6. Chapter III: Understanding the cross talk between autophagy and apoptosis during neurodegeneration

6.1 Introduction

The relationship between autophagy and apoptosis is quite complicated. In some instances autophagy proves to be a protective mechanism while in other cases it seems to induce death of cells. This differential functionality of autophagy seems to be dependent on circumstances. Past literature supports the idea of cross talk between apoptosis and autophagy to maintain cell homeostasis, which is the ultimate aim of cell during survival. This also indicates how intricately these are connected to decide the fate of cell [1, 2].

Autophagy and apoptosis are the two highly organized cellular processes that play vital role in embryonic development and tissue homeostasis. Dysregulation of these two processes have been associated with a number of pathological conditions, such as neurodegenerative diseases, autoimmune diseases and cancer. Various studies have very well established that autophagy and apoptosis extensively communicate with each other and the final outcome decides the fate of the cell during physiological and pathological conditions. Under stress conditions, cells initiate autophagy as a pro-survival mechanism to combat apoptosis. However, as stress increases towards a point of no return or threshold, cells block autophagy and initiate apoptotic cell death. The decision when to switch off this prosurvival autophagic process may mainly depend on the level of stress and is regulated by various proteins involved in autophagy and apoptosis.

Autophagy antagonizes the effect of apoptosis and the extent may vary with the stimulus, cell type, and stress level. Inhibition of autophagy has been reported to enhance sensitivity toward apoptosis. Indeed, inhibition of autophagy has been shown to enhance both caspase dependent and independent cell death. In contrast to bulk degradation, autophagy has been shown to selectively degrade active caspase 8 and inhibit the tumor necrosis factor (TNF) related apoptosis inducing ligand (TRAIL) induced apoptosis [3]. A recent study has demonstrated that Atg7 forms a complex with caspase 9 and keeps a check on its apoptotic activity [4]. At the same time, for effective onset of cell death, autophagy is inhibited by degradation of Atg proteins by apoptotic proteins such as caspases and calpains.

The interplay between Bcl 2 family proteins regulates both apoptosis and autophagy. Bax, a pro apoptotic protein was shown to inhibit autophagy by interacting with Beclin 1 [5]. Similarly, Bim inhibits Beclin-1 mediated autophagy, independent of its pro-apoptotic functions [6]. Autophagy adaptor protein p62 has also been found to play a role in caspase-8 associated cell death in U87MG cells treated with MG132 [7].

Cell death orchestrates life during development and also delivers the essence of life. Cell death via apoptosis can be of two main types, caspase dependent programmed cell death or apoptosis and caspase independent programmed cell death or necroptosis [8-10]. These may be interconnected by caspases which could activate non-caspase proteases, while these proteases could in turn activate the caspases.

Conversely some studies also highlight the importance of autophagy in activation of initiator caspase 8 without the involvement of receptor related signal from outside the cells. Puma and Bax induced autophagy have been demonstrated to contribute to the apoptosis in response to mitochondrial stress [11]. Another study concluded that poly ADP ribose polymerase (PARP) and receptor interacting protein 1 are required for effective induction of autophagy, which enhances the apoptotic cell death [12, 13].

Luo and Rubinsztein have shown that the interplay between Bcl-2 family proteins not only regulates apoptosis but autophagy as well. It binds to both the pro-apoptotic proteins (e.g. Bax) and pro-autophagic proteins (e.g. Beclin 1). Furthermore, mitochondria localized Bcl-2 inhibits activating molecule in Beclin1 regulated autophagy (AMBRA1) induced autophagy and ER localized Bcl-2 inhibits Beclin 1 induced autophagy [14]. Therefore, it is not surprising that the molecular crosstalk between these two pathways is very complex and sometimes contradictory as well.

P53 which was initially thought to be a transformation-associated protein, was later found to be involved in several other dimensions as in cell cycle control, DNA repair, cell differentiation, apoptosis and autophagy [15-19]. Profound study has been done in understanding the role of p53 in apoptosis. Irradiated mouse thymocytes were the first group of cells where p53 mediated apoptosis was first discovered [20]. Tasdemir et al show that deletion of p53 triggers autophagy in human and mouse cells. Further Du et al show that, p53 leads to proteasomal inhibition induced autophagy upregulation, which in turn has a negative effect on p53 mediated mitochondrial apoptotic pathway.

Besides the ability of Bcl-2 family proteins to regulate autophagy through the interaction with Beclin-1, caspases have been shown to inhibit autophagy via a mechanism mediated by the cleavage of autophagy-related proteins, such as Beclin1, PI3K, ATG5 and ATG4D. Their cleavage facilitates their localization to mitochondria where they serve new functions such as promoting mitochondria-mediated apoptosis. Wirawan et al. [21] reported that the N-terminal (Beclin-1-N, aa 1–133) and C-terminal (Beclin-1-C, aa 150–450) cleavage products of Beclin-1 failed to induce autophagy. More importantly, he proved that Beclin-1, once cleaved by caspases, can acquire a new apoptosis-promoting function. Indeed, Beclin-1-C translocated from the cytosol to mitochondria and amplified apoptosis induced by interleukin-3 deprivation [21]. We therefore sought to study the crosstalk between autophagy and apoptosis at the molecular level in cellular models for the study of neurodegeneration.

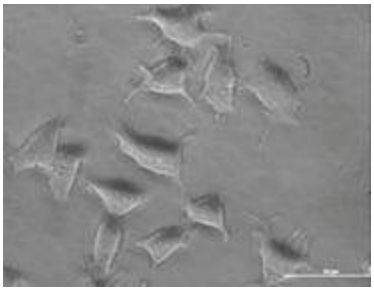
6.2 Results

6.2.1 Cellular models used for the study of neurodegeneration

Death of neurons occurs during development and about half of neurons die due to lack of target-derived trophic support such as caused by limiting supplies of Nerve growth factor (NGF) (Oppenheim 1991). It has also been recently shown that NGF deprivation causes the enhanced formation and release of Aβ which then interacts with cells to trigger an apoptotic pathway [22]. Among the major cell culture models used for studying death that is promoted by NGF deprivation, PC12 cells is the most accepted model. PC12 cells are dependent on NGF for their survival. When NGF signaling is initiated, they respond to the growth factor signalling by exiting the cell cycle, in response to the signalling they develop neurites and they also gain the ability to conduct electrical impulse [23]. When NGF is withdrawn from these cells, it is observed that they die via apoptosis [24]. Furthermore cell death induced in neuronally differentiated PC12 due to NGF withdrawal resembles that of sympathectic neurons [25]. We also employed neuronally differentiated PC12 cells treated with oligomeric Aβ as model of neurodegeneration. Aβ being the main pathologic species of the disease serves as the specific model for AD. The oligomeric forms of Aβ are more toxic than the monomers and the fibrillar species. Naïve PC12 cells were cultured in DMEM medium supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum (Fig. 1A). Cells were neuronally differentiated by NGF (50 ng/ml) in DMEM containing 1% Horse serum (Fig. 1B). On the fifth day of differentiation the differentiating medium was removed and the neuronally differentiated (primed) PC12 cells were washed with DMEM medium. Cells were then incubated with either Aβ (5 μM) (Fig. 1C), or DMEM containing anti-NGF (5 μg/ml) for 24 h (Fig.1D).

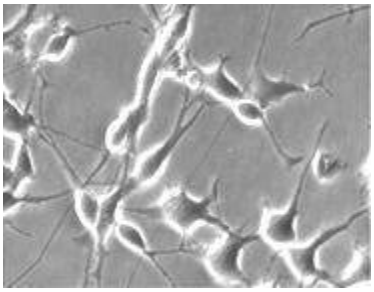
A.

Naïve PC12 cells



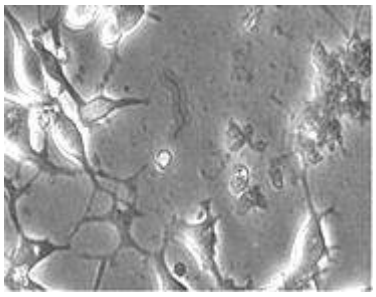
B.

Differentiated PC12 cells



C.

Aβ (5μM)



D.

αNGF (100ng/ml)

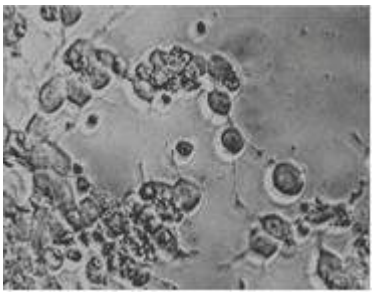


Figure C.3.1. Cellular models used to study neurodegeneration: ***(A)*** *Naïve PC12 cells.* ***(B)*** *PC12 cells were differentiated with NGF (50 ng/ml) for 5 days. Following differentiation the cells show morphological changes as development of long processes similar to those of neurons.* ***(C)*** *On the fifth day the neuronally differentiated PC12 cells were treated with oligomeric Aβ (5 μM).* ***(D)*** *Fully differentiated PC12 cells were subjected to NGF withdrawal on the fifth day of differentiation. Whereby NGF containing medium is removed and anti-NGF (5μg/ml) is added to the medium.*

6.2.2 Induction of both autophagy and apoptosis in neuronal PC12 cells in response to NGF deprivation and Aβ treatment

NGF-deprived differentiated PC12 cells die by both caspase3 dependent and independent pathways [24]. Our results corroborated with the above finding. We subjected differentiated PC12 cells to NGF deprivation for different time points. We observed that in response to NGF deprivation, there was increased LC3 punctate staining, which corresponds to increased autophagosome formation, at 16 h of treatment (Fig. 2A & B), while there was an increased cleavage of PARP1 with time, which indicates an increase in apoptosis (Fig. 2C & D). Neuronally differentiated PC12 cells were also treated with 5 μM Aβ for different time points. The cells were then fixed and processed for immunocytochemical staining with LC3 and pH2AX (a marker for DNA damage) antibodies. We observed an increase in both these markers with time (Fig. 2E).

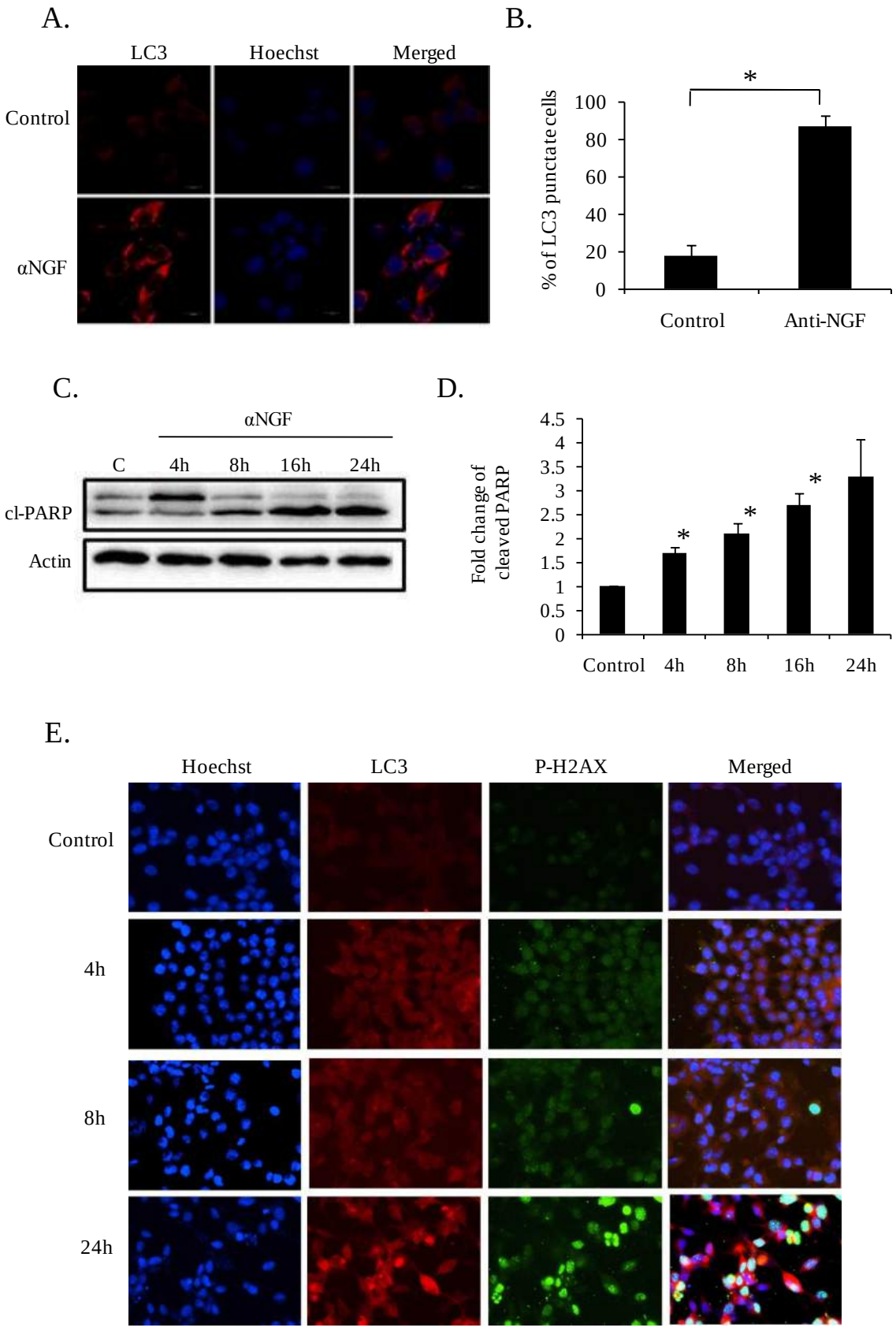


Figure C.3.2: Induction of autophagy and apoptosis in neuronal PC12 cells upon withdrawal of NGF or Aβ treatment. (A) *Primed PC12 cells were subjected to NGF withdrawal for 16h. Cells were fixed and immunocytochemical staining was performed with LC3 antibody, as a*

marker for autophagy. ***(B)*** *Graphical representation of percentage of cells stained with LC3.* ***(C)*** *Primed PC12 cells were subjected to NGF withdrawal for the respective time points. Cell lysate was collected and protein samples prepared for western blot analysis. Samples were run through an SDS-PAGE. Immunoblot was performed for PARP, a marker for apoptosis.* ***(D)*** *Graphical representation of cleaved PARP fold changes as quantified by densitometric analysis of western blots.* ***(E)*** *Primed PC12 cells were treated with Aβ for the respective time points. Immunocytochemical staining was performed for LC3 and pH2AX with the respective primary antibodies. Data represents mean ±SEM of three independent experiments: *p < 0.05.*

6.2.3 Selection of effective doses of Rapamycin and 3-Methyl Adenine to induce and inhibit autophagy respectively

In our endeavour to understand the crosstalk it was necessary to interfere with the process of autophagy. We therefore used Rapamycin (an inducer of autophagy) and 3-Methyl Adenine (3-MA, an inhibitor of autophagy). It was imperative to check the apt dose of Rapamycin and 3-MA which did not cause any toxicity to the cells. For this, differentiated PC12 cells were treated with varying concentrations of Rapamycin and 3-MA. Cell viability was checked by intact nuclear counting assay (as described in the Materials and Methods section) after 24 h of treatment. It was observed that for Rapamycin doses 200 nM and 500 nM (Fig. 3A & B), while for 3-MA doses 0.5 μM and 1 μM (Fig. 3C & D) did not cause significant toxicity to the cells. We next checked whether the selected doses of Rapamycin and 3-MA effectively induced and inhibited autophagy respectively. For this primed PC12 cells were treated with 200 nM, 500 nM of Rapamycin and 0.5 μM, 1 μm of 3-MA for 16 h. Following which western blot analysis was performed for Atg5 expression. Atg5, autophagy related gene 5 is upregulated when autophagy is induced [26]. It was observed that 500 nM of Rapamycin effectively induced autophagy compared to 200 nM while 1μM of 3-MA inhibited autophagy better than 0.5μM (Fig. 3E & F).

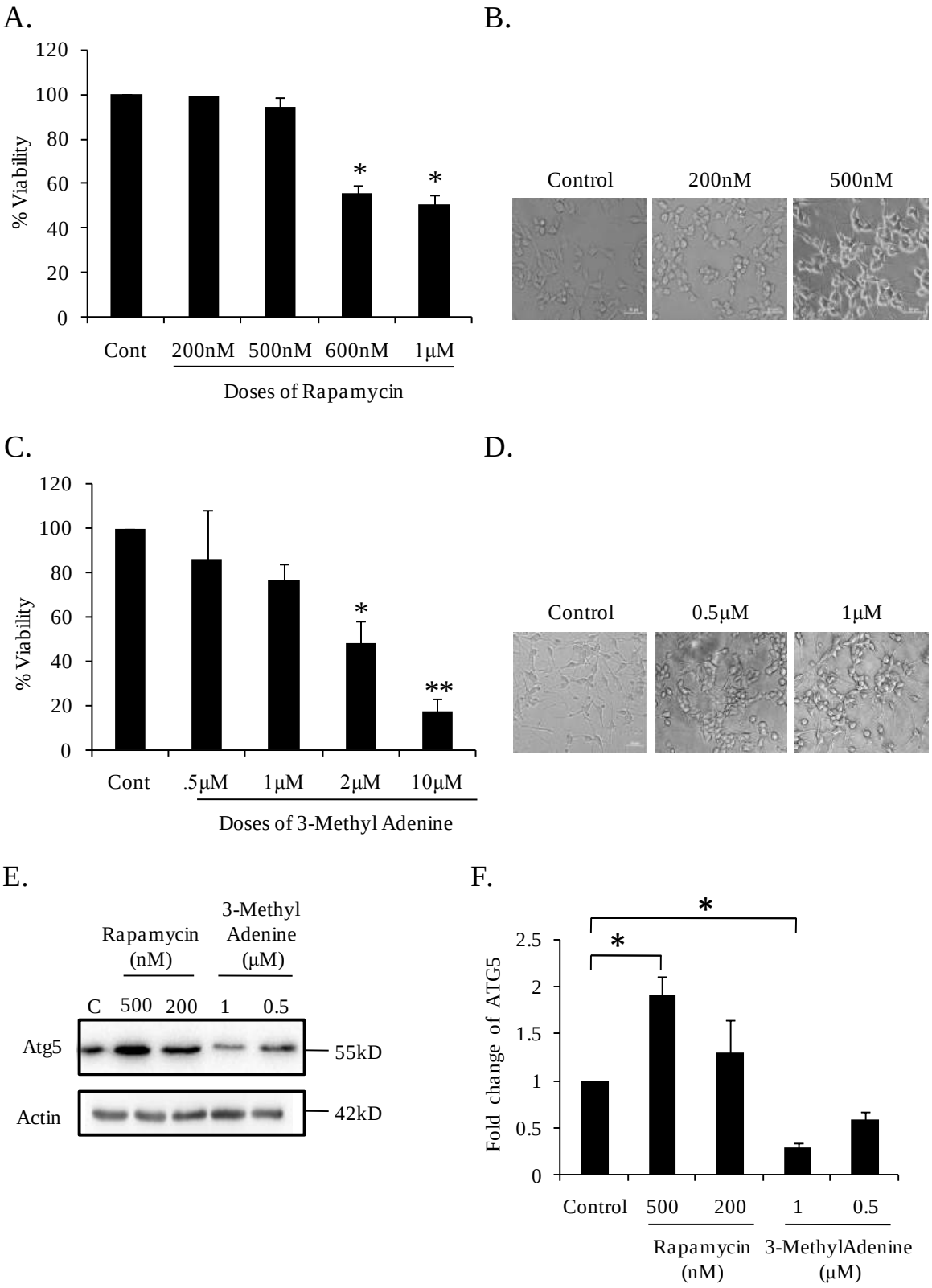
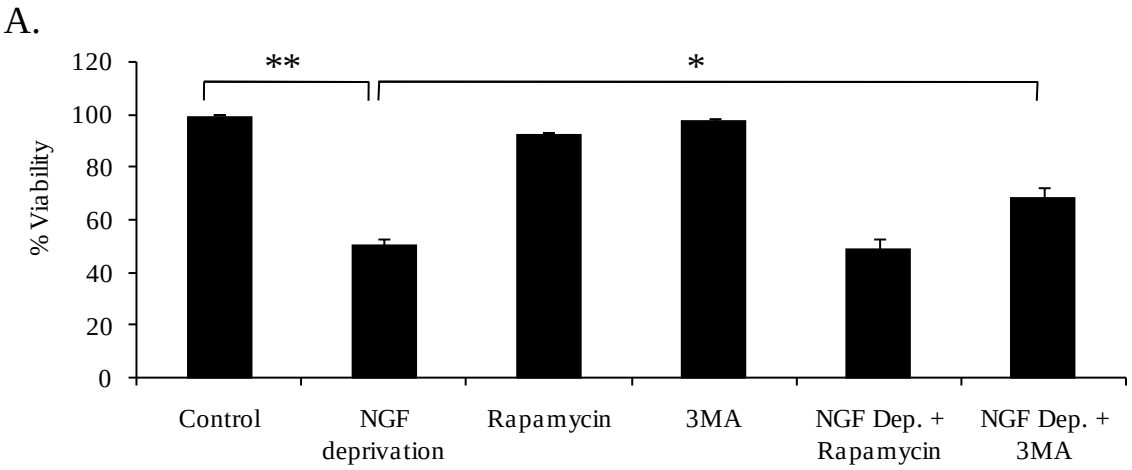


Figure C.3.3: Selection of doses of Rapamycin and 3-Methyl Adenine that do not harm cells. *Neuronally differentiated PC12 cells were treated with different doses of* ***(A)*** *Rapamycin and* ***(C)*** *3-MA. Nuclear counting assay was performed, whereby intact viable nuclei were counted which acts as an indication of survival. Graphical representation of percentage of cell viability following treatment with different doses of 3MA and Rapamycin respectively. Data represented*

*as mean ± SEM of three independent experiments performed in triplicates. The asterisk denotes statistically significant differences between indicated classes: *p<0.05.* ***Primed PC12 cells were treated with the selected doses of Rapamycin (B) & 3-MA (D).*** *Representative images show retention of neuronal processes in PC12 cells treated with the respective doses. Images have been taken in phase contrast mode under 20X magnification Scale bar corresponds to 50 µm.* ***(E)*** *Neuronally differentiated PC12 cells were treated with respective doses of Rapamycin and 3-MA for 16 h after which total cell lysate was subjected to western blotting analysis. Representative immunoblot showing Atg5 and Actin levels.* ***(F)*** *Densitometric analysis of Atg5 levels.*

6.2.4 Inhibition of autophagy provides protection to neuronal cells undergoing degeneration

Next, we determined whether interfering with autophagy could provide protection to neuronal cells from degeneration induced by NGF withdrawal or Aβ treatment. Primed PC12 cells were subjected to NGF withdrawal or Aβ along with 500nM of Rapamycin or 1µM of 3-MA for 16 h. Following which cell viability was checked by intact nuclear counting assay. We observed that inducing autophagy did not provide protection to cells, whereas, inhibition of autophagy by 3-MA provided significant protection to neuronal PC12 cells after NGF deprivation or Aβ treatment (Fig. 4A & B).



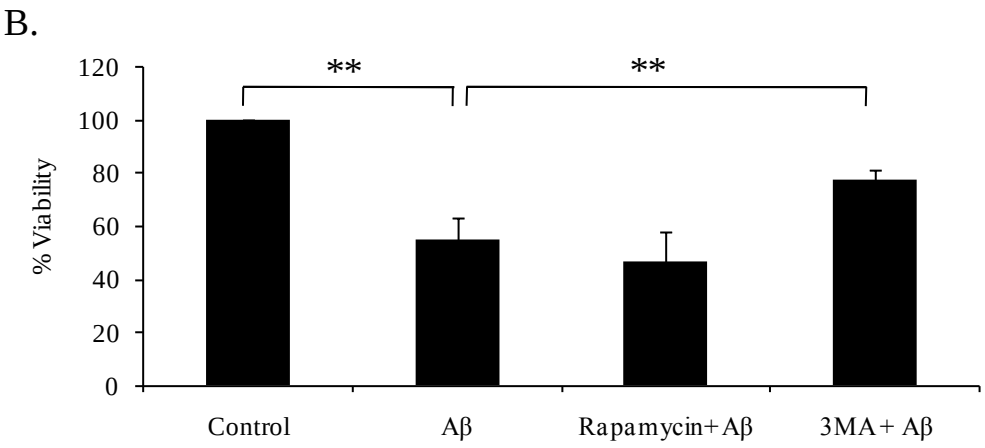


Figure C.3.4: Inhibition of autophagy provides protection against NGF deprivation and Aβ treatment: (A) *Graphical representation of cell survival following NGF deprivation with Rapamycin and 3-MA for 16 h.* ***(B)*** *Graphical representation of cell survival following Aβ treatment with Rapamycin and 3-MA for 16 h. Data represented as mean ± SEM of 3 independent experiments. *p<0.05, **p<0.001.*

6.2.5 Inhibition of both autophagy and apoptosis confers increased protection to neuronal cells

Massive death of neurons occurs in various neurodegenerative diseases. While apoptosis is a well known mechanism by which neuron death occurs, our findings suggest that induction of autophagy in response to NGF deprivation or Aβ treatment causes death of neurons as well. Thus we checked whether inhibition of both these processes could provide better protection to neuronal cells under these conditions. For this we deprived neuronally differentiated PC12 cells of NGF or treated with 5 µM Aβ. They were then treated with a pan-caspase inhibitor or 3-MA, or both together for 16 h. We observed that inhibition of both autophagy and apoptosis separately provided protection to neuronal cells under these conditions. Further, higher protection could be provided to cells when both apoptosis and autophagy were inhibited simultaneously, after NGF withdrawal (Fig. 5A) or Aβ treatment (Fig. 5B & C). A simultaneous blockage of both the death pathways proves to be sufficiently protective to Aβ treated and NGF deprived neuronal cells. This indicates the existence of a link between the two processes autophagy and apoptosis which orchestrates death in cells under stress.

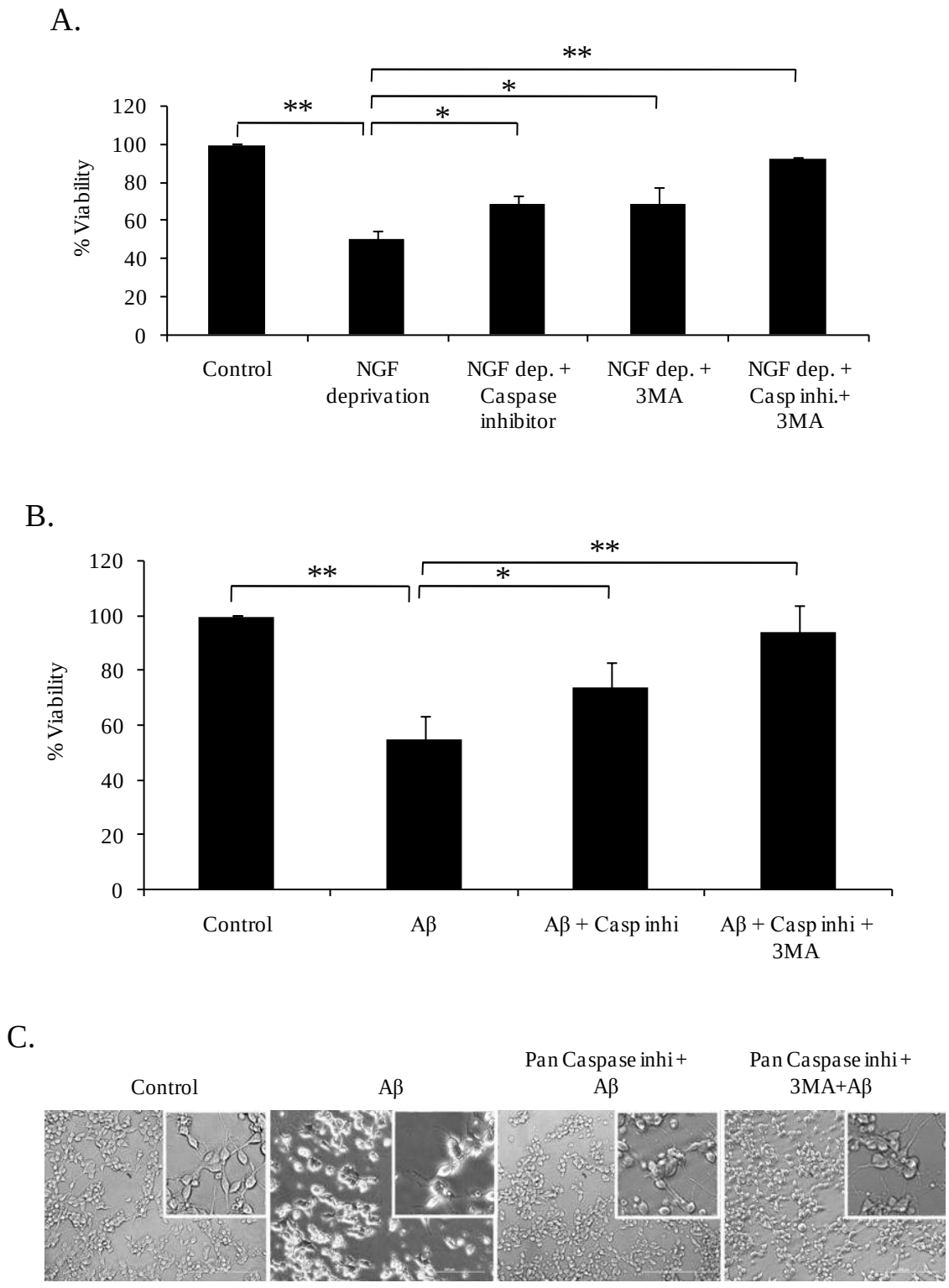


Figure C.3.5: Neuronal cells are better protected from death evoked by NGF deprivation or Aβ treatment when both autophagy and apoptosis are inhibited: (A) *Graphical representation of cell survival following NGF deprivation with pan caspase inhibitor and 3-MA, or both, for 16 h.* ***(B)*** *Graphical representation of cell survival following Aβ treatment with pan caspase inhibitor and 3-MA, or both, for 16 h. Data represented as mean ± SEM of 3 independent*

*experiments. *p<0.05, **p<0.001.* ***(C)*** *Representative Phase contrast micrographs of neuronal PC12 cells before and after treatment of Aβ for 16h.*

6.2.6 Beclin1 is cleaved by caspase3 in response to NGF deprivation or Aβ treatment

Our results indicate that inhibition of both autophagy and apoptosis provides increased protection to neuronal PC12 cells. This kindles a possibility of crosstalk between both these processes. Reports indicate the occurrence of several crosstalk mechanisms between these two phenomena. Of the several incidents of crosstalk, of interest here are the ones initiated by Beclin1. Beclin1 protein is essential for autophagy [16] possessing a potential role in the crosstalk between apoptosis and autophagy, whereby it is cleaved by caspases in cardiac diseases [27]. We therefore wanted to check whether there occurs cleavage of Beclin1 in our models of neurodegeneration. For this, primed PC12 cells were treated with anti-NGF for various time points and western blot was performed with Beclin1 primary antibody. We observed that with increase in time Beclin1 was cleaved into its 37 kD fragment (Fig. 6A & B). It has been reported that Beclin1 is cleaved at its C-terminal by active caspase3, leaving a cleaved fragment of 37 kD. This fragment translocates to the mitochondria where it initiates cytochrome-c release and hence apoptotic death of cells [21, 28, 29]. We next determined the involvement of caspase3 in cleaving Beclin1 in our model of neurodegeneration. For this we treated primed PC12 cells with anti-NGF in presence or absence of caspase3 inhibitor for 16 h. Western blot was performed with Beclin1 antibody. Our results revealed that Beclin1 was cleaved in response to NGF withdrawal, while this cleavage was significantly blocked when caspase3 inhibitor was used (Fig. 6C & D). Cleavge of PARP1 has been checked to see effective blockage of apoptosis by caspase3 inhibitor. The result indicates that active caspase3 could be involved in cleaving of Beclin1 in NGF deprivation condition. Another prominent caspase which has been reported in cleaving Beclin1 is caspase8. We checked whether caspase8 was involved in cleaving Beclin1 in response to Aβ treatment. For this, primed PC12 cells were treated with Aβ for 16h in presence or absence of caspase8 inhibitor and western blot was performed with Beclin1 primary antibody. We observed that caspase8 inhibitor blocked the cleavage of Beclin1 that occurred in the presence of Aβ (Fig. 6E & F).

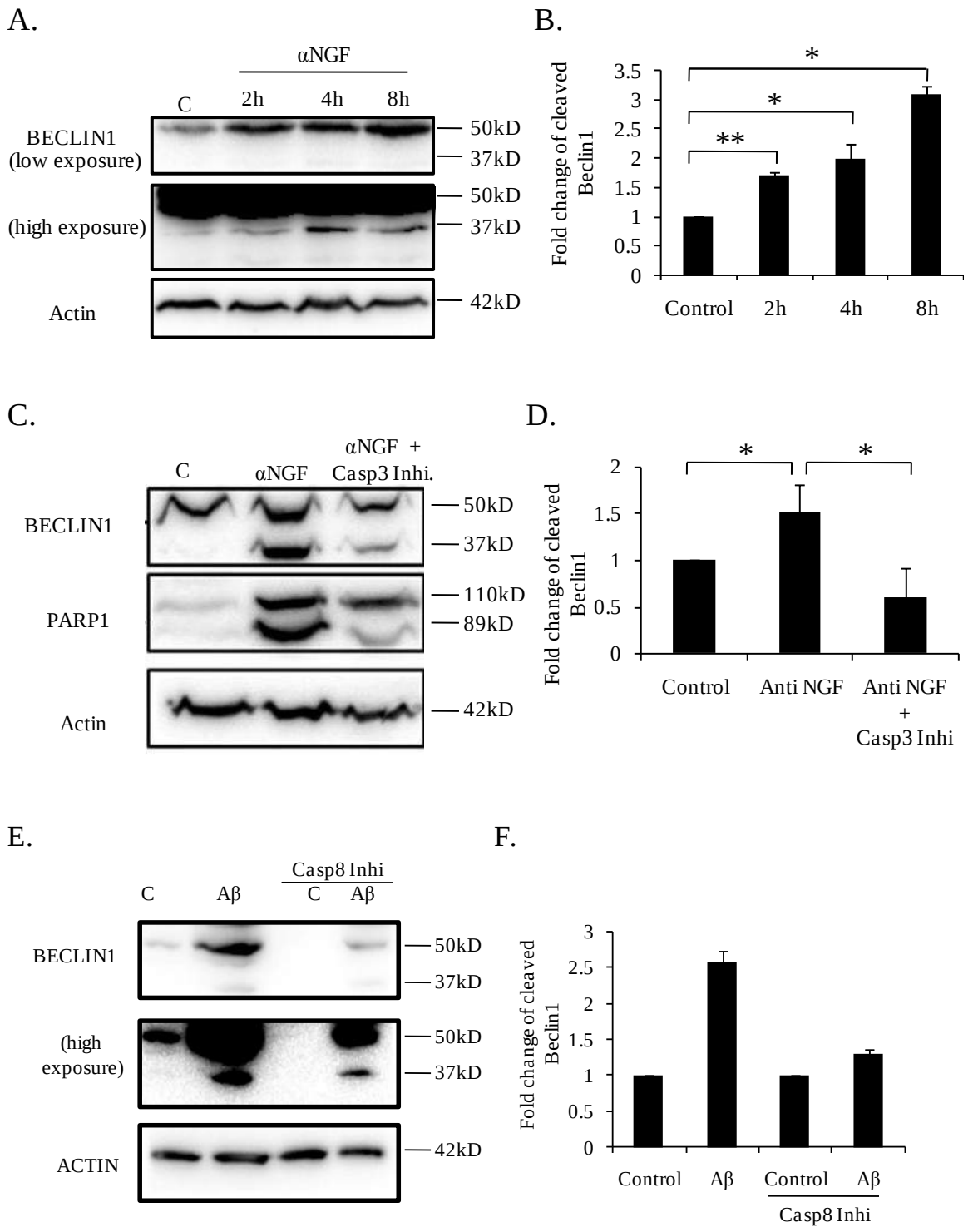
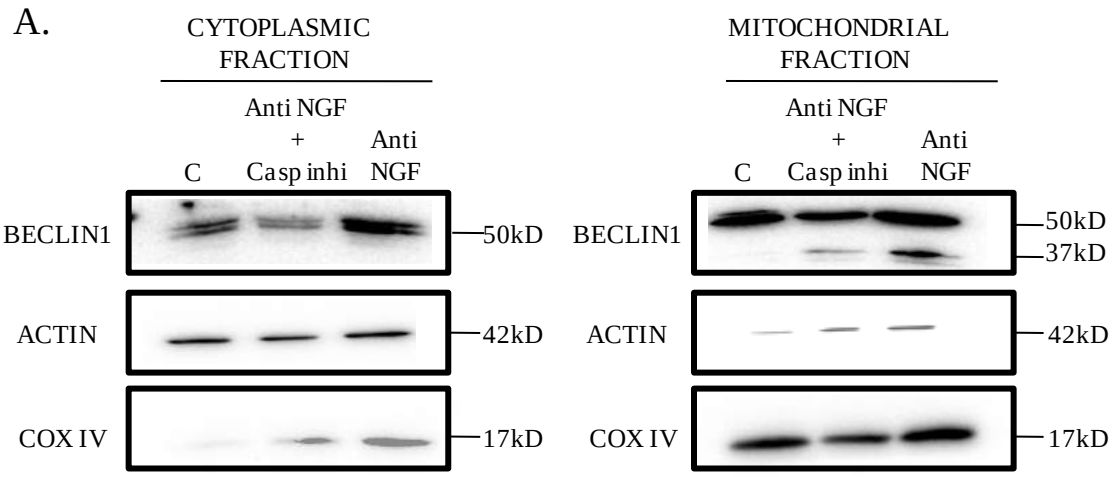


Figure C.3.6: Beclin1 is cleaved in response to NGF deprivation and Aβ treatment*: **(A)** Neuronal PC12 cells were subjected to NGF withdrawal for the indicated time points. After which they were processed for immunoblotting. A. Representative immunoblot of Beclin1 (upper panel: low exposure immunoblot, lower panel: higher exposure immunoblot. Representative image of 3 experiments with similar results are shown.* ***(B).*** *Densitometric analysis of immunoblot.* ***(C)*** *Neuronal PC12 cells were subjected to NGF withdrawal in presence or absence of caspase3 inhibitor for 16 h. After which they were processed for immunoblotting. Representative immunoblot of Beclin1, PARP1 and actin****. (D).*** *Densitometric*

*analysis of fold change of cleaved Beclin1 fragment. Result represented as mean ± SEM of 3 independent experiments. *p<0.05, **p<0.001. **(E)** Neuronal PC12 cells were treated with Aβ and caspase8 inhibitor for 16 h. After which they were processed for immunoblotting. Representative immunoblot of Beclin1.**(F)** Densitometric analysis of fold change of cleaved Beclin1 fragment. Result represented as mean ± SEM of 3 independent experiments. *p<0.05, **p<0.001. Densitometry?*

6.2.7 Cleaved fragment of Beclin1 translocates to the mitochondria and is essential for neuron death

As our results suggest, Beclin1 is cleaved by caspases in response to NGF deprivation and Aβ treatment, we determined whether the cleaved fragment of Beclin1 was translocating to the mitochondria in these models of neurodegeneration. For this we subjected the total cell lysate to subcellular fractionation and separated the mitochondrial fraction from the cytoplasmic fraction. Western blot analysis of the cytoplasmic and mitochondrial fractions revealed the presence of the cleaved fragment of Beclin1 in the mitochondria rather than the cytoplasm (Fig. 7A and B), thus indicating the translocation of the cleaved fragments to the mitochondria in our model of neurodegeneration. We further assessed the ability of the cleaved fragment in causing death of cells. For this we overexpressed the C terminal cleaved fragment of Beclin1 and used an empty vector as control. It was observed that the cleaved fragment of Beclin1 was sufficient to cause death of cells even in the absence of stress stimuli (Fig. 7C). Finally, we wanted to see whether downregulating Beclin1 could provide protection to cells. For this we used RNAi mediated knockdown of Beclin1. Neuronally differentiated PC12 cells were transfected with either shBeclin1 or shRand (control), and then cells were subjected to NGF withdrawal (Fig. 7D & E) or Aβ treatment (Fig. 7H) for 24 h and 48 h. Results showed significant protection, where Beclin1 was knocked down compared to cells transfected with shRand. Similar protection was obtained in cortical neurons treated with 1.5 μM Aβ upto 48 h (Fig. 7F & G). Knockdown of Beclin1 not only protects neuronal cells from death but also retains the overall neuronal morphology of neurons even after 48 h of Aβ treatment (Fig. 7F).


A.
CYTOPLASMIC FRACTION
MITOCHONDRIAL FRACTION
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BECLIN1
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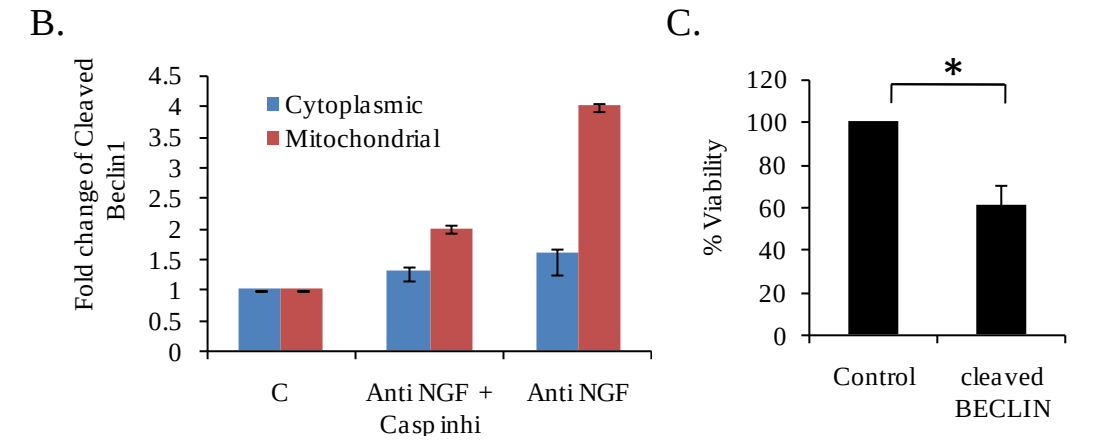
42kD
17kD


B.
Fold change of Cleaved Beclin1
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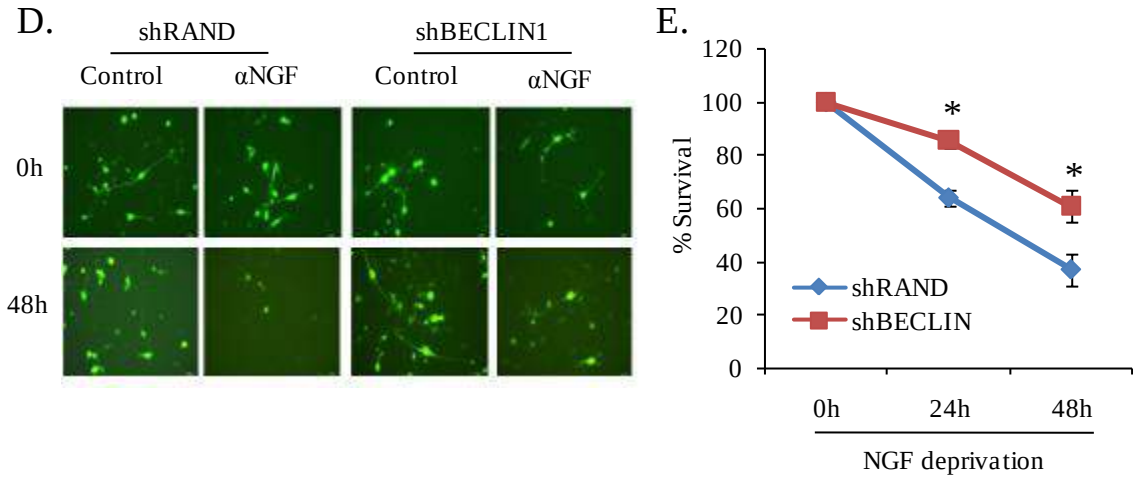
D.
shRAND
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E.
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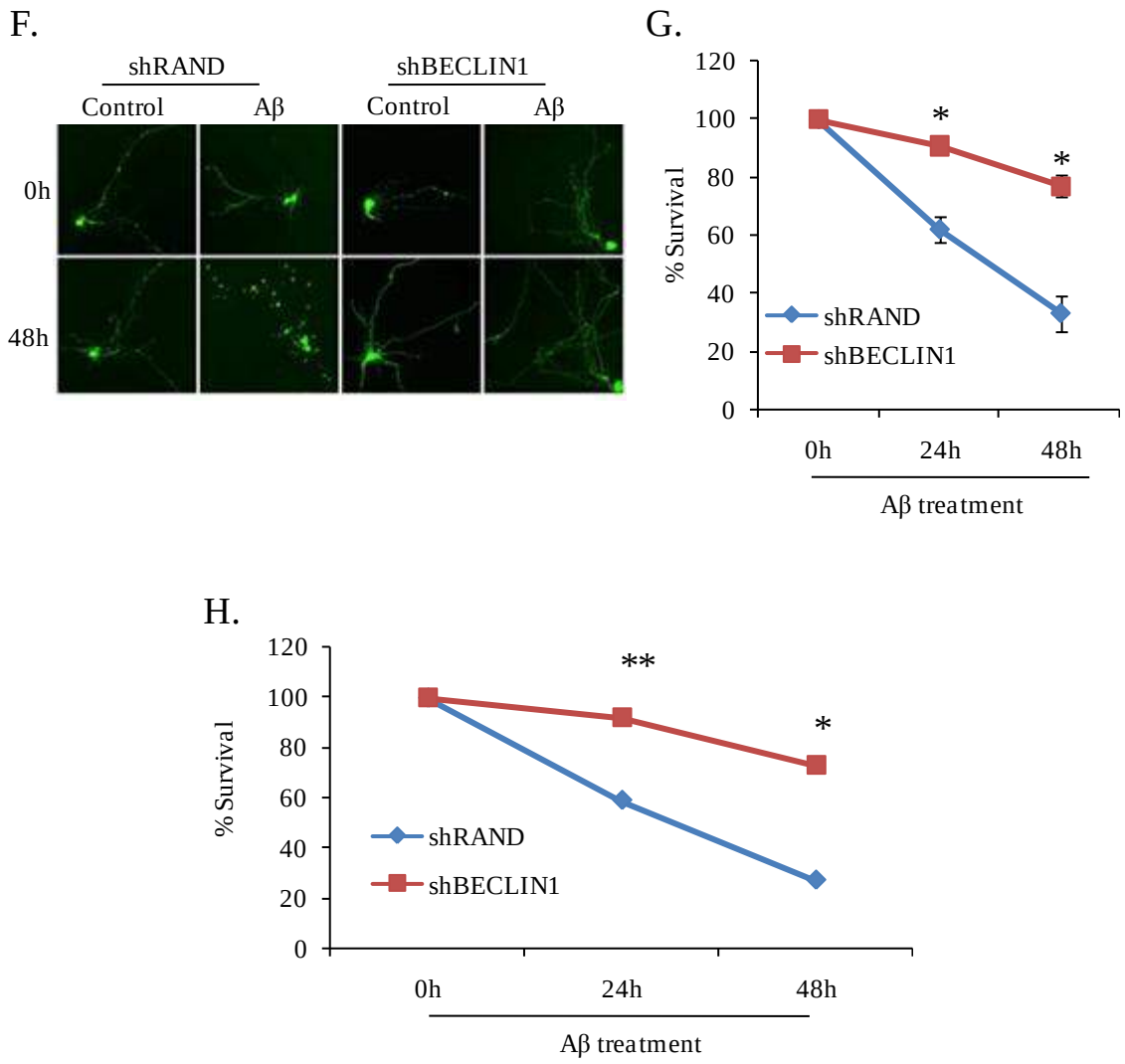
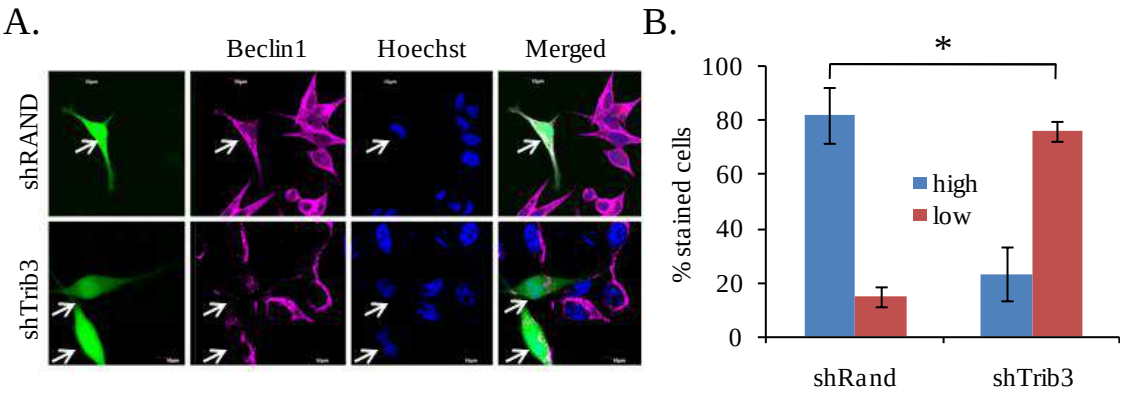


Figure C.3.7: Downregulating Beclin1 protects neuronal cells against NGF deprivation and Aβ treatment*: **(A)** N euronally differentiated PC12 cells were subjected to NGF deprivation for 16h. The cytosolic and mitochondrial extracts were isolated and proteins were analysed by western Blotting. Representative immunoblots showing change in Beclin1levels. **(B)** Graphical representation of change in cleaved Beclin1 levels.**(C)** Primed PC12 cells were transfected with pEGFP vector containing C-terminal cleaved fragment of Beclin1 expression vector and empty pEGFP vector as control. Live green cells were counted under fluorescence microscope after 36 h to analyze cell death due to Beclin1 cleaved fragment over-expression.**(D)** Neuronal PC12 cells were transfected with shBeclin1-zsgreen or shRand-zsgreen. 48 h post transfection cells were subjected to NGF withdrawal. Live green cells were counted at indicated times under a fluorescense microscope.**(E)**Graphical representation of percentage of viable cells**. (F)** Primary cultured rat cortical neurons (3 DIV) were transfected with shBeclin-zsgreen (shBeclin) or control shRand-zsgreen (shRand) and maintained for 48 h and then subjected to Aβ (1.5 μM) treatment for 48 h. Representative pictures of transfected neurons that were maintained in presence or absence of Aβ for indicated time periods are shown.**(G)** Graphical representation of percentage of viable green cells after each time point.**(H)** Neuronal PC12 cells were transfected with shBeclin1-zsgreen or shRand-zsgreen. 48 h post transfection cells were treated with Aβ. Live green cells were counted at indicated times under a fluorescense microscope. Graphical representation of percentage of viable cells.Data are from three independent experiments, each with comparable results and are shown as mean ± SEM,*

*performed in triplicates. The asterisks denote statistically significant differences from control (shRand) at corresponding time points: *p< 0.05; **p<0.001.*

6.2.8 Beclin1 is regulated by Trib3 in neuronal cells upon Aβ treatment

Finally, we e determined whether Beclin1 is under the control of Trib3 in neuronal cells exposed to Aβ. We transfected differentiated PC12 cells with shTrib3 or shRand, exposed them to Aβ followed by immunocytochemical analysis for Beclin1 expression. Results revealed that downregulating Trib3 resulted in reduction of total Beclin1 expression (Fig. 8A & B), as well as regulated its cleavage too (Fig. 8C & D).



Further, we performed western blotting analysis with Trib3 knockdown PC12 cells for Beclin1 level following Aβ treatment. We observed a significant upregulation of Beclin1 following Aβ exposure in shRand transfected cells. But interestingly this upregulation was significantly blocked in shTrib3 transfected cells even after Aβ treatment (Fig. 8C & D). These findings thus indicate that Trib3 regulates Beclin1 expression upon Aβ treatment.

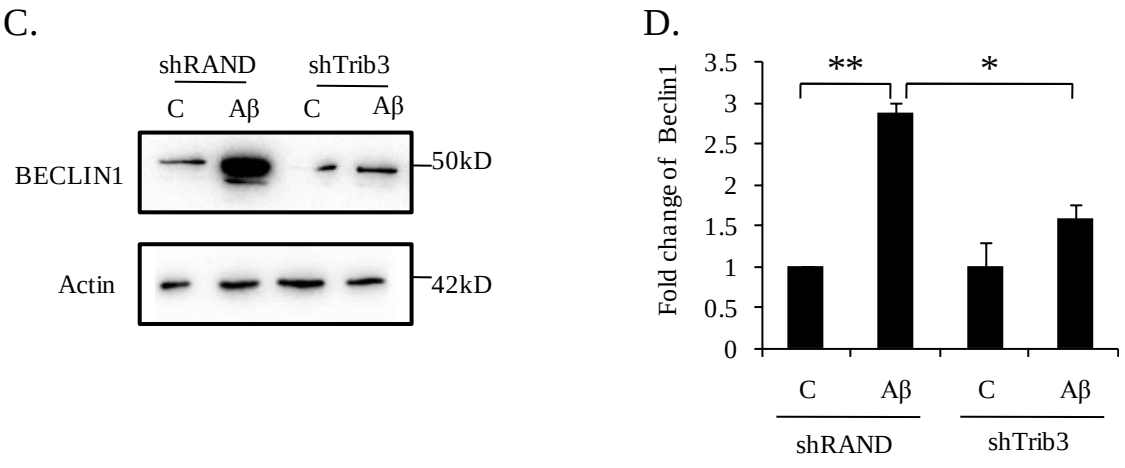


Figure C.3.8: Trib3 regulates Beclin1 in neuronal cells upon Aβ treatment (A) *Neuronally differentiated PC12 cells were transfected with shTrib3 and shRand, the cells were maintained for the next 48 h and then treated with Aβ 5 μM for 16 h, after which they were immunostained with Beclin1 antibody (Red).* ***(B)*** *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) Beclin1 immunoreactivity levels after treatment with Aβ. Data represents mean ± SEM of three experiments. Number of cells evaluated per culture are 50 (approx). The asterisks denote statistically significant differences between low staining cells and high staining cells: *p<0.01.****(C)*** *PC12 cells were transfected with shTrib3 or shRand, primed and then treated with 5μM Aβ. Downregulation of endogenous Beclin1 levels was analysed by western blotting using Beclin1 antibody****.*** ***(D)*** *Graphical representation of fold change of Beclin1 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*

6.3 Discussion

NGF deprivation leads to the activation of the amyloidogenic pathway and causes subsequent apoptotic death of neurons [30-32]. NGF deprivation in sympathetic neurons is reported to induce autophagy and mediate caspase independent neuronal death [33-35]. Evidence proves that differentiated PC12 cells die in response to NGF deprivation by both caspase3 dependent and independent pathways [24]. We used cellular models of neurodegeneration to study the crosstalk between autophagy and apoptosis. In corroboration with the above observations we find that withdrawal of NGF leads to upregulation of both autophagy and apoptosis in differentiated PC12 cells.

Multiple connections exist between autophagy and apoptosis, and both these processes together decide the fate of the cells [36, 37]. Both autophagy and apoptosis may occur independently through a common stimulus, sometimes the stimulus may result in a combination of both these processes, or, sometimes, each process may in an exclusive manner decide the fate of the cell [38]. We interfered with the process of autophagy to understand whether regulating it could elucidate the crosstalk between the two processes. In order to study this, two chemicals, namely Rapamycin (Autophagy inducer) and 3-Methyladenine (Autophagy inhibitor) were used after proper optimization to ensure minimal cell toxicity and proper induction or inhibition of autophagy. We find that 500 nM of rapamycin, caused induction of autophagy with least cell toxicity. Similarly in case of 3-MA, 1 μM is found to inhibit autophagy with least cell toxicity. Interestingly, survival assay revealed that inhibition of autophagy by 3-MA, rather than its induction, is protective, even under NGF deprivation conditions, which indicates that autophagy may have a role in killing cells. When we used a combination of 3-MA and pan-Caspase inhibitor and checked survival of neuronal cells against NGF deprivation and Aβ, we found significantly high protection of cells. These results led us to look into the crosstalk between these two processes at the molecular level. Recently Wirawan et al (2010) reported Caspase mediated cleavage of Beclin1 in Ba/F3 cells in response to IL3 deprivation [21]. Caspase3 is involved in cleavage of Beclin1 in HeLa cells in response to staurosporine treatment [38]. We find that Beclin1 is cleaved with time after NGF deprivation, while inhibiting Caspase3 and Caspase8 with specific inhibitors reduced cleavage of Beclin1 significantly. This led to the

conclusion that Caspase3, Caspase8 and Beclin1 might be involved in the cross talk between autophagy and apoptosis in NGF withdrawal conditions. We further observe that downregulating Beclin1 leads to significant protection of neuronally differentiated PC12 cells, primary cortical neurons upto 48 hrs of NGF deprivation or Aβ treatment.

In chemotherapy-induced apoptosis, Beclin1 is cleaved by Caspase8 thereby inhibiting autophagy [39, 40]. Therefore it appears quite relevant to investigate whether other caspases are also involved in cleaving Beclin1 in response to NGF deprivation. Further investigation into the key players involved in cleavage of Beclin1 will elucidate the complexities of the crosstalk between the two processes.

The initial discovery of Beclin-1 as a Bcl-2 binding partner [41] hinted that the Bcl-2-Beclin-1 complex may serve as a point of crosstalk between the apoptotic and autophagic signaling pathways. It was then found out that the interaction is through a BH3 domain present in Beclin1 [42, 43]. Interestingly, this interaction inhibits Beclin-1-mediated induction of autophagy in nutrient-sufficient conditions in a location-dependent manner. Although Bcl-2, Bcl-xL, and Mcl- 1 are largely localized to the mitochondria, where they serve to inhibit mitochondrial outer membrane permeabilization, only endoplasmic reticulum (ER)-localized Bcl-2 family members can inhibit starvation-induced autophagy [38, 44]. During starvation or other stress conditions, however, Bcl-2 and Bcl-xL must be displaced from Beclin-1 to permit autophagy [45]. The dissociation of this complex can be achieved through JNK mediated phosphorylation of Bcl-2, death-associated protein kinase (DAPK)-mediated phosphorylation of Beclin-1, translocation of the nuclear protein high-mobility group box 1 (HMGB1) to the cytosol, or competition with other BH3-only proteins for Bcl-2 binding [14, 46-48]. Intriguingly, while the Bcl-2/Bcl-xL-Beclin-1 interaction inhibits Beclin-1-dependent autophagy, it does not affect the anti-apoptotic function of Bcl-2 [49].

Another link between autophagy and apoptosis has been reported recently by Lee et al. (2009) [50, 51], who showed that Flip (Flice inhibitory protein), which is a prototypic inhibitor of death receptor-mediated apoptosis (and represses caspase-8 activation) can suppress autophagy. Both cellular Flip and viral Flip orthologs compete with LC3 for Atg3 binding, thereby preventing Atg3- mediated autophagosome elongation. Interestingly, mutational analyses indicated that this anti-autophagic role of Flip can be dissociated from its anti-apoptotic and nuclear factor-kB-stimulatory activities.

Therefore, as seen for Bcl-2, Flip inhibits autophagy through a direct interaction with essential autophagy-relevant proteins [50]. Conversely, Bcl-2 and Flip are two anti-apoptotic proteins that inhibit autophagy by forming complexes with Beclin 1 and Atg3, respectively.

The effector proteases of apoptosis have also been shown to inhibit autophagy through cleavage of autophagy-related proteins. The first direct evidence that apoptosis-associated proteases regulate the balance between apoptosis and autophagy was published in 2006, when Yousefi and colleagues detected calpain 1- and calpain2-mediated cleavage of Atg5 in human neutrophils undergoing spontaneous apoptosis [52]. An N terminal Atg5 cleavage product was detected in multiple cell types and found to translocate to the mitochondria, where it associated with Bcl-xL to induce cytochrome c release [52]. Furthermore, overexpression of this N-terminal Atg5 cleavage product was sufficient to cause nuclear condensation, demonstrating the ability of cleaved Atg5 to directly induce apoptosis. In contrast, the N-terminal cleavage product was not sufficient to induce autophagy [52].

Similar to the calpain mediated cleavages, caspases also followed alike strategies. The first important study that highlighted the role of caspases in mediating crosstalk between autophagy and apoptosis through the cleavage of Atg proteins was by Cho *et al*., 2009, which was followed by other researches that discovered the cleavage of Beclin-1 by caspases following either intrinsic or extrinsic apoptotic stimulation [53, 54].

Wiwaran et al showed that Beclin 1 and class III phosphatidyl inositol-3-kinase, two components of the autophagy-inducing complex, are direct substrates of caspases. The cleavage of Beclin 1 and class III phosphatidyl inositol-3-kinase by caspases was observed in response to different inducers of the intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways of apoptosis and occurred in several distinct cell types. Thus, Beclin 1 and class III phosphatidyl inositol-3-kinase are novel substrates of caspases 3, 7 and 8, highlighting a possible cross talk between autophagy and apoptosis. It was reported that the N-terminal (Beclin 1-N, aa 1–133) and C-terminal (Beclin 1-C, aa 150– 450) cleavage products of Beclin 1 failed to induce autophagy in conditions, in which the positive control, full-length Beclin 1 clearly stimulated an autophagic response. More importantly, Wirawan et al. (2010) [21] proved that Beclin 1, once cleaved by caspases, can acquire a new apoptosis-promoting function. This pro-apoptotic effect appears to be directly mediated by cleaved Beclin-1, as a C-terminal

cleavage product of Beclin- 1 translocated to the mitochondria and induced the release of cytochrome c.

Sirois *et al*. have demonstrated the potential role of caspase 3 in the regulation of maturation and release of autophagic vesicles (AVs) in human endothelial cells. Under nutrient deprivation condition, LC3 conversion increase and AVs were released with concomitant activation of caspase 3 and cleavage of PARP.

Caspase 8 is a predominant initiator caspase involved in the extrinsic apoptotic pathway and has been shown to be an important modulator of autophagy. Inhibition of caspase 8 [55], induces autophagy, which initiates the cell death program. Therefore, autophagy may act as an alternative form of PCD, where apoptosis is compromised. Cleavage of Atg3 at amino acid 166 to 169 has also been shown by caspase 8, which leads to inhibition of autophagy.

Juie Han and group in 2014 provided evidence that caspase-9 facilitates the early events leading to autophagosome formation. It forms a complex with Atg which is not a direct substrate for caspase-9 proteolytic activity and that, depending on the cellular context, Atg7 represses the apoptotic capability of caspase-9, whereas the latter enhances the Atg7-mediated formation of light chain 3-II. The repression of caspase-9 apoptotic activity is mediated by its direct interaction with Atg7, and it is not related to the autophagic function of Atg7. They proposed that the Atg7·caspase-9 complex performs a dual function of linking caspase-9 to the autophagic process while keeping in check its apoptotic activity.

6.4 References

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