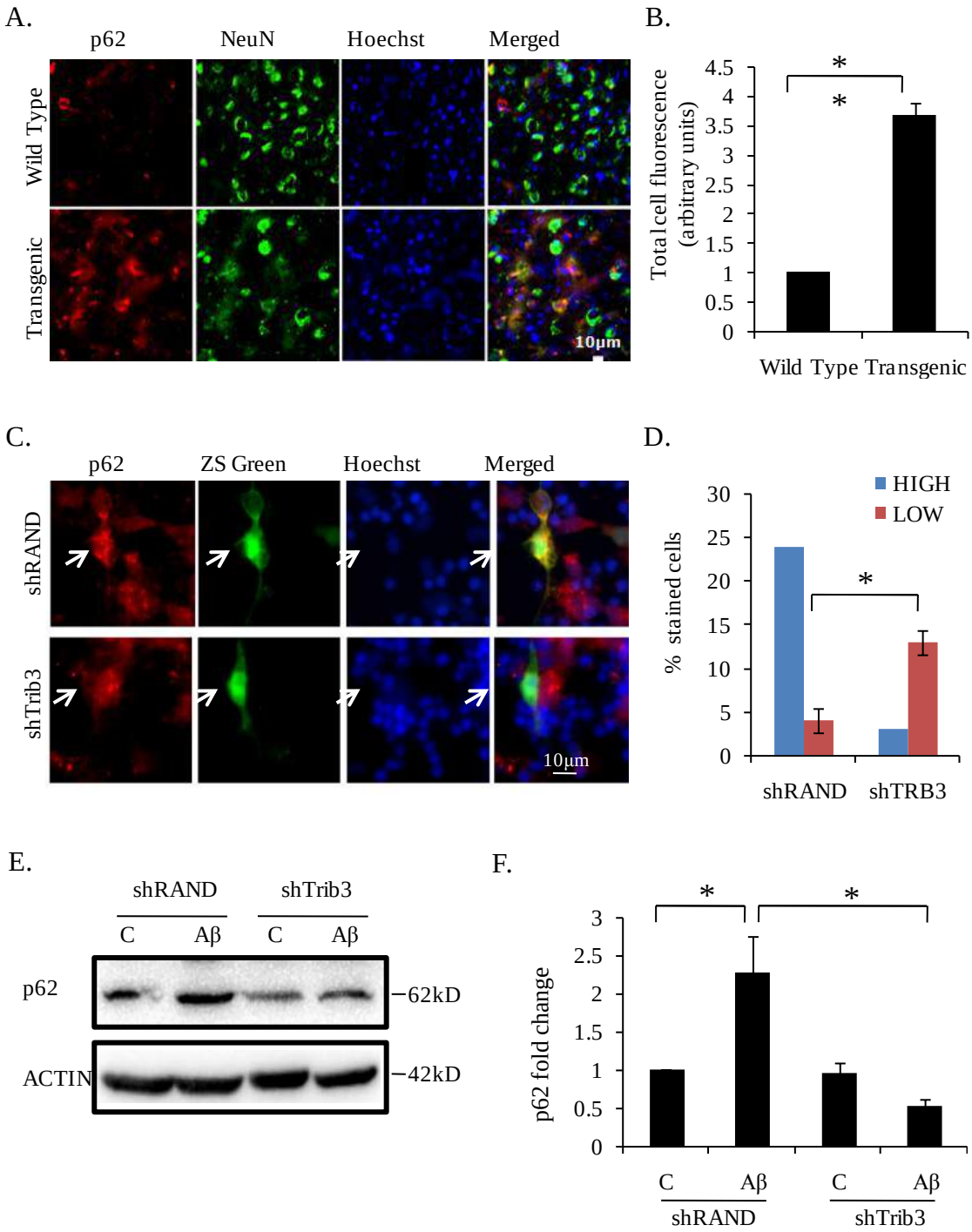


Figure C.2.3. Trib3 accumulates in PC12 neuronal cells in response to* Aβ:** ***(A) *Differentiated PC12 cells were treated with 5 μM Aβ for 24 h. immunocytochemical staining was performed with P62 antibody.* ***(B)*** *Graphical representation of % of cells positive for P62 staining. Data represents mean ± SEM of three independent experiments.*p < 0.05.*

We also checked the levels of p62 in APP/PS1 transgenic mice by performing immunohistochemical staining of the brain sections from APP/PS1 transgenic mice and wild type mice. We observed that there was an increased staining of p62 in transgenic mice as compared to wild type mice (Fig. 4A & B). Interestingly, the increase in p62 levels in cortical neurons in response to Aβ treatment was blocked when Trib3 was downregulated (Fig. 4C, D, E & F). Downregulation of Trib3 using a shRNA against it, also showed a decrease in the levels of p62 in those cells even under Aβ treatment. On the other hand, when Trib3 was overexpressed, by using an overexpression vector of it, and a PWPI as a control for the overexpression, an enhanced accumulation of p62 was observed in the cells overexpressing Trib3 as compared to the non transfected cells, and the PWPI transfected cells (Fig. 4G & H). Collectively, these results indicate that Trib3 induces autophagy in Aβ-treated cells. However, it culminates in a frustrated autophagy due to decreased autophagic flux as seen by accumulation of p62.


A.
p62
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Hoechst
Merged
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Transgenic
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4
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*
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shTrib3
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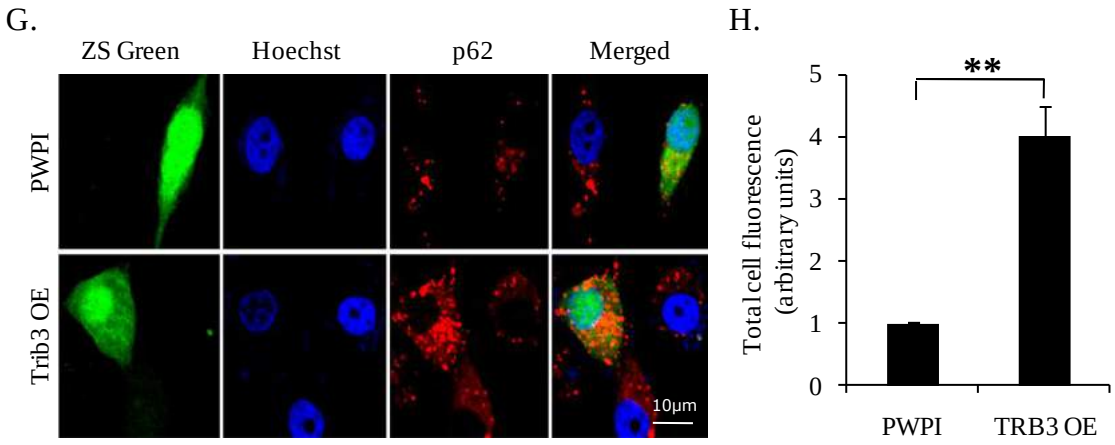
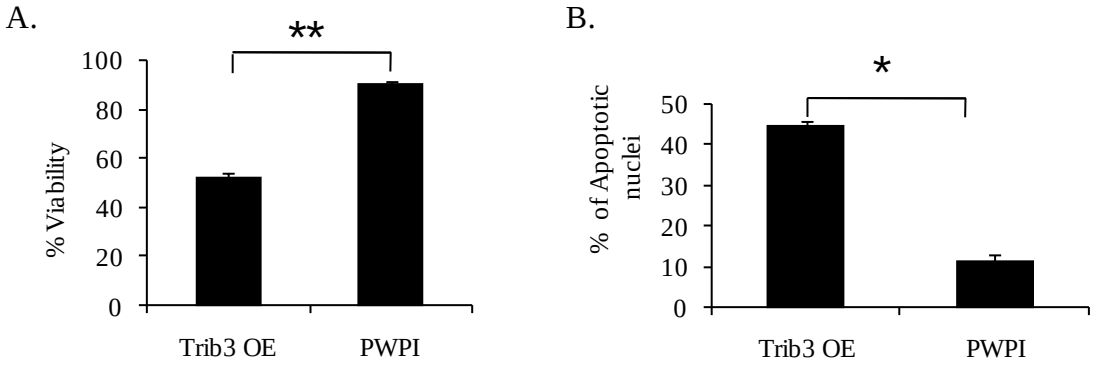


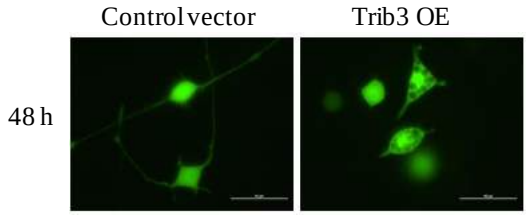
Figure C.2.4. Trib3 causes impaired autophagic flux in neurons: (A) *Brain sections obtained from AβPPswe-PS1de9 transgenic mice and control littermates were stained with p62 antibody. Nuclei were stained with Hoechst. Representative image of one of the brain sections with similar results in each case is shown.* ***(B)*** *Graphical representation of corrected total cell fluorescence of p62 in transgenic and wild type brain sections. Difference in intensity of p62 staining is quantified by NIH imageJ as described in experimental procedures. Data represent mean ± SEM of thirty different cells from three independent experiments. *p < 0.05.* ***(C)*** *Cortical neurons were transfected with shTrib3 or shRand, 48 h post transfection they were treated with 1.5 μM Aβ. p62 levels were determined by immunocytochemical staining using p62 antibody following the protocol mentioned in the methods and materials section above.* ***(D)*** *The Percentage of stained cells indicate the proportions of transfected cells (green) with high (more or equal than the neighbouring non-transfected cells) or low (less than the neighbouring non transfected cells) p62 immunoreactivity levels after treatment with Aβ. *p<0.05.* ***(E)*** *PC12 cells were transfected with shTrib3 or shRand, primed and then treated with 5 μM Aβ. After 16 h of Aβ treatment total cell lysate was isolated and samples prepared according to the protocol mentioned in the materials and methods section. The levels of p62 were analysed by western blotting using p62 antibody.* ***(F)*** *Graphical representation of fold change of p62 levels by densitometric analysis upon transfection with shTrib3 or shRand in presence or absence of Aβ (5μM). Data represents mean ± SEM of three independent experiments.*p < 0.05.* ***(G)*** *Primed PC12 cells were transfected with Trib3 overexpression vector or PWPI, as control. 48 h post transfection cells were fixed and p62 levels were checked by immunocytochemical staining using p62 primary antibody overnight staining, and Alexa 488 conjugated secondary antibody was used.* ***(H)*** *Graphical representation of corrected total cell fluorescence of p62 in differentiated PC12 cells transfected with PWPI or Trib3 OE vector. Difference in intensity of p62 staining is quantified by NIH imageJ as described in experimental procedures. Data represent mean ± SEM of thirty different cells from three independent experiments. *p < 0.05.*

5.2.4 Overexpression of Trib3 is sufficient to cause neuronal cell death whereas downregulating it promotes neuronal survival

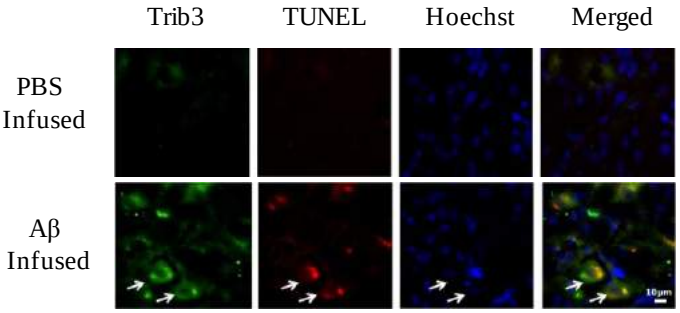
Our previous experiments reveal the critical role of Trib3 in inducing both autophagy and apoptosis in neuronal cells in AD model. We therefore wanted to study the specificity of death inducing ability of Trib3 by overexpressing it. For this primed PC12 cells were transfected with an overexpression vector of Trib3 and the empty PWPI vector was used as control. 36 h post transfection cells were observed for both apoptosis and autophagy. Trib3 overexpression was sufficient to kill cells by 48 h of transfection (Fig. 5A). Trib3 overexpression accounted for both, induction of apoptotic nuclei (Fig. 5B) as seen by Hoechst staining and the occurrence of autophagic vacuoles (Fig. 5C). In our *in vivo* data we observe that there occurs a co-localisation between increased Trib3 expression and TUNEL positive cells which go onto indicate a dual role of Trib3 in causing death of neurons upon Aβ treatment (Fig. 5D). To check the role of Trib3 in promoting death of neurons we downregulated Trib3 by using a short hairpin RNA against it and checked for several parameters. We observed that neurons in which Trib3 was downregulated showed retention of the synaptic membrane integrity as stained by a post synaptic membrane protein marker, PSD95 (Fig. 5E). They also showed maintenance of mitochondrial membrane potential as compared to the non transfected cells or the rand transfected cells (Fig. 5F).While we also observed a decreased expression of cleaved caspase 3 in cells in which Trib3 was downregulated as compared to the rand transfected cells indicating a decreased apoptotic activity in cells in which Trib3 was downregulated even upon Aβ treatment (Fig. 5G).These experiments therefore indicate the potential role of Trib3 to cause death of neuronal cells by both autophagy and apoptosis.



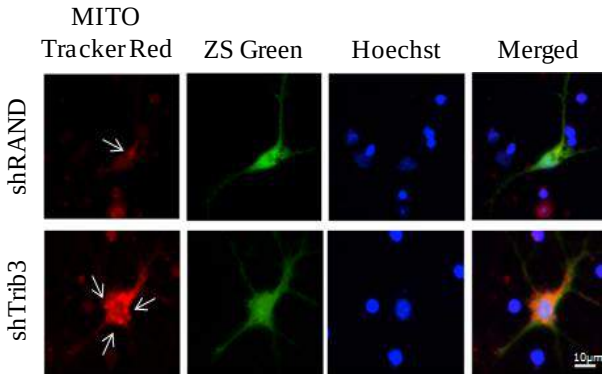
C.


Control vector
Trib3 OE
48 h

D.


Trib3
TUNEL
Hoechst
Merged
PBS Infused
Aβ Infused

E.


MITO Tracker Red
ZS Green
Hoechst
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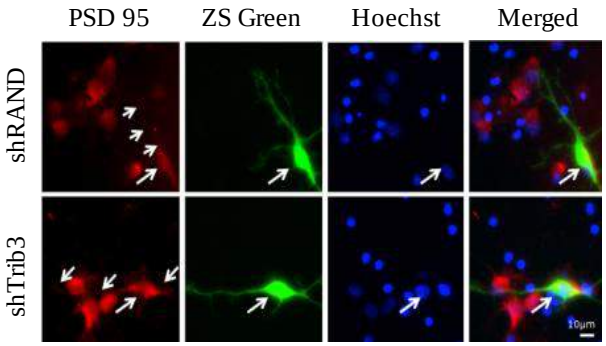
shRAND
shTrib3

F.


PSD 95
ZS Green
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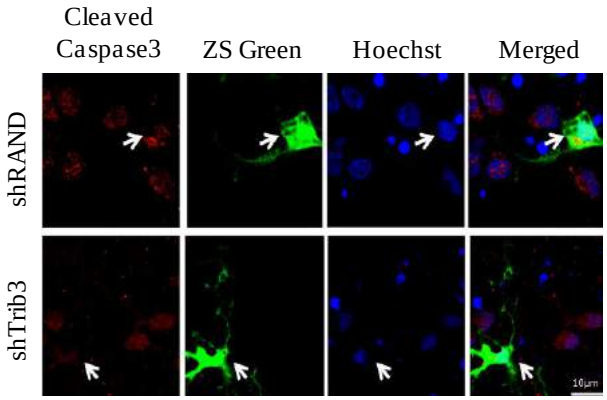

Figure C.2.5. Overexpression of Trib3 is sufficient to cause neuronal cell death. (A) *Primed PC12 cells were transfected with Trib3 overexpression vector or PWPI, as control. Live green cells are counted under fluorescence microscope after 36 h of transfection. Graphical representation of percentage viability.* ***(B)*** *Primed PC12 cells were transfected with Trib3 overexpression vector or PWPI, as control. 36 h post transfection cells were stained with Hoechst dye. Apoptotic nuclei were counted under the microscope. Graphical representation of percentage of apoptotic nuclei.* ***(C)*** *Primed PC12 cells were transfected with Trib3 overexpression vector or PWPI, as control. 48 h post transfection cell were observed under the microscope for autophagic vacuoles. Levels by densitometric analysis upon transfection with shTrib3 or shRand in presence or absence of Aβ 5µM****.(D)*** *Brain sections from Aβ and PBS infused rats were analysed for TUNEL assay and then immunostained for TRB3. Nuclei were stained with Hoechst. Representative images of one of the brain sections with similar results in each case are shown.* ***(E)(F)&(G)*** *Cultured cortical neurons (5DIV) were transfected with shTrib3 and shRand, the cells were maintained for the next 48 h and then treated with 1.5 µM Aβ for 16 h, after which they were immunostained with PSD95 (E), Mito tracker red dye (F), or (G) Cleaved caspase3 antibodies.*

5.3 Discussion

In this study, we investigated the role of Trib3 in inducing autophagy in Aβ induced neurodegeneration. A number of our experimental observations indicate that Trib3 promotes autophagy in response to Aβ. We found that Trib3 expression leads to phosphorylation and hence activation of autophagy gene Ulk1 in neuronal cells thus initiating the cascade of autophagy following Aβ treatment. A similar induction of autophagy was also observed in the APP/PS1 double transgenic mice model. The result from these transgenic mice also showed an elevated level of LC3 marked autophagosomes and an accumulation of p62 within the cells. Moreover, knocking down Trib3 by using shRNA in cortical neurons showed less induction of LC3 marked autophagosomes and also showed decreased accumulation of p62. Overexpression of Trib3 showed an increased aggregation of p62 in the neuronal cells, corroborating with the previous reports which indicate a physical interaction between Trib3 and p62.

Mechanistic studies reveal that Trib3 causes dephosphorylation of mTOR thus inactivating it. Here it must be mentioned that mTOR usually remains phosphorylated and active in normal cells where it regulates autophagy at a very basal level. Dephosphorylation of mTOR inactivates it; hence its repression on autophagy is lost. This leads to the initiation of the autophagic cascade in the cells. Interestingly, reports indicate that mTOR usually inhibits autophagy by phosphorylating Ulk1 at serine-757. This is the inactivating phosphorylation of it. We observe that upon Aβ treatment, this inactivating phosphorylation of Ulk1 is lost, which goes onto indicate an induction of autophagy in Aβ treated condition. Further, downregulating Trib3 restores the inactivating phosphorylation of Ulk1 indicating the role of Trib3 in activating Ulk1.Immunohistochemistry of transgenic mice brains also reveal a decrease in the inactivating phosphorylation of Ulk1 at serine-757. Most important markers of autophagy are LC3 and p62. In recent years, there have been several reports indicating their usage as a marker for autophagy. We find that there is an increase in LC3 punctated staining in the APP/PS1 transgenic mice brain sections as compared to control. Moreover downregulating Trib3 leads to a decrease in LC3 stain pointing towards a decrease in autophagosome formation in neuronal cells. Further our western blot results reveal that LC3 is cleaved upon Aβ treatment into LC3-I and LC3-II, while downregulating Trib3 reduces the cleavage of LC3 into its smaller fragments. This

potentiates the role of Trib3 in autophagy induction in neuronal cells upon Aβ treatment.

p62, which as mentioned earlier is called sequestosome 1 (SQSTM1) in humans, is also referred to as A170 in mouse, ZIP in rats, and Ref 2(p) in Drosophila melanogaster. P62 binds to and accumulates ubiquitinated protein aggregates formed in various neurodegenerative diseases, liver diseases, and myofibrillar myopathies [45-50]. p62 interacts with LC3 through a short LC3 interaction region (LIR). This facilitates interaction not just with LC3 but also with GABARAP-family members and this causes p62 to be degraded specifically by autophagy [51, 52]. The protein p62 is expressed in several cells and tissues and functions as a scaffold binding protein or adapter protein in a multitude of signaling pathways [42]. Of interest is the fact that since p62 degradation is autophagy dependent, whenever there is inhibition of autophagy the level of p62 increases [53]. It has been reported that p62 accumulates in cells and tissues from autophagy-deficient mice [54, 55]. Accumulation of p62 has been used extensively as a marker for inhibition of autophagic degradation or defects in autophagic flux [7, 50, 51, 55-60]. We find that there is an increased accumulation of p62 in the transgenic mice brain sections as compared to the control sections. There occurs increased accumulation of p62 upon Aβ treatment while when Trib3 is downregulated there is a decreased aggregation of p62 in these cells as compared to the non transfected cells. Finally, we find that overexpression of Trib3 alone can lead to increased accumulation of p62 as compared to the non transfected cells.

We further find that overexpression of Trib3 leads to occurrence of autophagic vacuoles in the cells while downregulating it leads to retention of synaptic membrane integrity, maintains mitochondrial membrane potential and shows decreased caspase activity. Therefore absence of Trib3 promotes overall neuronal cell survival. Our observation of the *in vivo* data indicates the role of Trib3 in initiating both autophahgy and apoptosis in AD model.

Hence our study points out to the dual role of Trib3 in causing neuron death upon Aβ treatment, via both death paradigms apoptosis and autophagy.

5.4 References

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