7. Summary

7.1 Summary of Chapter I

Chapter I deals with studies on the induction of Trib3 and the feed forward regulatory loop between Trib3 and FoxO in β-Amyloid (Aβ) induced neuron death, the salient findings are as follows:

- Trib3 is induced in response to Aβ toxicity both at transcript and protein level in primary cultured rat cortical neurons.
- Trib3 is also seen to be upregulated in AD *in vivo* models: both in Aβ-infused adult rat brain as well as AD transgenic mice (AβPPswe-PS1de9).
- Trib3 knockdown by the use of specific shRNA prevents cortical neuron death evoked by Aβ toxicity and the protection is achieved for a longer time (upto 72 h).
- A significant number of shTrib3 expressing hippocampal neurons survived with intact neurites even after extended exposure of Aβ compared to control.
- Trib3 is also seen to negatively regulate Akt by physically interacting with it and inhibiting its activities.
- Trib3 regulates activity and nuclear translocation of FoxO via Akt. Downregulation of Trib3 by its specific shRNA reduces FoxO level.
- Aβ induced toxicity leads to dephosphorylation and activation of FoxO which undergoes nuclear translocation and regulates Trib3 levels transcriptionally. FoxO directly binds to the Trib3 promoter and enhances its expression.
- A feed forward regulatory mechanism occurs between Trib3 and FoxO which regulate each other upon Aβ treatment.
- Trib3 upregulates the expression of Bim via the transcription factor FoxO and thus mediates the apoptotic death of neurons induced by Aβ

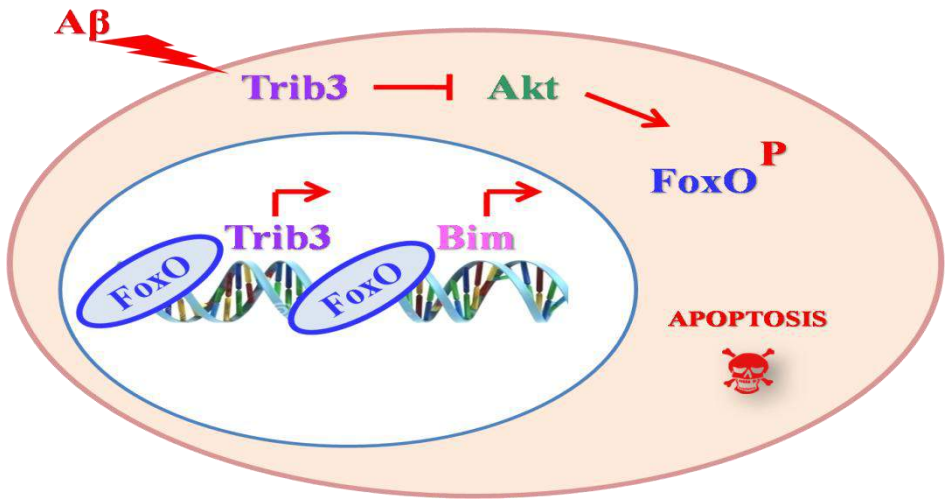

Figure S.1: Schematic representation of Trib3 mediated apoptotic neuron death in response to Aβ.

7.2 Summary of Chapter II

Chapter II deals with the role of Trib3 in inducing autophagy in Amyloid β (Aβ) induced neuron death. The salient findings are as follows:

- Trib3 induces autophagy in neurons evoked by Aβ via the Akt-mTOR pathway.
- Downstream of mTOR, Trib3 activates ULK1 by dephosphorylating it at S-757 and thus initiates the autophagic cascade.
- The inhibitory phosphorylation of ULK1 is also decreased in transgenic mice brain sections, indicating initiation of autophagy in the APP/PS1 double transgenic mice.
- Trib3 induces formation of autophagosomes in neuronal cells in response to Aβ as seen by the abundant presence of LC3 positive autophagosomes.
- Increased LC3 puncta are also observed in the APP/PS1 transgenic mice brain sections; implying induced autophagy.
- Trib3 causes impaired autophagic flux in neurons exposed to Aβ, by causing accumulation of autophagosomes marked by the aggregation of p62.
- Accumulation of p62 is also observed in the APP/PS1 transgenic mice which indicates impaired autophagy flux in the transgenic mice.
- Overexpression of Trib3 causes occurrence of autophagic vacuole, apoptotic nuclei and is sufficient to cause neuronal cell death.
- Downregulating Trib3 promotes neuronal survival even in the presence of Aβ.

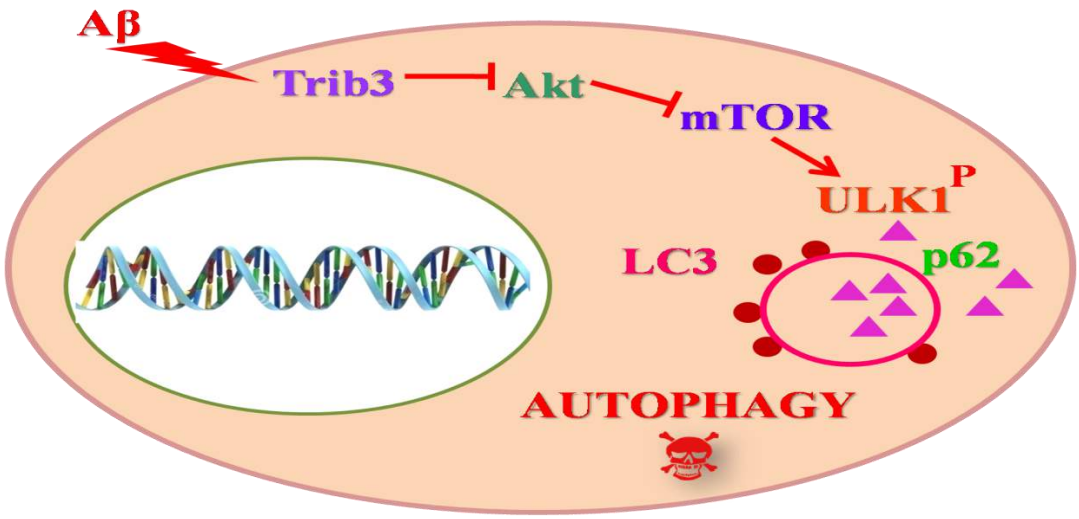

Figure S.2: Schematic representation of Trib3 mediated autophagic neuron death in response to Aβ.

7.3 Summary of Chapter III

Chapter III deals with studies on the crosstalk between autophagy and apoptosis at the molecular level. The salient findings are as follows:

- NGF deprivation induces both autophagy and apoptosis in neuronal PC12 cells.
- 500nM of Rapamycin induces autophagy, while 1μM of 3MA inhibits autophagy in neuronal cells.
- Inhibition of autophagy rather than inducing it provides protection to the neuronal cells even in the presence of NGF deprivation condition.
- Simultaneous inhibition of both autophagy and apoptosis provides significant protection to the NGF deprived neuronal cells as well as in Aβ insult.
- Beclin1 is upregulated in the neuronal cells upon NGF withdrawal conditions, there also occurs sequential cleavage of Beclin1 with time in NGF deprived neuronal cells.
- Caspases are involved in cleaving Beclin1 in NGF deprived condition.
- Cleaved fragment of Beclin1 translocates to the mitochondria in stress conditions.
- Downregulating Beclin1 provides significant protection to cortical neurons treated with Aβ, as well as to neuronal PC12 cells subjected to NGF deprivation condition.

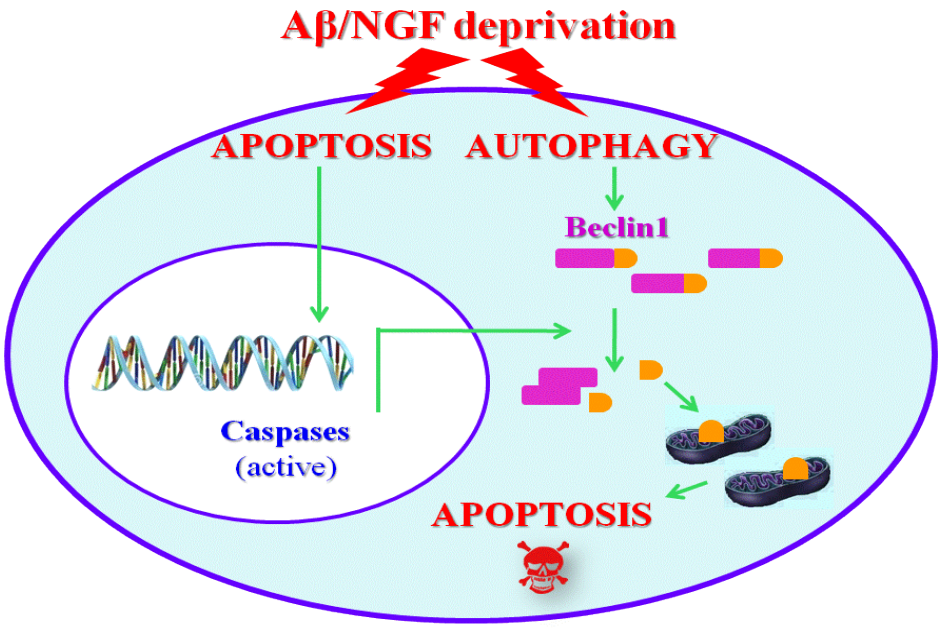

Figure S.3: Schematic representation of crosstalk between autophagy and apoptosis

8. Conclusive Discussion

Conclusive Discussion

Alzheimer's Disease (AD) is a complex disease and neurodegeneration underlies the pathology of this disease. Among several cell death modalities, both apoptosis and autophagy play important roles in neurodegeneration seen in AD. It suggests that there may be a common regulatory mechanism that initiates both these processes in AD.

In this study, we investigated the role of Trib3 in neuronal cell death in models of Alzheimer's disease. Our experimental observations indicate that Trib3 induces neuronal death by both apoptosis and autophagy in response to Aβ. We find that Trib3 expression is upregulated in neuronal cells both at transcriptional and translational levels upon Aβ treatment. A similar induction of Trib3 is also observed in the Aβ infused rat model and in APP/PS1 transgenic mice brain which overexpresses human Aβ. Moreover, downregulating Trib3 by using shRNA in cortical or hippocampal neurons protects cells from Aβ induced cell death. Sholl analysis also reveals the retention of neuronal processes as well as preservation of the overall neuronal morphology in neurons in which Trib3 has been downregulated, even after Aβ treatment.

Studies reveal that Trib3 physically interacts with and negatively regulates survival kinase Akt. It has also been shown that overexpression of Trib3 reduced phosphorylation of Akt at S473 and T308 [22] and knocking down of Trib3 restored phosphorylation of Akt in tunicamycin treated PC12 cells [27]. Interestingly, recently it has been shown that induction of Trib3, inhibition of Akt and activation of FoxO are involved in a self-amplifying loop that results in death of neurons evoked by withdrawal of NGF [22]. We have also observed that inhibition of PI3K/Akt signalling by LY294002 leads to upregulation of Trib3. Recently, FoxO transcription factors have been shown to play an important role in neurodegeneration [37, 38]. Reports suggest the presence of FoxO binding sites on the Trib3 promoter [48-51]. Our results revealed that there occurs enhanced binding of FoxO1 on the Trib3 promoter which in turn regulates its expression upon Aβ insult. These findings clearly indicate that a feed-forward regulatory mechanism is active between Trib3, Akt and FoxO1 in Aβ-treated neuronal cells as well. Next, we investigated the target of Trib3, downstream, that mediates neuronal death. We observed that downregulating Trib3 blocks the upregulation of Bim upon Aβ exposure suggesting the potential role of Bim in synchronising apoptotic death of neurons upon Trib3 induction. Bim is a transcriptional target of FoxO and has also been implicated in neuronal apoptosis in AD [39, 66-68].

Recent reports revealed that Trib3 was responsible for autophagic death in cancer cells [6, 7, 14]. Autophagic failure in AD has also been studied extensively [69-72]. There exist multiple connections between autophagy and apoptosis, and both these processes together decide the fate of the cells. Both autophagy and apoptosis may occur independently through a common stimulus, sometimes it may result in a combination of both these processes, or, each process may decide the cell fate in a mutually exclusive manner. The master regulator of autophagy is mTOR [54, 73]. The signalling cascade

PI3K/Akt/mTOR is inhibited by Aβ [74]. Trib3 inhibits the Akt/mTOR axis and leads to autophagy in human glioma cells [7]. In corroboration to previous findings we also found that Aβ causes dephosphorylation of mTOR and thus inactivates it. Downregulating Trib3 blocked the dephosphorylation of mTOR after Aβ treatment. Moreover, we also found that Ulk1, a direct target of mTOR, is activated upon Aβ treatment. It is required to initiate the autophagic cascade. Furthermore, we found an increased punctuated staining of the autophagosome marker LC3 upon Aβ treatment. Interestingly, downregulating Trib3 blocked the activation of Ulk1 and also decreased the increase in LC3 II following treatment with Aβ. This exemplifies the fact that Trib3 plays a crucial role in Aβ-induced autophagy. We checked another important marker for autophagy flux, p62. Accumulation of p62 within the cell occurs when the vacuoles are unable to fuse with the lysosomes, or when there is abnormal protein degradation [57]. We observed that following Aβ treatment there occurs an accumulation of p62; downregulation of Trib3 blocked this induction while overexpressing Trib3 increases the aggregation of p62. It has recently been reported that accumulation of p62 can mediate apoptosis via caspase-8 [75]. However, accumulating evidence indicates that high levels of autophagy can lead to autosis which is an autophagy-dependent non-apoptotic cell death [76]. This suggests that Trib3 could also be involved in autophagic death induced by Aβ by increasing the aggregation of autophagic vacuoles with decreased clearance from the cells. Having observed the dual modality by which Trib3 could lead to neuronal death, we further looked into the crosstalk between these two mechanisms in models of neurodegeneration. Recently Wirawan *et al* 2010 (ref) reported Caspase mediated cleavage of Beclin1 in Ba/F3 cells in response to IL3 deprivation. We found that Beclin1 is cleaved with time after NGF deprivation, while inhibiting Caspase3 with a specific inhibitor reduced cleavage of Beclin1 significantly. This led to the conclusion that Caspase3 and Beclin1 might be involved in the cross talk between autophagy and apoptosis in NGF withdrawal conditions. We observed similar results in Aβ model. We also found that the cleaved fragment of Beclin1 translocates to the mitochondria in stress condition. We further observed that downregulating Beclin1 leads to significant protection of PC12 neuronal cells from NGF deprivation and cortical neurons from Aβ upto 48 h of treatment.

In conclusion, this study suggests the multi-faceted mechanism by which Trib3 could be regulating death of neurons. Trib3 inhibits Akt, hence activates FoxO which can induce apoptosis via pro-apoptotic protein Bim. FoxO can also induce Trib3, hence a feed forward loop operating in degenerating neurons. Trib3 also induces autophagy by inhibiting mTOR, hence activating autophagy inducer ULK-1. Moreover, our study indicates a crosstalk between autophagy protein Beclin1 and apoptotic death executioner caspase3/caspase8.