**Master Thesis**

**entitled**

# Investigating the role of autophagy in mammalian neurons

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## A. Introduction

### A.1 What is autophagy?

Autophagy is an evolutionarily conserved catabolic process, which is crucial for the control and maintenance of energy balance and homeostasis across species. Cells are often required to undergo major "restoration" to cope with changing conditions or environments. During these events the cell must degrade pre-existing molecules and produce building blocks for new synthesis. Unlike the Ubiquitin-Proteasome System (UPS) which is restricted to degrading specific proteins, autophagy facilitates the recycling not only of proteins but also other macromolecules(such as lipids and nucleic acids) as well as of superfluous or damaged organelles, by delivering them to lysosomes for degradation. Therefore, autophagy is a major route via which the cell can produce new building blocks and energy to safeguard its homeostasis. Accordingly, it is not surprising that impairment of autophagy has been tightly associated with numerous pathological states in humans, including in particular neurodegenerative, metabolic and inflammatory diseases(Mizushima & Komatsu, 2011).

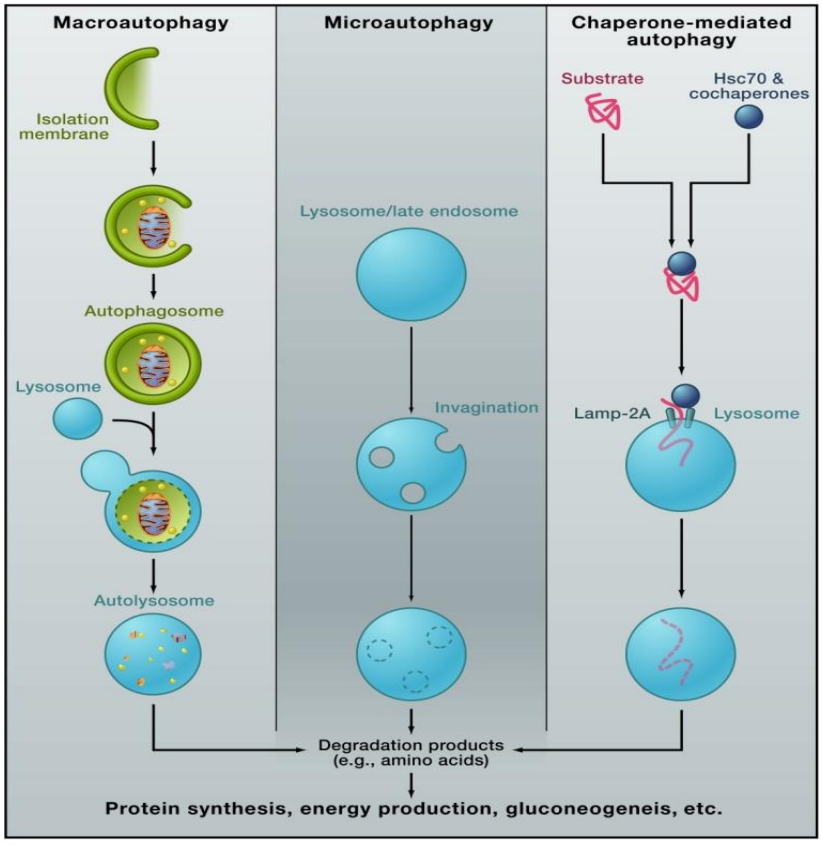



**Figure 1. The three different types of autophagy**.

There are macroautophagy, microautophagy and the chaperone-mediated autophagy. All types are involved in degradation of proteins in order to synthesize proteins, produce energy and contribute to gluconeogenesis (adapted from Mizushima& Komatsu, 2011).

There are three distinct types of autophagic degradation, which differ from each other in many ways, including the cargo they degrade, the mechanisms involved, and their regulation. These are microautophagy, chaperone-mediatedautophagy andmacroautophagy, as summarized in Figure

1.Briefly, microautophagy involves the entrapment of small molecules on the lysosome and their direct engulfment via invagination of the lysosomal membrane. In the case of chaperone-mediated autophagy, cargo proteins contain a KFERQ-like pentapeptide signal which is recognized by the cytosolic Hsc70 and co-chaperones. These chaperones lead the selected proteins to the lysosome by binding to the lysosomal protein Lamp-2A (Figure 1)(Kim & Lee, 2014; Mizushima & Komatsu, 2011). Finally, macroautophagy represents the most sophisticated mechanism, whereby cargo is recognized either by virtue of its sequence or of its post-translational modifications by specific cargo recognition proteins or adaptors and enclosed by a membrane (known as the phagophore). The phagophore undergoes nucleation and elongation, as described below, to create the double membrane autophagosome. Degradation of the autophagosomal cargo along with the inner membrane of the autophagosome occurs upon fusion with a lysosome(Kim & Lee, 2014; Mizushima & Komatsu, 2011).The work described here concerns the study of macroautophagy, which will be referred to as “autophagy” for convenience.

### A.2 Molecular mechanisms of autophagy

There are five highly conserved steps in autophagy:

1. Initiation
2. Vesicle nucleation
3. Vesicle elongation
4. Fusion with lysosome
5. Degradation.

#### A.2.1Initiation

In mammalian cells, key players in autophagic mechanism are the autophagy-related (ATG) proteins. Autophagy starts with the formation of a phagophore from the endoplasmic reticulum (ER). In many cellular conditions, the inhibition of mTOR kinase can induce autophagy, activating the ULK1 (or ATG1) complex (including Atg20, Atg24, Atg11, Atg17, Vac8, Atg13 as well as Atg1). The active ATG1 complex is delivered to the ER and activates the class III phosphatidylinositol (PtdIns) 3-kinase complex (including Ambra1, Beclin 1, Atg14(L)/barkor, Vps15 and Vps34).The activation of PtdIns3 complex signals the start of vesicle nucleation.

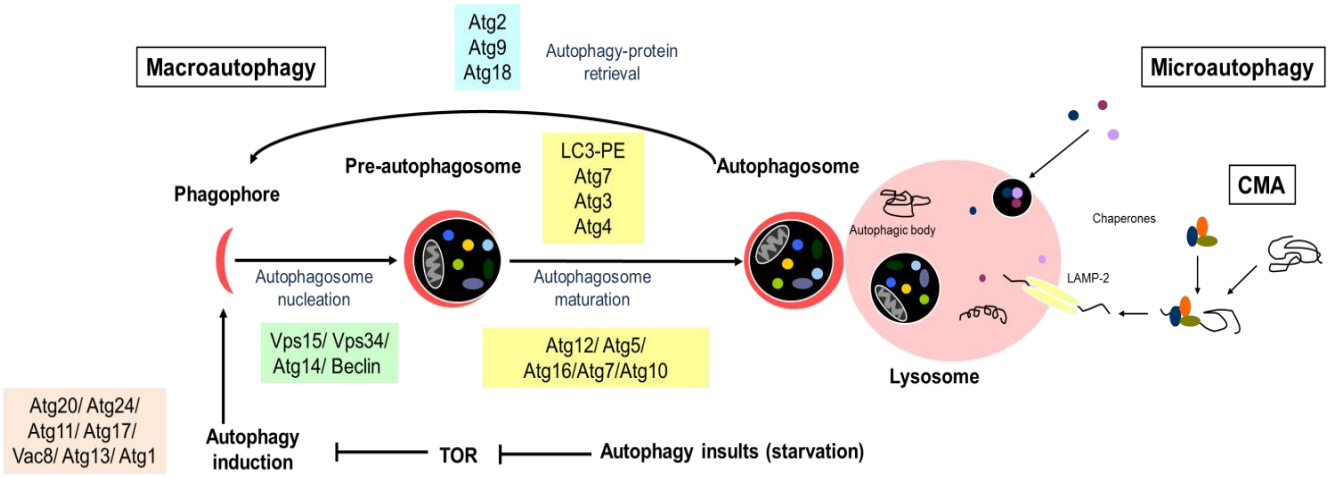



**Figure 2. The molecular mechanism of autophagy**

Upon starvation, mTOR is inhibited, hence inducing autophagy. ULK1 complex is activated, delivered to ER and activates the class III phosphatidylinositol (PtdIns) 3-kinase complex which induces the autophagosome formation helping by ATG proteins. The mature autophagosome is fused with lysosome and the autophagic cargo is degraded.

### A.2.2 Vesicle nucleation

The components of the active complex mediate a series of actions. First of all, PIP3 recruits the double FYVE-containing protein-1 (DFCP1) and WIPI (WD-repeat protein Interacting with Phospholnositides). These molecules are essential for the omegasome formation, a membrane portion of the ER that serves as a platform for the formation of the phagophore and the initiation of double membrane vesicle nucleation (Kim & Lee, 2014; Mizushima & Komatsu, 2011).Moreover, Beclin 1 can interact with BCL-2 protein familyin ER, playing an essential role in the autophagy and cell death. Under induction of autophagy, JNK1 is activated and releases Beclin 1 from the Bsc-2. The last prevents the induction of autophagy by binding in Beclin 1(Fullgrabe, Klionsky, & Joseph, 2014).

### A.2.3 Vesicle elongation

In vesicle elongation, two basic ubiquitin-like conjugated complexes are involved, (complex1:ATG12/ATG5/ATG16/ATG7/ATG10 and complex2: ATG7/ATG3/ATG4). There is one pathway whichcontributes to the covalent conjugation of ATG12 to ATG5, aided by ATG7 and ATG10 (complex2), and the non-covalent conjugation of ATG12-ATG5 with ATG16-like 1(ATG16L1). The second pathway contributes to the conjugation of phosphatidylethanolamine (PE) to LC3. Pro-

LC3 (ATG8) maturates into LC3-I by the ATG4 protease and then,LC3-I is conjugated to phosphatidylethanolamine (PE) by ATG7 and ATG3. The PE-LC3 is integrated in the pre-autophagosome and involves in creation of mature autophagosome as well as in cargo recognition (will be mentioned in detail below) (Fullgrabe et al., 2014).PE-LC3, also known asLC3-II,is used as a marker of autophagy. In addition, because of the crucial role of ATG proteins in autophagy, these proteins are used as tools for the development of mutants exhibiting impaired autophagy. In our project, we use *Atg5* deficient mice, which are known to be practically autophagy-deficient (Hara et al., 2006).

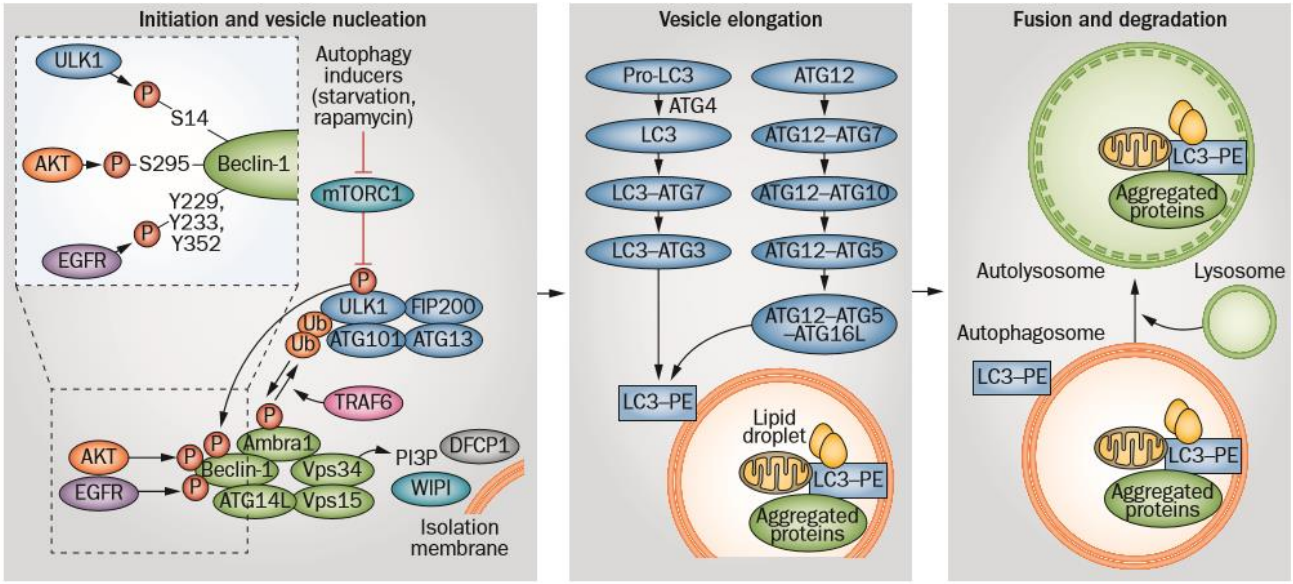



**Figure 3.The five steps of molecular autophagic mechanism.**

Graphic illustration of five steps of autophagy: Initiation, Vesicle nucleation, Vesicle elongation, Fusion and Degradation (adapted from Kim et al,. 2014).

### A.2.4. Fusion with lysosome

When the autophagosome reaches complete formation, proteins involved in autophagosome formation are released and recycled. Upon maturation, the complete autophagosome fuses with a lysosome to form an “autolysosome”.

### A.2.5. Degradation

When the autolysosome is formed, lysosomal enzymes digest the autophagic cargo. There is a wide variety of lysosomalenzymes (approximately 60 different proteins), synthesized in the ER and

containing hydrolytic enzymatic activity. In addition to the cargo, lysosomal enzymes also degrade the inner membrane of the autophagosome. Therefore, unlike the autophagosome which is a double-membraned vesicle, the autolysosome is single membrane.

### A.3 Regulation of autophagy

There are many factors inducing autophagy, most notable among which are starvation, limitation of nutrients, glucose levels, amino acids, growth factors, oxygen, energy and in general different types of stress. These are the cues for inhibition of the serine/threonine protein kisane mTORC1 (mammalian target of rapamycin complex 1) and induction of autophagy. On the other hand, the existence of sufficient amino acids and glucose activates the mTORC1 and block autophagy. There is a crosstalk between nutrients levels and autophagy via mTOR complex. However, mTOR is not the only regulator of autophagy. Below is a brief description of mTOR-dependent and independent pathways which regulate autophagy (Figure 4).

#### A.3.1 TOR-dependent signaling

Growth factor such as insulin, binding to their receptors in cell membrane, can activate the PI3KC1a/Akt/TSC/mTORC1 pathway, causing activation of mTOR and inhibition of autophagy. Therefore, nutrient limitation and the ensuing decrease of insulin can induce autophagy by mTOR (Sarkar et al, 2013). Moreover, PI3KC1a/Akt/TSC/mTORC1 pathway and especially Akt can phosphorylate the FOXO3 transcription factor, hence inactivating it. This transcription factor has crucial role of triggering apoptosis and autophagy. Foxo3 normally activates autophagy, therefore in presence of growth factors, FOXO3is inactivated by Akt, thus inhibiting autophagy (Altman et al, 2016). Finally, it is known that stress signals decrease the level of nitric acid (NO) which blocks autophagosome synthesis. Generally, NO decreases AMP activity, the last increases mTOR activity and as a result autophagy is blocked(Park et al., 2016). Moreover, NO inhibits Jun-N-terminal kinase (JNK) which activates autophagy (C. Shen, Yan, Erkocak, Zheng, & Chen, 2014). Therefore, under stress conditions, NO reduces autophagy via two distinct pathways.

#### A.3.2 TOR-independent signaling

There are four basic pathways, the inositol signaling, the calcium/calpain pathway, the cAMP and the JNK signaling pathways which regulate autophagy in mTOR-independent manner. Firstly, activation of phosphoinositide phospholipase Cγ (PLCγ) leads to the production of Inositol

trisphosphate (IP3) and diacylglycerol (DAG). IP3 provokes the release of calcium from ER and blocks autophagy (Parys, Decuypere, & Bultynck, 2012). On the other hand, intracellular changes of calcium can regulate autophagy. The increase of calcium activates calpains which are calcium dependent cystein proteases. These block autophagosome formation as well as the fusion between autophagosome and lysosome (Kuro et al., 2011). Furthermore, cyclic adenosine monophosphate (cAMP) has an unclear role in regulation of autophagy. On the one hand, increase of cAMP increases autophagy (Ugland, Naderi, Brech, Collas, & Blomhoff, 2011),on the other hand increase of cAMP inhibits autophagy by blocking the formation of phagophore(Noda & Ohsumi, 1998; Sarkar, 2013). Finally, Jun-N-terminal kinase (JNK) activates autophagy independently of TOR (Zhou, Li, Jiang, & Zhou, 2015). Upon starvation, JNK is activated and phosphorylatesBeclin 1, hence inducing autophagy.

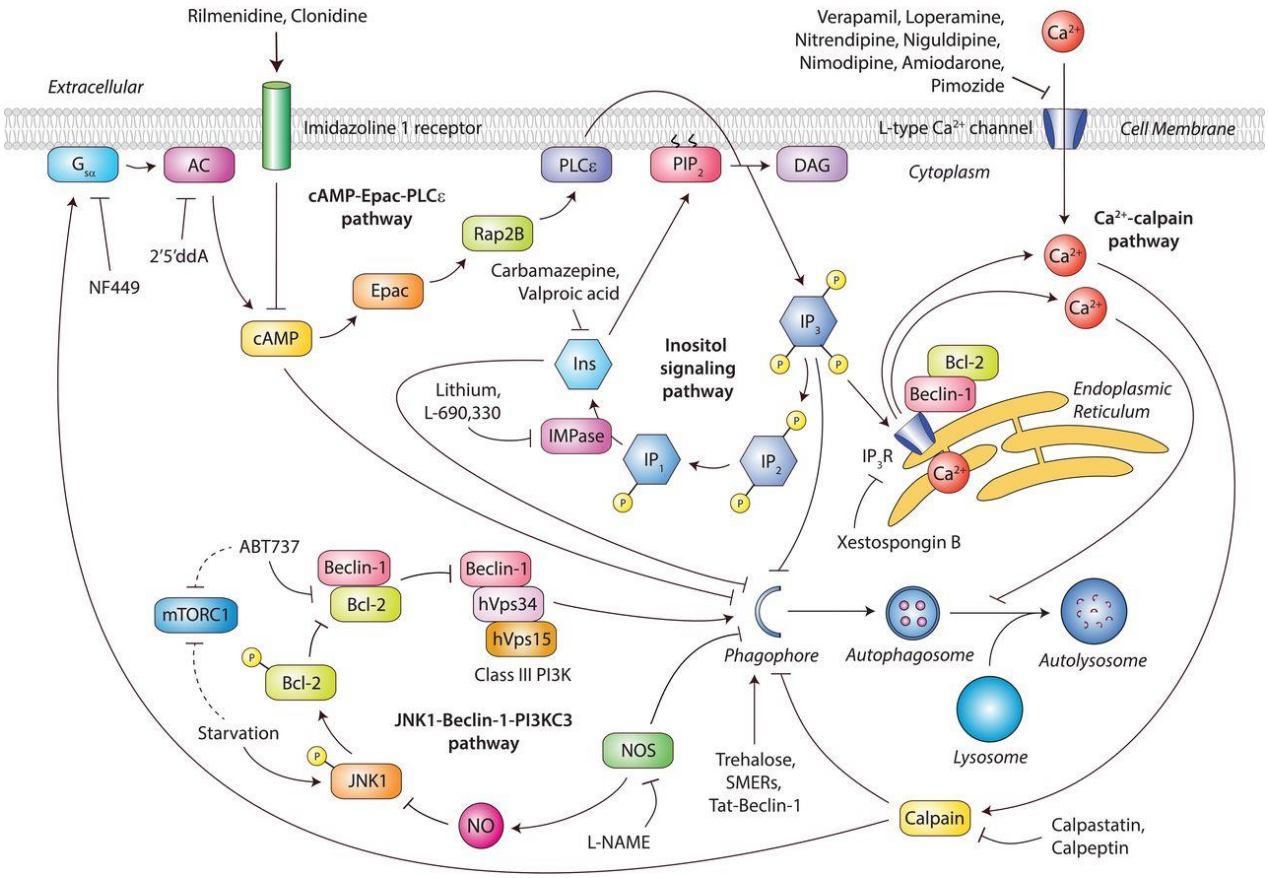



**Figure 4. Regulation of autophagy by TOR-independent pathways**

Graphic illustration showing the inositol signaling, the calcium/calpain pathway, the cAMP and the JNK signaling pathways regulating autophagy (adapted from Sarkar 2013)

### A.4 Cargo recognition

For years, autophagy was considered a bulk process, but now it is clear that autophagy has a high degree of selectivity (Stolz, Ernst, & Dikic, 2014). The most direct way of cargo recognition is mediated by an LC3-interacting region (LIR) motif on autophagic protein substrates, which is identified by LC3-II on the autophagosomal membrane. This LIR motif is characterized by sequences WXXI/L/V (X: any amino acid) (Birgisdottir, Lamark, & Johansen, 2013). A second, more indirect, manner of cargo recognition involves the use of autophagic adaptors, such as p62 (also known as sequestosome-1/SQSTM1), optineurin, NDP52, NBR1 and Alfy. p62 is by far the best studied autophagic adaptor. p62 interacts with LC3 via its LIR domain, while it recruits proteins modified by K63-linked poly-ubiquitin chains or mono-ubiquitination via its UBA domain, hence bridging them to the autophagosome. Thus, ubiquitinilation is used as a general signal for destruction. It is well-documented that in conditions of reduced autophagic activity, p62 is accumulated and it forms aggregates with ubiquitin (Komatsu et al., 2007).These aggregates have been also observed in neurodegenerative disorders as Alzheimer's (Mizushima & Komatsu, 2011).

### A.5 Autophagy in neurons

Genetic studies have firmly established that prolonged autophagy impairment results in progressive neurodegeneration in mice. In particular, mice with conditional deletion of *Atg5* in the neural lineage (obtained by crossing $Atg5^{f/f}$ and *Nestin-Cre* animals) develop widespread neurodegeneration and progressive motor deficit .These defects are accompanied by accumulation of protein aggregates and inclusion bodies in neurons (Hara et al.,2006) This observation suggests that the basal autophagy is essential for neuronal integrity. Similarly, mice with conditional deletion of *Atg7* in the neural lineage display comparable phenotypes(Komatsu et al., 2007). In our project, we use the conditional *Atg5* deficient mice for the neuronal lineage.

### A.6 Crosstalk between autophagy and apoptosis

There are numerous studies suggesting that autophagy, apoptosis and necrosis share common pathways and that there is interplay among them. Apoptosis is a process of programmed cell death which is observed in physiological conditions while necrosis is caused by external factors such as trauma, infection, toxins etc. Apoptosis is useful and beneficial for the organism and helps in maintenance of balance while necrosis is harmful and leads to inflammation. Furthermore,

apoptosis can be triggered by cell surface death receptor, such as tumor necrosis factor-α (TNFa), Fas (CD95/APO1) and TNF-related apoptosis-inducing ligand (TRAIL) receptors or mitochondrial signaling. Apoptosis is associated with activation of cysteine aspartyl proteases, called caspases, which provoke mitochondrial membrane permeabilization, chromatin condensation and DNA fragmentation(Nikoletopoulou, Markaki, Palikaras, & Tavernarakis, 2013). These are some basic differences between apoptosis and necrosis while there are apparent morphological differences. For instance, in apoptosis apoptotic bodies are created, the cell is shrinking, the cell membrane has bulge and the nuclear collapses. The last means chromosomal DNA fragmentations, nuclear fragmentations and chromatin condensation. These events create a characteristic morphology in these cells. On the other hand, in necrosis, cells are swelled and ruptured, the cell membranes are disrupted and hypoxia, ATP depletion and inflammation are observed (Nikoletopoulou et al., 2013).

Apoptosis, as mentioned above, is a caspase-dependent mechanism, while necrosis is caspase-independent (Nikoletopoulou et al, 2013). Furthermore, there is a third kind of programmed necrotic death, necroptosis which is calpain-dependent and is observed in neurodegenerative diseases and ischemia.

There is a strong connection between autophagy and cell death. Numerous studies in different contexts, either in diseases or during normal development present strong evidence for this interplay. Both apoptosis and autophagy share common pathways and are activated by similar cues. For instance, metabolic stress, starvation, elimination of growth factor and nutrients can induce autophagy and promote cell survival via AMPK pathway. This pathway influences the cyclin-dependent kinase inhibitor $p27^{kip1}$(or CDKN1B) which can block or slow down the division of the cells. In mutant of this kinase is observed increased apoptosis (Liang et al., 2007). Furthermore, ER stress can influence both autophagy and apoptosisin a tissue-dependent manner (Nikoletopoulou et al., 2013).For this reason, it is so difficult to be clear whether they cooperate or antagonize.

Moreover, the role of mTOR in autophagy is well established but now it is investigated whether it acts as an inhibitor or an enhancer of apoptosis. mTOR influences the tumor protein p53 (tumor suppressor), BAD and BcL-2 proteins which have a pro-apoptotic role (Castedo, Ferri, & Kroemer, 2002). As described earlier, Beclin 1 interacts with the anti-apoptotic protein, Bcl-2. However ,Beclin 1 cannot promote apoptosis by modulating the anti-apoptotic effect of Bcl-2 (Ciechomska, Goemans, Skepper, & Tolkovsky, 2009).

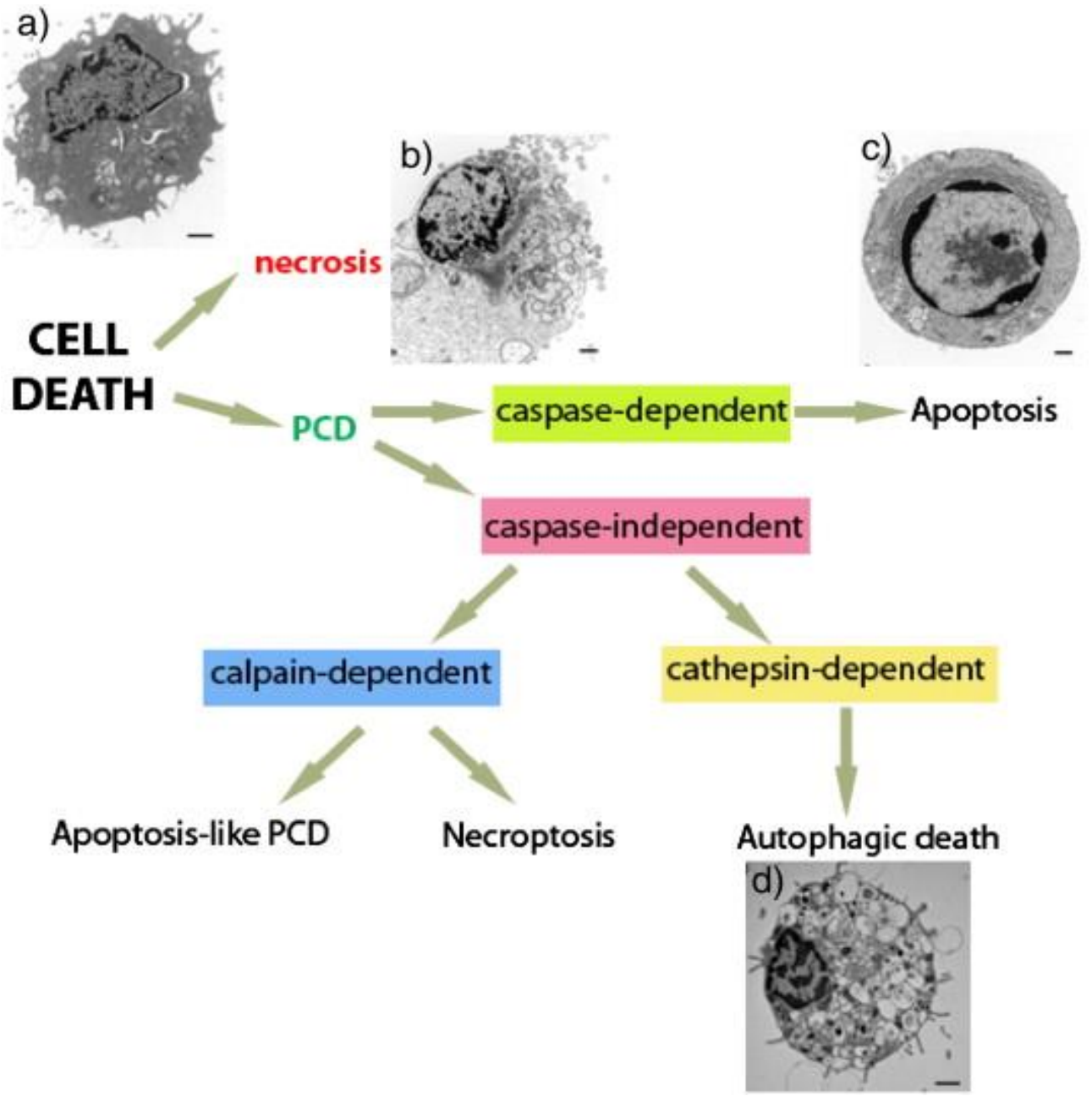



**Figure 5. The different types of cell death and their morphological characteristics**

a) a healthy cell, b) a necrotic cell, c) an apoptotic cell and d) an autophagic cell (adapted from Nikoletopoulou et al., 2013)

Furthermore, extrinsic signaling as FASL (tumor necrosis factor), TRAIL (or death receptor 5), and TNF (Tumor Necrosis Factor) can induce cell death. These receptors activate the FADD (FAS Associated Death Domain) which in turn activates caspase-8. The last, activates caspase-7 and caspase-3 and promotes apoptosis. In addition, there is an intrinsic pathway in which BH-3-only proteins are involved in apoptotic events. These proteins can provoke DNA damage, ER stress and activation of BAX/BAK (pro-apoptotic proteins) in outer mitochondrial membrane (MOM). These proteins form pores in MOM, permeabilize the mitochondria and release BAX/BAK proteins. These can block autophagy (Nikoletopoulou et al, 2007).

Moreover, caspases can also mediate the cleavage of Beclin 1 and PI3K and as a result Beclin 1 loses its autophagic activity. Beclin 1 is also localized in mitochondria and cleavage of its C-terminus increases apoptosis (Wirawan et al., 2010). Finally, there is a connection of Beclin 1 and Bax which is an apoptotic protein (Germain et al., 2011). Caspases are also correlated with autophagy. Caspase-3 cleaves ATG4D (a protease which cleaves pro-LC3 to LC3) and promotes cell survival under starvation conditions. However, overexpression of cleaved ATG4D can increase apoptosis. Caspases cleave ATG5 protein and this truncated form induces apoptosis by mitochondrial pathway (Yousefi et al., 2006). To sum up, caspases increase autophagic activity via ATG4D while they turn off autophagy, inducing apoptosis via Beclin 1 and ATG5. Finally, autophagy has an influence on caspases. Especially, autophagy blocks the activation of caspase 8 and thus blocking the activation of caspase 3 (Figure 6) (Nikoletopoulou et al, 2013).

In conclusion, there is an indisputable interplay between apoptosis and autophagy, however it is not absolutely clear. This interplay depends for the cellular context.

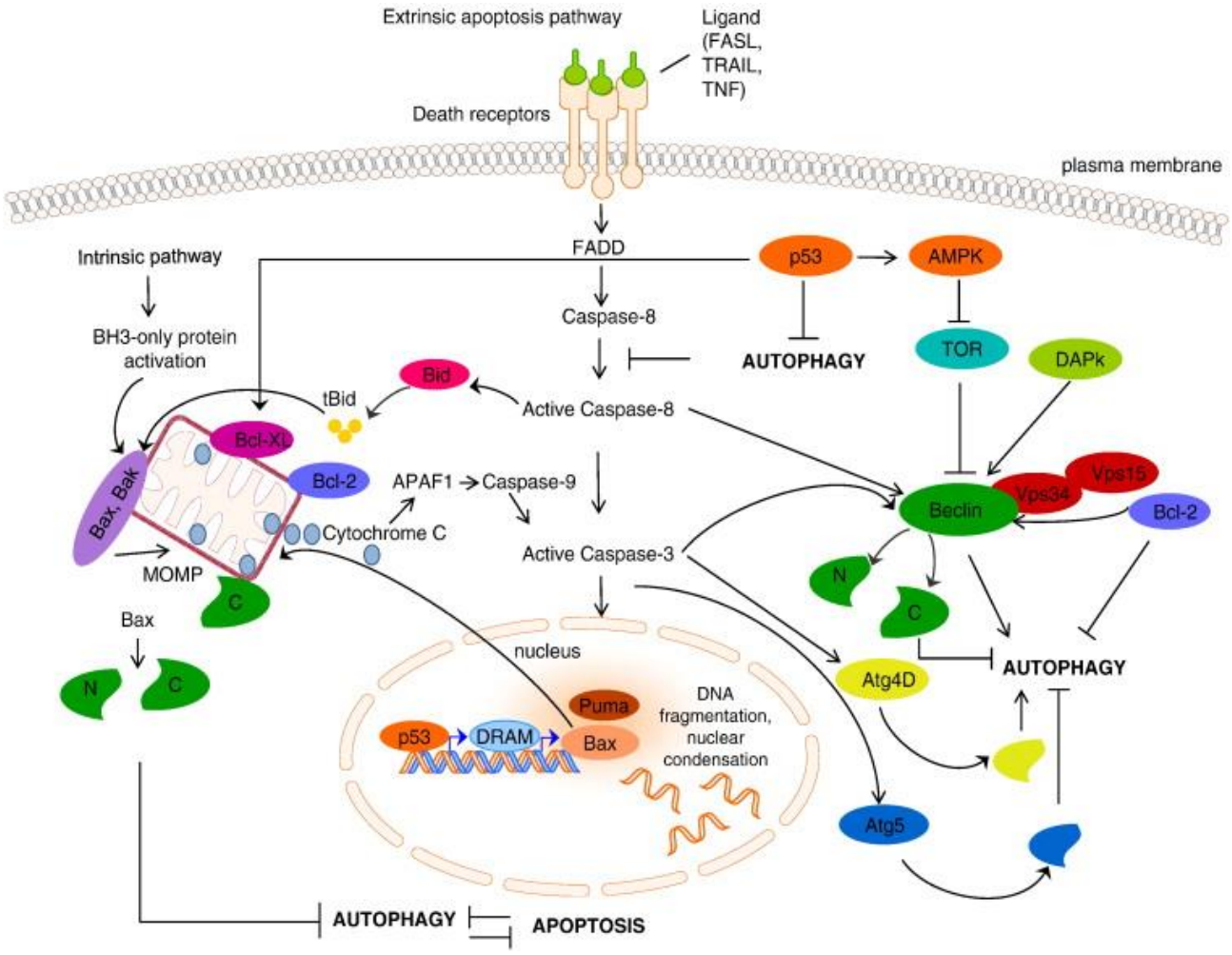



**Figure 6. Molecules which are involved in the interplay between autophagy and apoptosis**

It is illustrated in the figure the death receptor with their ligands, such as FASL, TRAIL or TNF. These can activate the FADD which activate caspase-8. The active caspase-8 cleaves the caspace 7 and 3, therefore promoting apoptosis. Moreover, stress can active BH3-only proteins and the last activate the BAX/BAK at MOM. The MOM is permeabilized and apoptotic molecules such as cytochrome c are released. Cytochrome c activates caspase-9,whch is cleaved and activate caspase 3 and 7. That was the apoptotic pathway, but caspase 3 can influence the autophagy. Caspase 3 cleave Beclin 1 and ATG5, thus blocking autophagy. Moreover, this caspase cleaves the ATG4D, hence inducing autophagy. Interaction between Beclin 1 and Bcl-2 is also crucial for this interplay. When Beclin 1 released from Bcl-2 induces autophagy. In addition, P53 is an essential molecule in order to upregulate pro-apoptotic proteins such as BAX, PUMA and BID and induce autophagy inhibiting mTOR (adapted fromNikoletopoulou et al., 2013).

### A.7 ThePeripheral Nervous System (PNS)

The most essential difference between neurons of the central nervous system (CNS) and of the peripheral nervous system (PNS) is their origin. CNS neurons arise from the neuroepithilium while PNS from the neural crest. In the first embryonic days (E2-E4) three brain vesicles are created (proencephalon, mesencephalon and rhombencephalon-see Figure 7A) where cells are divided rapidly and additional vesicles are formed. Each vesicle will form the regions of the mature CNS (forebrain, midbrain and hindbrain).

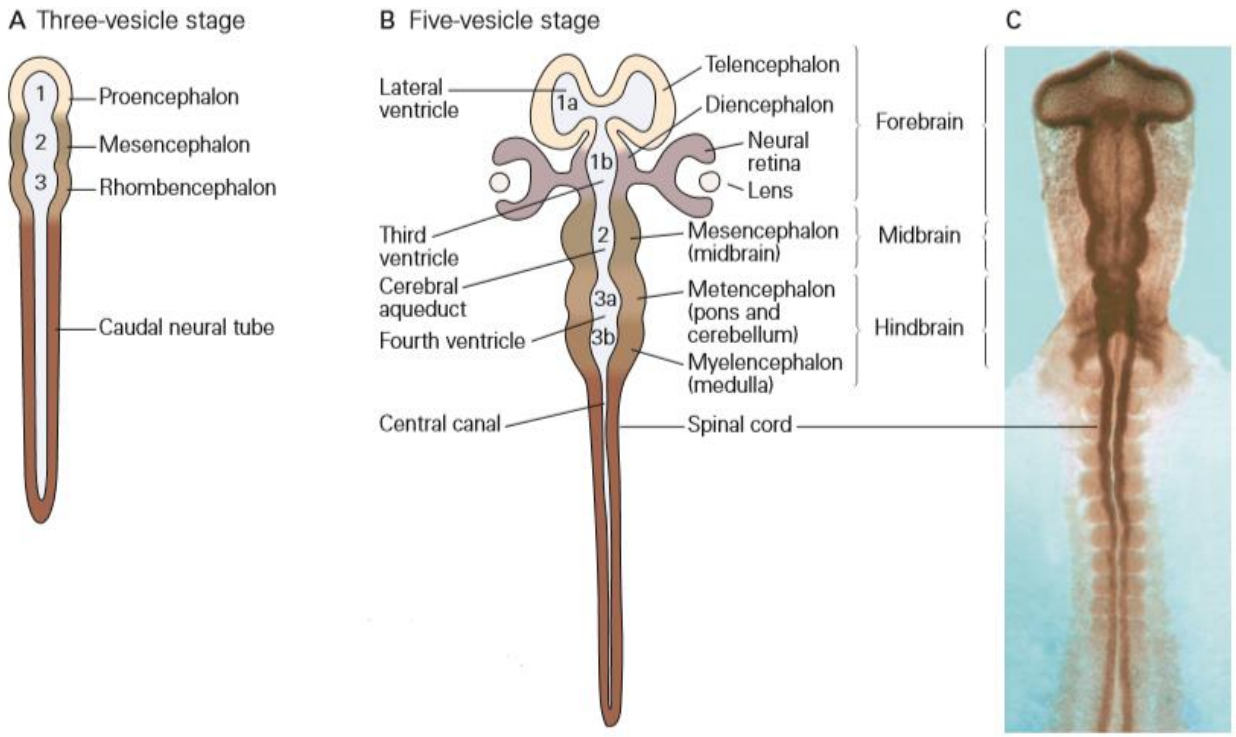



**Figure 7. Stages of neural tube in development**

A) The first days of development, the three-vesicles are created. B) The three vesicles are developed in five vesicles and create the brain. C. Chicken neural tube (adapted from Principles of Neural Science, 5th Edition)

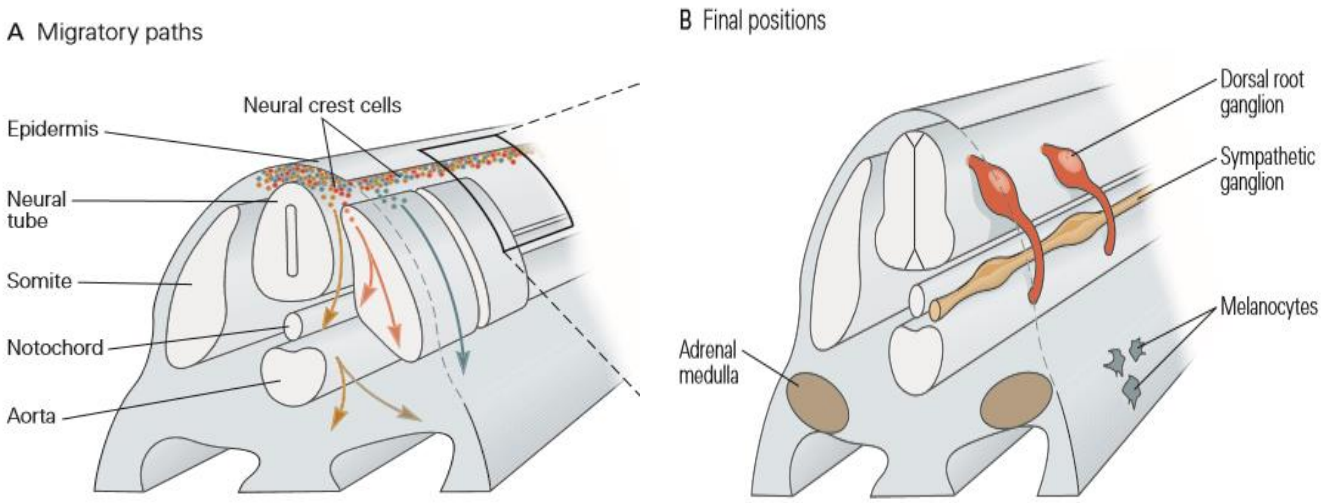



**Figure 8. Migration of neural crest cells to the peripheral nervous system**

A).Main pathways of neural crest cells. B).The final positions of these cells after migration and differentiation (adapted from Principles of Neural Science, 5th Edition)

By contrast, PNS neurons are derived from neural crest stem cells, which are a group of cells that delaminate from the neural tube, and specifically between the dorsal side of neural tube cells and epidermal ectoderm (see Figure 8A). After induction by specific cues, neural crest stem cells are transformed to mesenchymal cells, delaminate from neural tube and migrate freely. These cells do not use scaffold proteins as CNS neurons do, therefore this migration is called free migration and is based on cell adhesive changes. In this process, there are many significant adhesive molecules such as BMP (Bone Morphogenetic Proteins), Wnt signals, ephrins protein and many others.

In Figure 8A, neural crest cells, neural tube, epidermis and the different migratory pathways are illustrated while in Figure 8B the final positions of sympathetic ganglia, dorsal root ganglia (DRGs), adrenal medulla and

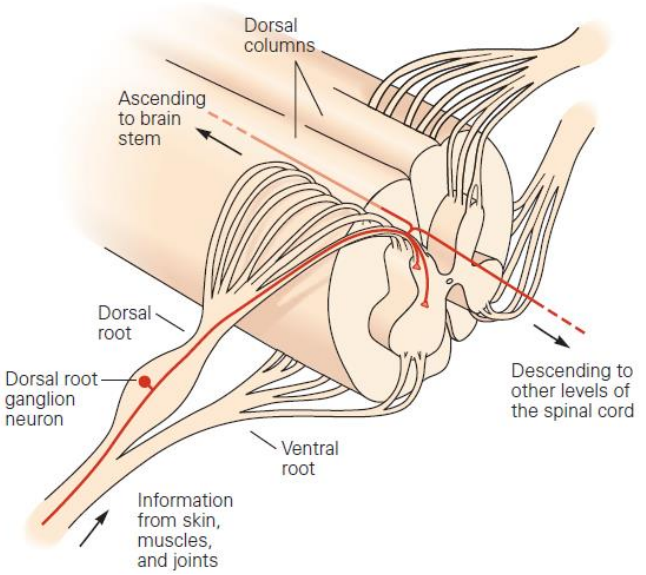



**Figure 9. Dorsal root ganglia and different spinal nerve roots**

DRGs, dorsal root, ventral root and spinal cord are illustrated (adapted from Principles of Neural Science, 5th Edition)

melanocytes which have origin from neural crest cells.

Notably, we will focus on DRGs which mediate communication of the brain with the rest of the body, skin, musclesand so on. DRGs include the cell bodies of neurons which integrate the sensory information from the peripheral body. The axons of DRGs neurons are split into peripheral and the central branches. The last enters in the dorsal horn of the spinal cord (Figure 9).

The neuronal fate of these cells depends on the expression of specific transcription factors. Numerous studies indicate the powerful factors which determine the neuronal fate. In Figure 10, there is a brief overview of these. Focus is given on sensory neurons, neurogenin 1 and 2 (Ngn1 and Ngn2) which belong to bHLH proteins and can produce precursor sensory neurons. Runt-related transcription factor, Runx1 and Runx3 as well as the tyrosine kinase receptor, TrkA and TrkC can transform these precursors to nociceptive and proprioceptive neurons.

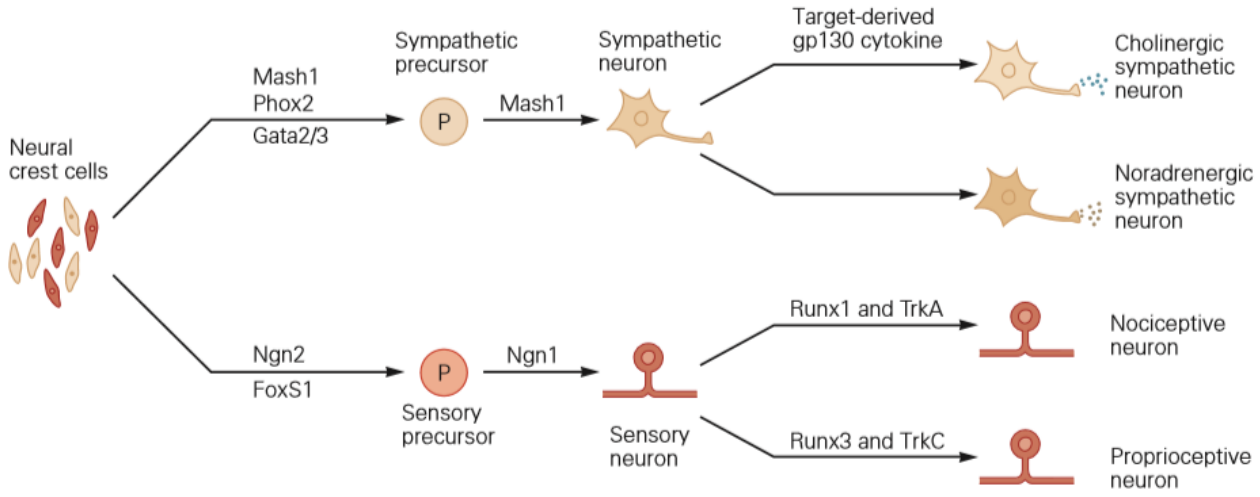



**Figure 10. Neural fate determined by transcription factors**

Neural crest cells are differentiated by transcription factors to sensory and sympathetic neurons. Ngn1 and 2 participate in the development of sensory neurons while Runx1 and 3 in the development of nociceptive and propioceptive neurons respectively (adapted from Principles of Neural Science, 5th Edition).

One other important characteristic of PNS neurons is the overproduction of these and their selection. During development, a far greater number of peripheral neurons are produced and later via programmed cell death this overproduction is corrected. In this mechanism Nerve Growth Factor (NGF) and the other neurotrophins have a crucial role. Rita Levi-Montalcini who has been honored for this work with the Nobel Prize, discovered NGF and established the Neurotrophic

Hypothesis. Peripheral neurons extend their axons to target cells, which secrete low levels of NGF. Neurons receiving more of this factor, will survive, while those that do not die through a programmed cell death. This death can determine the connections between cells in PNS. Remarkably, this is the system in which apoptosis has a decisive role under physiological conditions.

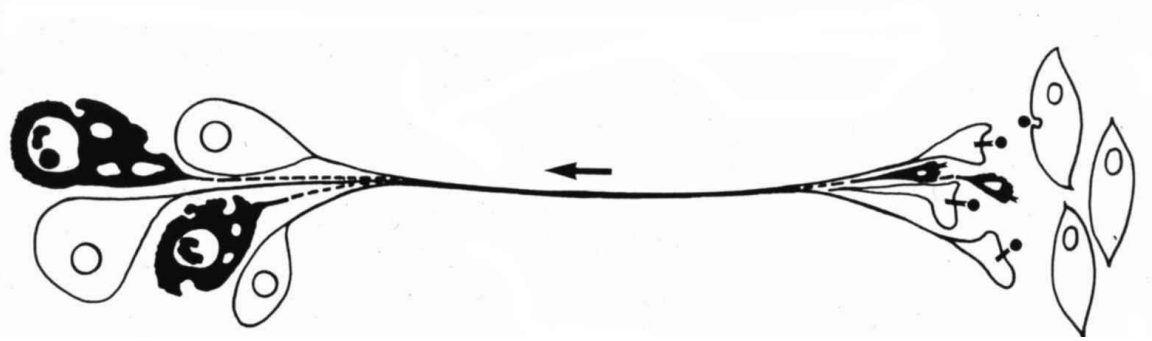


**Figure 11: Neurotrophic Hypothesis**

Neurons extend their axons to target cells, which secret low level of NGF. These receiving more will survive, while the others die (adapted from Bibel and Barde, 2000 genes and Dev)

Based on this hypothesis, a new field develops and neurotrophins and their receptors are studied on a great scale. There are four basic neurotrophic factors: NGF, NT3, BDNF and NT4. These can bind to p75 death receptor (p75NTR) and this binding provokes axon pruning and neuronal cell death. Same neurotrophins have also their 'unique' receptor in which they bind with higher affinity. The couples are illustrated in Figure 12. NGF binds to TrkA, NT3 to TrkC and BDNF and NT4 to TrkB. Trk receptors provokes the neuronal survival and growth. I would like to focus on their role in the control of the development of specific subpopulations in sensory neurons.

The action of neurotrophins establishes the three basic modalities in sensory neurons. NGF is essential for the survival of small neurons with unmyelinated or thinly myelinated axons and for nociceptive neurons general. In particular, NGF mouse deficient have severe pain insensitivity (Capsoni et al., 2011). Notably, TrkA receptor is the major receptor expressed in DRGs. BDNF affects the development of mechano-receptors while NT4/5 affects the deep hair cell afferents. Finally, NT3 is important for DRGs neurons survival early in development and affects the thickly myelinated axons which innervate muscles spindles (Bibel & Barde, 2000) (Figure 13).

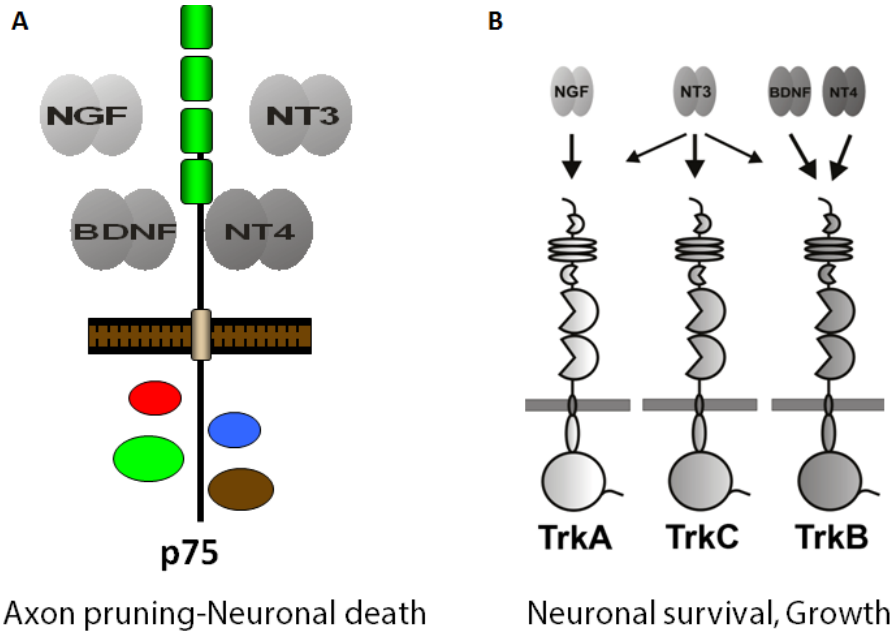



**Figure 12. Diverse functions of neurotrophins**

A) NGF, NT3, BDNF and NT4 bind to p75. B) NGF binds to TrkA, NT3 to TrkC and BDNF and NT4 to TrkB.

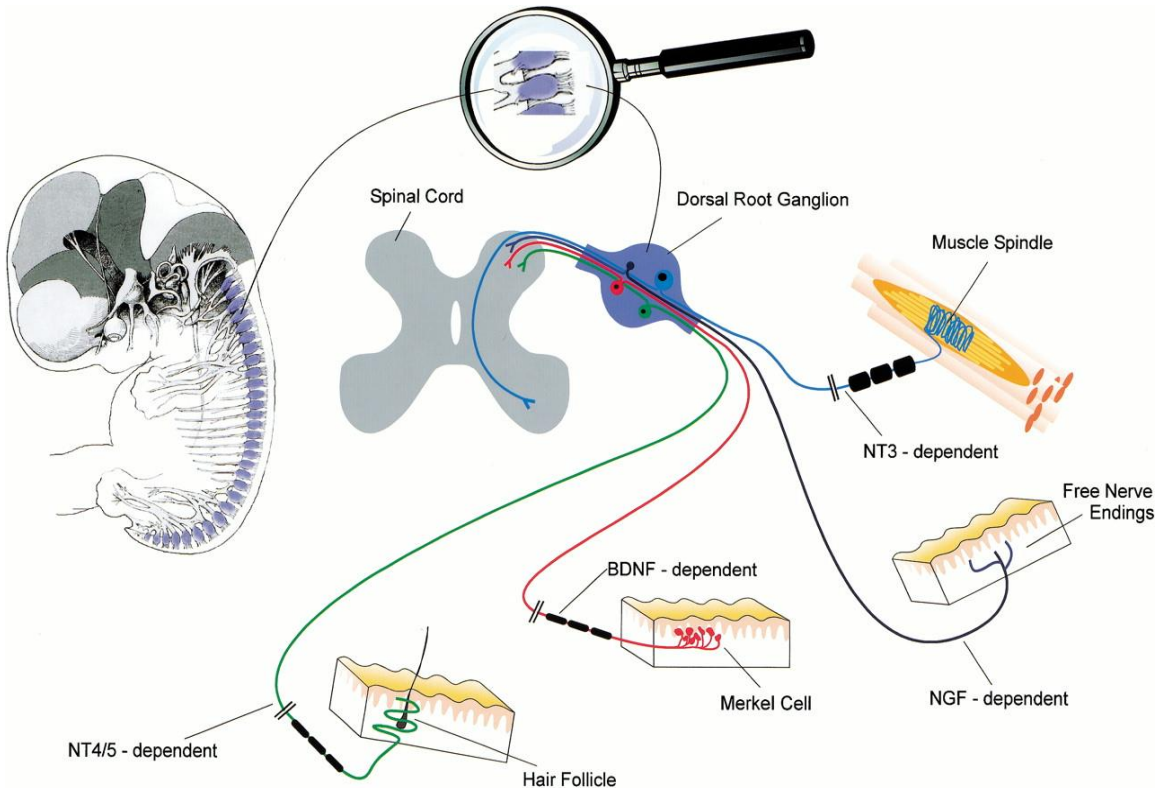



**Figure 13. Three seonsory modalities in DRGs mediated by neurotrophic factors**

BDNF, NGF, NT3, and NT4/5 establishe the three basic modalities, nociception, proprioception and mechano-reception (adapted from Bibel&Barde, 2000).

Besides these, Ret receptor in DRGs, a tyrosine kinase receptor of GDNF (Glial cell-derived neurotrophic factor), transduces signals for glial cells. This receptor defines an additional subpopulation of DRGs. In Figure 14, Runx1 transcription factor influences Ret expression and many other ion channels and receptors (TRP class thermal receptors, $Na^+$ channels, ATP-gated, and $H^+$ gated channels, the opioid receptor MOR, and Mrgpr class G protein coupled receptors) which are important for the specification of these neurons(Kramer et al., 2006). However, it is not clear which sensory modalities they transduce. Besides that, there are some specific peptides and proteins which influence the threshold of nociceptor activation. For instance, bradykinin, substance P, nerve growth factor (NGF), ATP, histamine, serotonin, prostaglandin, leukotrienes and acetylcholine are some examples (Principles of Neural Science, 5th Edition).

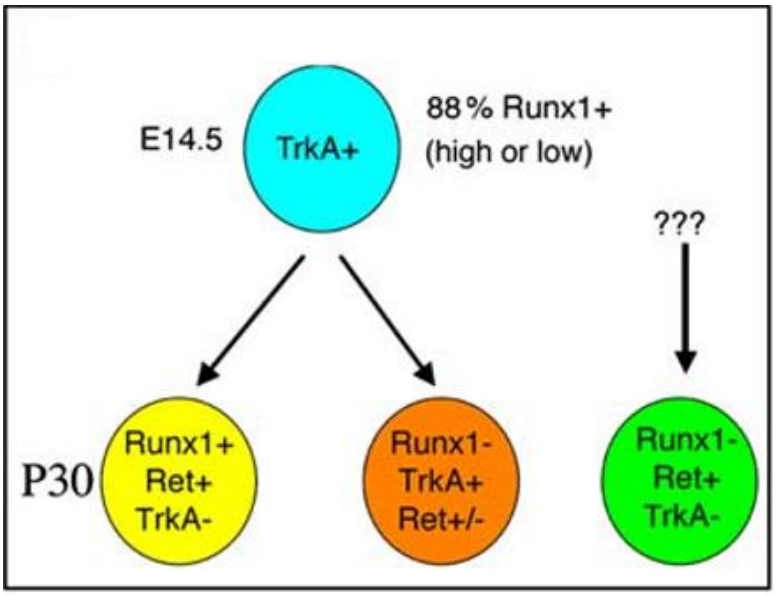


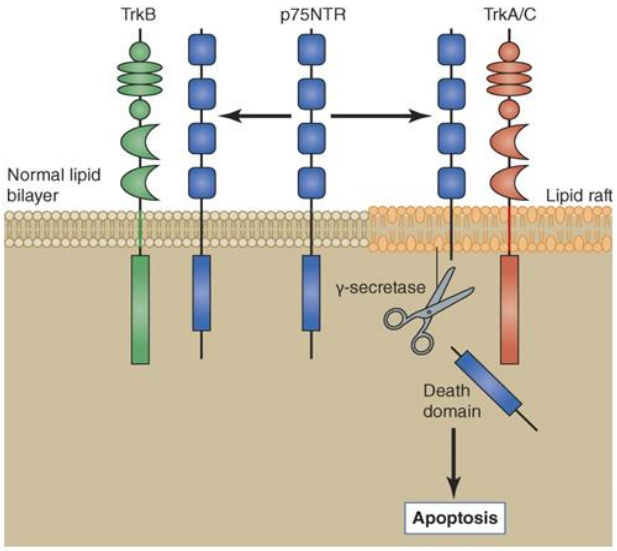

**Figure 14. Expression pattern of TrkA, Runx1 and Ret.**

Runx1 expresses in E14.5TrkA+ neurons and Ret receptor expression (adapted from Kramer et al., 2006)

Firstly, the cells are chosen by cell death and later in development all these receptors and proteins give them a specification. Cell death is defined by p75 while survival by Trk receptors, as mentioned earlier. However, Dr. Nikoletopoulou proved that TrkA can act as a dependence receptor, thus inducing cell death. Notably, TrkA and TrkCcan associate with p75 and then γ-secretase-proteases cut the intracellular death domain of p75 inducing apoptosis (Bibel, Hoppe, & Barde, 1999; Nikoletopoulou et al., 2010) (Figure 15). Moreover, Patel and their colleagues showed that apoptosis inducing by TrkA is Bax dependent in DRGs (Patel, Jackman, Rice, Kucera, & Snider, 2000) (Figure 15). For this reason the double mutant of Bax and TrkA presents apoptosis similar to the wild type, while TrkA mutant has reduced apoptosis (Figure 15)(Patel et al., 2000)

**A**

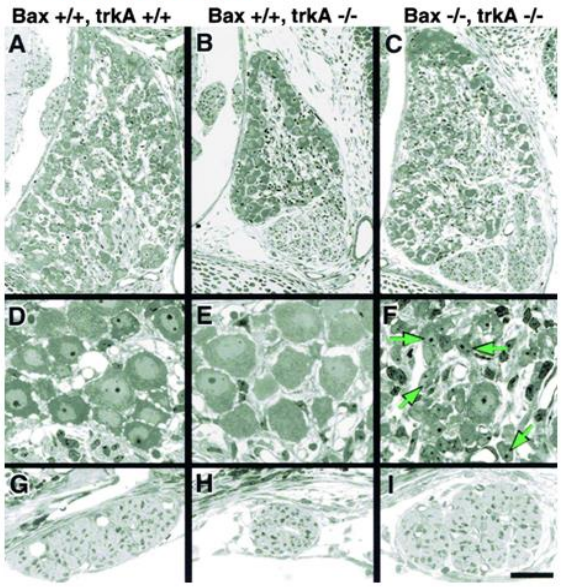
TrkA acts as a **dependence** receptor: it causes cell death in the absence of NGF




(Nikoletopoulou et al., 2011)

**B**

Death of nociceptive (TrkA+) neurons is apoptotic and **Bax-dependent**




(Patel et al., 2000)

**Figure 15. Cell death inducing by TrkA and Bax dependent**

A) TrkA acts as a dependence receptor B) TrkA$^{+}$ neurons die via a Bax dependent way ( adapted from Nikoletopoulou et al., 2013, Patel et al., 2000)

## B. Hypotheses

### B.1 Hypothesis 1: **Does autophagy play a role in death or survival decisions in the PNS during development?**

The aforementioned work highlights the numerous examples where autophagy has a modulatory role in cell death paradigms, most of which are associated with pathologies. They also raise the question whether autophagy is essential for the modulation of cell death processes in physiologically relevant conditions. One of the best examples of naturally occurring cell death is the apoptosis of a large proportion of peripheral neurons during embryogenesis. Our work investigates the interplay of autophagy and apoptosis in this physiological paradigm, which determines the correct number of peripheral neurons to match the needs of their targets in the periphery.

### B.2 Hypothesis 2: **Autophagy has different roles in CNS neurons**

In the second part of my work, we investigate the different role of autophagy in the CNS and examine its contribution in synaptic function.

## C. Results

### C.1 PNS

#### C.1.1 Increasing autophagy during development with pharmacological tools

We set out to investigate the effect of increasing autophagy during development on PNS neurons. To this end, we used a pharmacological tool, Torin2, which has been shown to increase autophagy by selectively inhibiting mTORC1.Torin2was administrated by intravenous injections in pregnant mothers at 11.5, 13.5 and 16.5 *post coitum* and the embryos were analyzed at E17.5. Immunofluorescence was performed on horizontal section of the lumbar region of the spinal cord. Staining with a nuclear dye (Hoechst) revealed that Torin 2 treatment caused severe morphological defects (Figure 16). Even at very low doses (3mg/kg), Torin 2 severely impacted the physiological development of embryos and resulted in the shrinkage of the spinal cord, most prominently of ventral structures, such as the ventral horns which were practically eliminated. A probable explanation of this effect is that inhibiting mTOR signaling also blocks protein translation which is important for development. Immunostaining with antibodies against p75NTR and TrkA revealed in reduced expression of these receptors in Torin2-treated embryos compared to controls (Figure 17-18), which was also confirmed by western blot analysis with a p75NTR antibody in lysates of lumbar DRGs. However, given the gross morphological defects caused by Torin2, these findings are difficult to interpret. More generally, these studies indicated that pharmacological tools are not appropriate for studying autophagy during development. Instead, we focused the rest of the work on loss of function paradigms and particularly the analysis of a conditional mutant of autophagy, as described below.

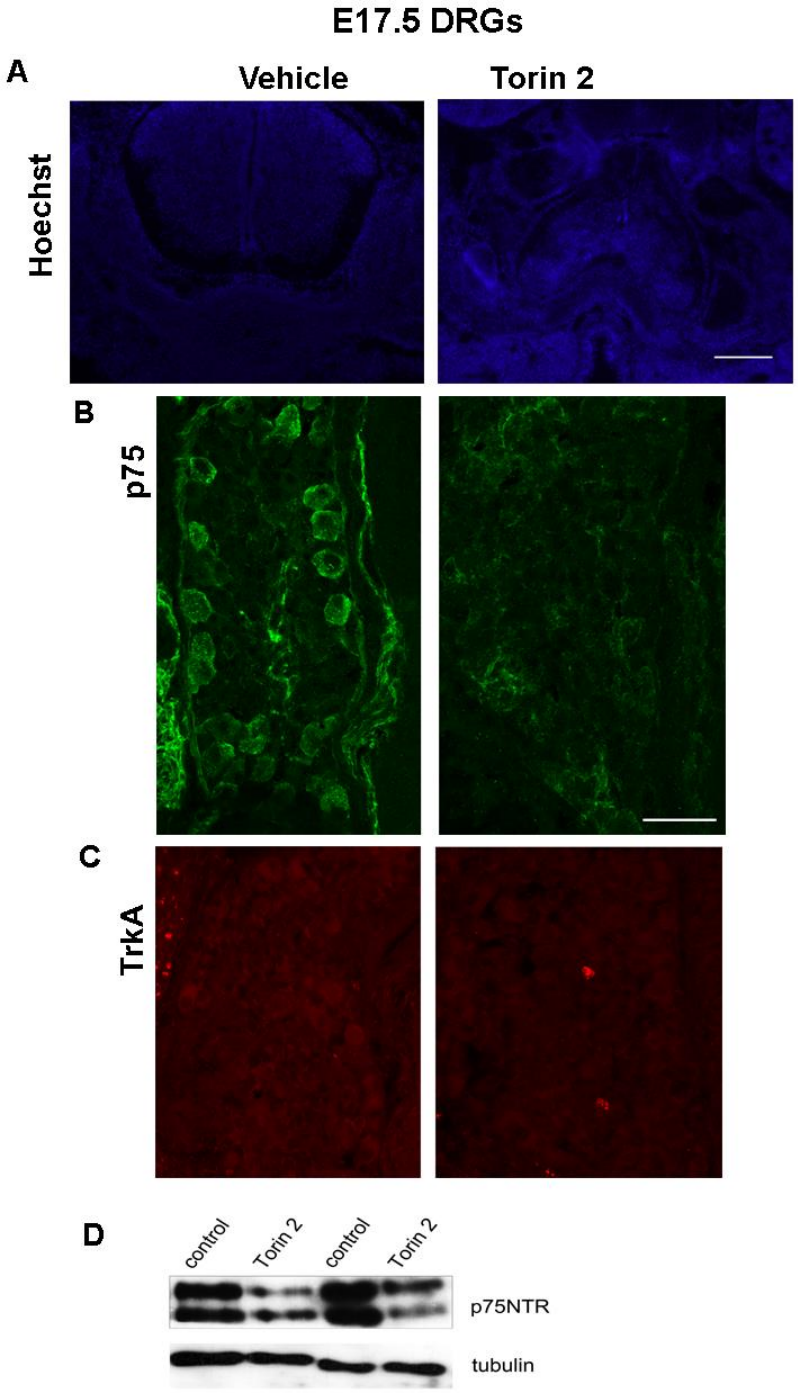



**Figure 16. DRGs at E17.5 after administration of Torin 2**

A) Administration of Torin 2 in embryos during development provokes morphological defects in DRGs and spinal cord. Hoechst immunofluorescencestaining are showed from dissected embryos B)Immunofluorescence staining of p75 C) Immunofluorescence staining of TrkA and D) Western blot of decreased p75 in DRG. Scale bars: (A), 200 µm; (B-C), 100 µm

### C.1.2 Generation of conditional autophagy-deficient animals

We next sought to analyze mice with conditional deletion of *Atg5* in the neuronal lineage. These mice were generated by crossing*Atg5*$^{f/f}$ with *Nestin-Cre* deleter and result in the ablation of*Atg5* in all neurons and glia, with the exception of microglia that have a mesodermal origin (Hara et al., 2006). *Nestin cre;Atg5*$^{f/f}$ mice were born normally and survived, but they have growth retardation (1.5 times lower weight). Furthermore, these mice develop progressive neurodegeneration and motor and behavioral deficits after P21 (postnatal) such as ataxic walking pattern and they cannot extent their limbs. Finally, a tremor is observed in three months old mice (Hara et al., 2006).

### C.1.3 Autophagy deficiency in the neuronal lineage prevents developmental death of peripheral neurons

These conditional mutants were used in order to investigate whether there is a crosstalk between autophagy and apoptosis in DRGs during development. Embryos were dissected at embryonic day E13.5, E17.5 and the first day of birth (PO) (see methods) and sections from lumbar region were analyzed. Assessment of nuclear integrity by Hoechst at lumbar DRGs at E13.5 revealed a reduction in the number of apoptotic nuclei in the conditional mutants compared to littermate controls. Next, we used TUNEL staining in order to identify apoptotic cells (see methods). In line with our Hoechst data, significant differences were observed among control, heterozygous and conditional knockout animals (Figure 19A). At E13.5, the number of apoptotic neurons in the DRG was dramatically decreased by loss of autophagy. As developmental death of DRG neurons occurs between E13.5 and E17.5 in DRG, we sought to determine whether this apparent inhibition of cell death in the conditional mutants reflected diminished apoptosis or just a delay. Therefore, we measured apoptosis by TUNEL at E18.5, a time point when developmental cell death of peripheral neurons in the control has been completed. Interestingly, both control and conditional knockout animals contained no apoptotic cells in the DRG at E18.5, indicating that loss of autophagy in the neural lineage blocks developmental cell death of peripheral neurons. These results identify autophagy as a novel mechanism required for the death of peripheral neurons during development.

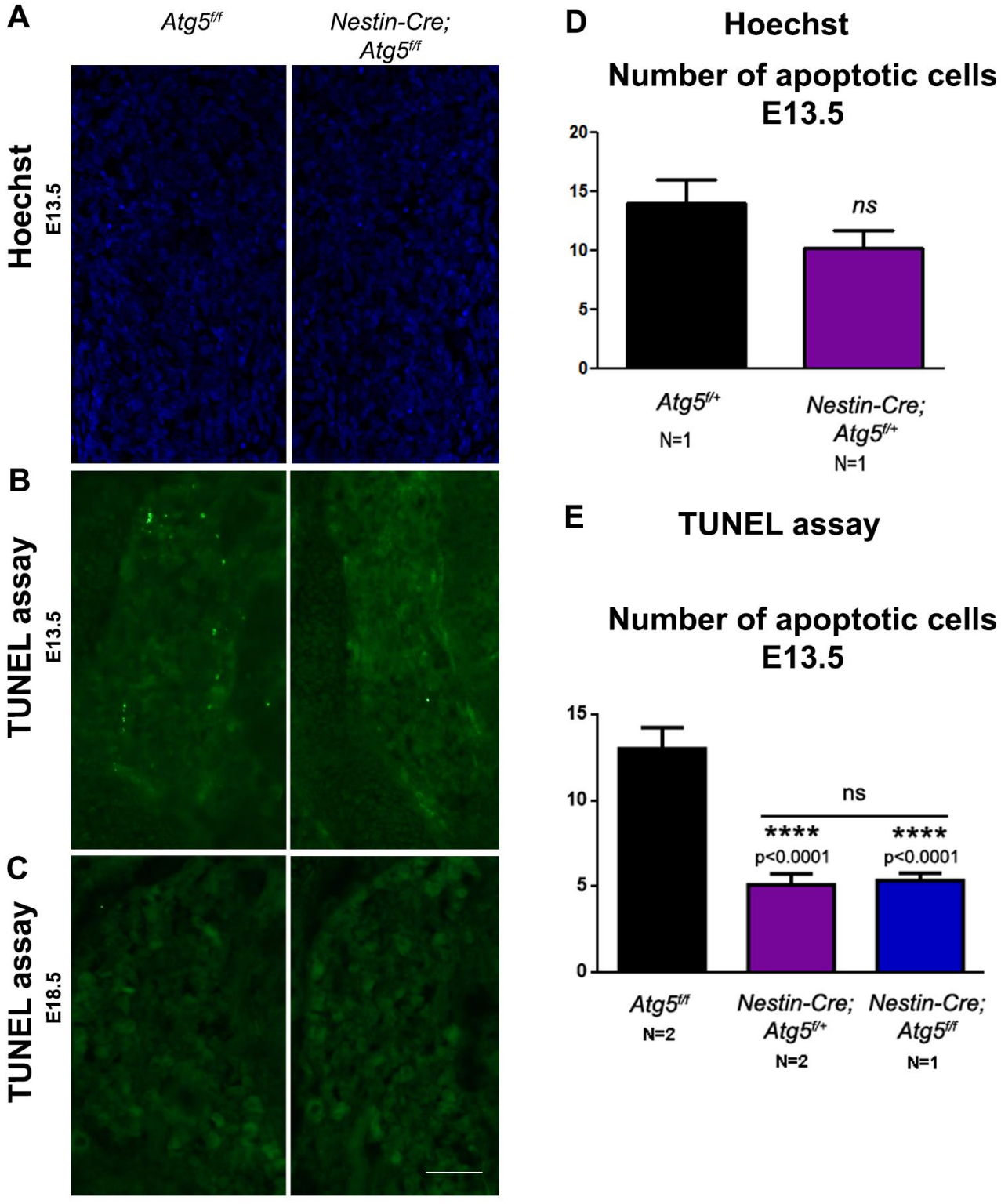



**Figure 19. Autophagy deficiency in the neural lineage prevents developmental cell death in DRGs at E13.5**

A) Hoechst was used to stain DNArevealingnuclear integrity at E13.5 DRGs. B) TUNNEL staining at E13.5 DRGs, significant differences present between control and conditional knockout mice. B) TUNNEL staining in DRGs at E18.5 where no apoptotic events are presented C) The quantification of apoptotic cells at E13.5 DRGs by Hoechst and by TUNEL assay (D), where significant reduction of apoptotic cells is observed in conditional knockout compared with littermate control animals (N indicates number of animals). Scale bars represent 100µm. Statistical analysis: Student's t test (D) $p<0.1$ and (E) $p<0.0001$ 15DRGs per group in every animal

### C.1.3 Mice with conditional ablation of autophagy in the neural lineage exhibit increased nociception

We next sought to determine the functional consequence of blocking cell death in the DRGs by ablation of autophagy. As the majority of DRG neurons are TrkA$^{+}$ nociceptive neurons responsible for the relay of noxious stimuli such as heat, we carried out a nociception test in one month-old mice, based on the use of a hotplate.

The term nociception (from the Latin *nocere*, "to harm") is developed by the Nobelist Sherrington, at the beginning of the twentieth century. The term nociceptive refers to the potential of a stimulus to produce a tissue lesion and a reaction from the organism. The hot plate technique is used for nociception studies and the licking, the withdrawal, immobility and vocalization are recording, demonstrating nociceptive behavior. In our case, we used a hotplate at 48°C and for one minute recorded the withdrawal and licking of paws.

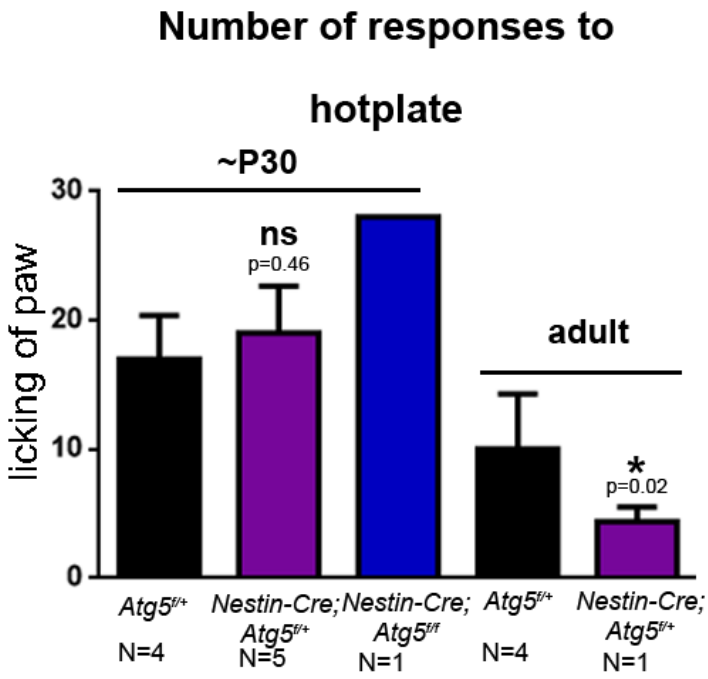



**Figure 21. Decreased nociception in adult deficient mice while increased in younger**

Hotplate test is performed in adult and one month year old mice. Younger mice have an increased nociception which is not significant. However this is result of the limited number of animals. In addition, adult mice show an important reduction of nociception. More behavioral experiments are in progress. Statistical analysis: Student's t test $p<0.05$

Conditional autophagy mutants exhibited significantly increased nociception compared to their littermate controls in a dosage dependent manner. Ablation of one allele of *Atg5*already showed a trend towards increased nociception, which was further reinforced in the mutant. These findings are in line with the presence of more neurons in the DRG of the conditional mutants, as apoptosis of superfluous neurons is practically abolished. However, adult conditional mutant mice exhibited decreased, instead of increased nociception, compared to age-matched controls (Figure 21).This finding, although it may

seem contradictory at first, it reflects the requirement of neurons for baseline autophagy in order to maintain their homeostasis and integrity and is in line with the progressive neurodegeneration observed in the CNS.

**C.2 Analysis of CNS-deficient neurons**

In addition to examining the role of autophagy in the PNS, we also sought to investigate its contribution to higher CNS functions, such as memory and learning. While in the PNS autophagy participates in life and death decisions, as revealed by our work, in the CNS it has a role that is modulatory. This notion is supported by the fact that autophagy-deficient animals are born with intact numbers of CNS neurons and that neurodegeneration is late onset. Moreover, it has become increasingly apparent that autophagy modulates CNS synapses (D. N. Shen, Zhang, Wei, & Yang, 2015). Preliminary data from our laboratory indicates that acute ablation of autophagy in neurons leads to increased numbers of dendritic spines, the post synaptic sites of glutamatergic excitatory neurons, increased long-term potentiation (LTP) of hippocampal Schaffer collaterals, and enhanced memory and learning (Nikoletopoulou et al., submitted).However, as memory and learning rely on the ability of synapses to be plastic, we wondered about the effects of prolonged autophagy ablation on these functions.

To this end, we examined the memory of adolescent and adult autophagy-deficient mice, where autophagy has been chronically ablated in neurons since their birth, as compared with control littermates. Memory was measured in a contextual fear conditioning paradigm, where animals are conditioned to associate a specific environment with a foot shock and their response to the memory of this association is measured a day later by recording the percentage of their freezing when presented with the same environment (Figure 22).

Our results indicated that both adolescent and adult conditional mutants exhibit a severe loss of memory, which is currently further documented by analyzing a larger cohort of animals. Animals with conditional deletion of one *Atg5* allele displayed more varied responses. In the adults these heterozygotes had a tendency for reduced memory, while in adolescent mice they showed a trend for improved memory. These differences are not suprising given the postnatal pruning and maturation of synapses that occurs in adolescence, however, more mice are needed before definite conclusions can be reached (Figure 23).

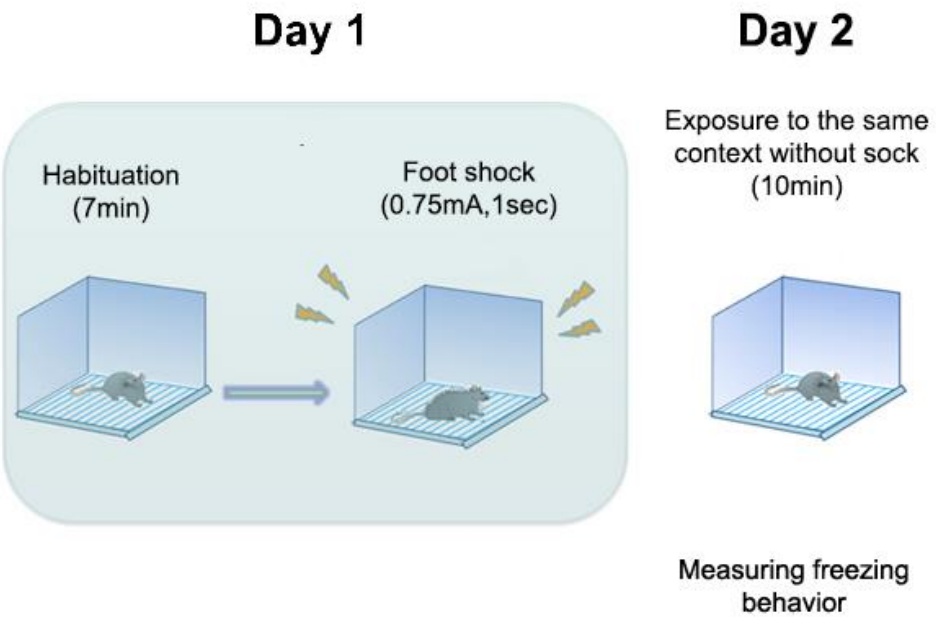



**Figure 22.** **Contextual fear conditioning**

On day one, the animal is placed for 10 minutes in a cage which has a specific non aversive stimulus, like a smell, colors or light. After 7 minutes, the animal is placed in the cage, it receives a foot shock. On day two, the animal is placed again in the same cage, and the behavior of freezing is recorded. Day 2 is free of foot shocks. The freezing response is evidence of fear. Therefore, the non aversive stimulus is paired with the aversive stimulus which was the foot shock.

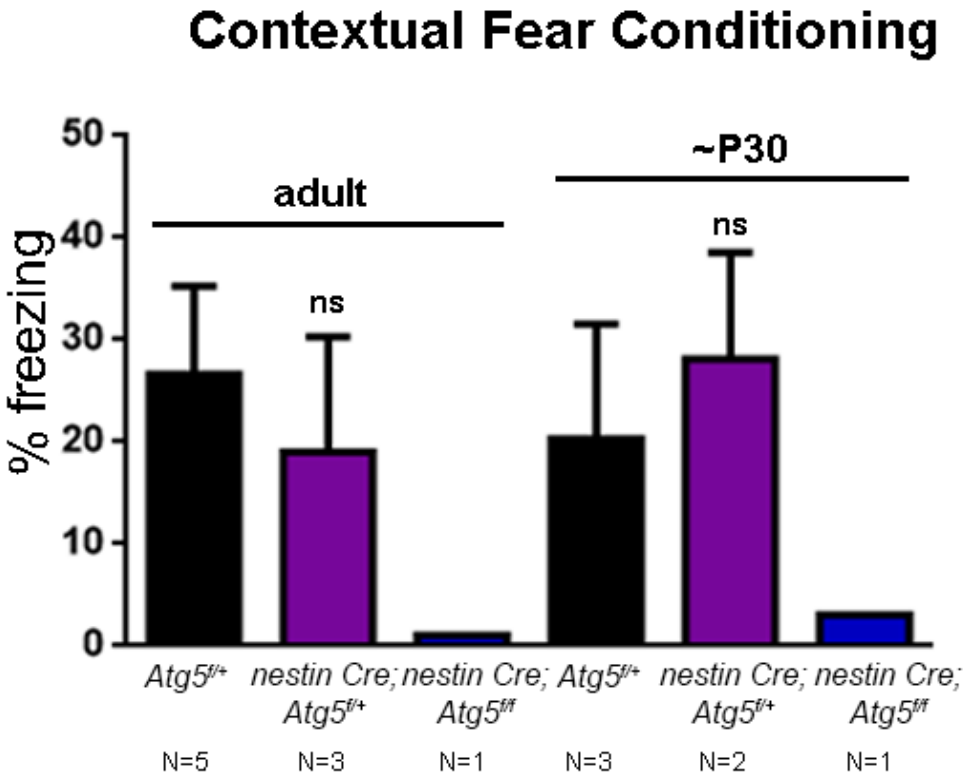



**Figure 23.** **Contextual fear conditioning in autophagy deficient mice in neural lineage**

Because of limited number of animals, no significant difference is observed but just a trend. These experiments are in progress. Statistical analyses: Student's t test

## D. Discussion

### D.1 Autophagy deficiency in the neural lineage prevents developmental death of peripheral neurons.

While our results have firmly established that autophagy is required for the death of PNS neurons during development, the mechanisms by which autophagy interacts with the apoptotic pathway remain unknown. To gain mechanistic insight, we are commencing a proteomic analysis comparing DRGs from autophagy-deficient and control littermates at E14.5, an embryonic stage where apoptosis is at its peak. This analysis will highlight the differences in expression of proteins and will reveal groups of proteins that are deregulated and may fall within mechanistically-relevant pathways. As autophagy is a degradative pathway that has specific protein substrates, the deregulated group of proteins may reflect important substrates that need to be degraded by autophagy during development to facilitate the apoptotic program.

In addition, we are in the process of increasing the number of animals analyzed in our nociception studies to gain confidence regarding the behavioral consequences of impaired autophagy in the PNS.

Finally, we want to investigate in a broader context whether autophagy is required for the proper specification of neurons in the PNS. DRGs contain distinct types of neurons that have unique profiles and serve three different modalities: nociception, mechanoreception and proprioception. Each modality is specified by a distinct developmental program, as outlined in Figure 13. Given that autophagy ablation blocks naturally occurring cell death, hence leading to the maintenance of the superfluous neurons, the question arises as to the specification of these excessive neurons. To this end, we aim to characterize the identity of these neurons using a variety of proteomic and biochemical tools. Taken together these experiments will fully elucidate the requirement of autophagy in PNS development and physiology.

### D.2Autophagy and CNS synaptic function

Our results in the CNS indicate that prolonged inhibition of autophagy results in impaired memory. Following up on this finding we want to analyze the cellular events that mediate this behavioral

deficit by counting the number of dendritic spines and electrophysiological properties of autophagy-deficient neurons.

In addition to highlighting the differences between acute and prolonged autophagy inhibition on synaptic function, our results also suggest that there may be critical periods during which autophagy serves different processes of neuronal maturation. To this end, we will cross the *Atg5*$^{f/f}$mice with a tamoxifen inducible deleter (*SLICKH-Thy-1-Cre*$^{ERT2}$), which allows the ablation of autophagy specifically in pyramidal neurons upon addition of tamoxifen. Using this tool, we will ablate autophagy at early postnatal stages (P10), and before (P20) or after (P30) the period of developmental dendritic spine pruning. The effects of autophagy ablation at these different developmental windows will be assessed both on a cellular and a behavioral level. Finally, we plan to study the effects of neuronal activity on the autophagic flux in primary cultures of hippocampal neurons.

Taken together, these experiments will give us novel insight on the role of autophagy synaptic function and behavior during different critical windows that are commonly associated with substance use disorders, autism, and psychiatric disorders such as schizophrenia, affective or anxiety disorder and bipolar among others (Giedd et al., 2008, Thomas et al., 2015).

## E. Materials and methods

### Mice

Mice were housed at a 12 hours light/dark cycle with ad libitum access for food and water.All experiments were performed in accordance with the guidelines of FORTH ethics committee and received the approval of Veterinary Directorate of Prefecture of Heraklion.
In order to specify the embryonic day, the time of plug was counted as day 0.5. All plugs were detected by the special vet assistant of FORTH.

*B6.Cg-Tg(Nes-cre)1Kln/J* mice, also known as *Nestin-Cre* mice, were used. This line was generated from Jackson laboratoryand expresses CRE recombinase in central and peripheral nervous system, as well as in neuronal and glial cell precursors. This CRE recombinase is active after embryonic day 11. These mice were hemizygous for this transgenic insert. Crosses between mice were done with *Atg5* deficient mice in order to generate *Nestin-Cre; Atg5$^{f/f}$*, *Nestin-Cre; Atg5$^{f/+}$* as well as *Atg5$^{f/+}$*and *Atg5$^{f/f}$*, the last two being our control mice. All mice are in C57BL/6 background.

### Genotyping

Mouse genomic DNA was isolated from tail biopsies following overnight incubation at 55°Cin 700ul lysis buffer (1M Tris HCL pH 8.0, 0,5M EDTA pH 8.0, 5M NaCl and 20% SDS) and 20ul proteinase K (10mg/ml).Following procedure include addition 700ul chloroform, vortex and centrifugationfor20min at 13.200rpm. 500ul of upper phase is transferred to a new tube and 350ul isopropanol is added. The solution is mixed by inverting and centrifuged for 25 min at 13.200 rpm. Supernatant is removed, washed with 350ul 75% ethanol and centrifuged for 5min at 10.000rpm. Pellet is left to dry at 55°C for 20min and 75ul TE buffer (1mTris-HCl pH 6.8, 0.5 EDTA pH=8.0) is added

The primers in the below were used in order to detect the *Atg5* flox allele and the existence of *Nestin-Cre*. Note that these primers confirm the existence of CRE recombinase (no information given for heterozygosity)

| | |
|---|---|
| *Cre-F* | 5’-AGG TGT AGA GAA GGC ACT CAG-3’ |
| *Cre-R* | 5’-CTA ATC GCC ATC TTC CAG CAG-3’ |
| *MEH-F* | 5’-AAG TGA GTT TGC ATG GCG CAG C-3’ |
| *MEH-R* | 5’-CCC TTT AGC CCC TTC CCT CTG-3’ |
| *Atg5-1* | 5’- ATA TGA AGG CAC ACC CCT GAA -3’ |
| *Atg5-2* | 5’-AAC GTC GAG CAC AGC TGC GCA A-3’ |
| *Atg5-3* | 5’-ACT GCA TAA TGG TTT AAC TCT T-3’ |

**Tissue fixation and cryo-protection**

All mice were sacrificed by cervical dislocation according to the guidelines of FORTH ethics committee. Embryos from pregnant female mice were dissected and fixed in 4% PFA (4% PFA in PBS pH 7.4) for 24-48 hours at 4°C and incubated in a cryo-protection solution of 30% sucrose (30% sucrose in PBS) at 4°C until complete penetration of sucrose into the embryonic tissue. Embryos were then embedded in Tissue-Tek (Tissue-Tek O.C.T. Compound) and stored at 80°C forcryo-sectioning. All embryos were cut into 14μm horizontal sections while brains were cut into coronal sections and were selected on Superfrost Plus slides.Tissue slices were dried at 37°C and stored at 80°C.

**TUNEL staining**

TUNEL staining was performed in sections of embryos, using the Roche In Cell Death Detection Kit (Cat.No.11 684 809910).The apoptotic cells in DRGs were identified and measured via. This is a specific kit which can detect and quantify the apoptotic cells, via labeling DNA strand breaks. Fluorescence microscopy was used to analyze the signal.

**Immunofluorescence**

Tissue sections were re-hydrated with PBS (15min washes) and incubated for 1hour in blocking solution (10% fetal calf serum-FCS and 0.2% Triton in PBS-the second only if the antibody demands permeabilization) at room temperature. Sections were incubated with primary antibodies and

blocking solution overnight at 4°C. The sections were rinsed with PBS (3 washes X10 min) and were incubated with the secondary antibody with PBS for one hour. Finally, the sections were rinsed with PBS and mounted. The primary antibodies used were the following: $p75^{NTR}$ extracellular domain (1:1500, Cell signaling), TrkA (1:250, Santa Cruz Biotechnology), both without permeabilization. As secondary antibodies were used the following: anti-rabbit conjugated to Alexa Fluor 488 or 594, anti-mouse conjugated to Alexa Fluor 488 or 594, anti-goat conjugated to Alexa Fluor 594 (1:10000) and Hoechst 33342 (1:5000)dye which (nuclear dye).

**Western blot**

Tissues were lysed in Ripa Buffer (50mM Tris-Cl pH 7.4, 150mM NaCl, 1mM EDTA, 1% TritonX-100, 0,1% SDS and 0,1% sodium deoxycholate, Rauskolb et 2010) containing protease inhibitors (Roche). Tissues were sonicated for 20seconds on ice. Tissues were then incubated overnight on ice and were centrifuged for 30min at 14,000rpm. Bradford method was used to measure the concentration of proteins in samples and the proteins were separated using (7.5-15%) polyacrylamide gel and were transferred to a nitrocellulose membrane which has a 0.2µm pores size. The membrane was incubated in 5% milk for 1hour and overnight in the primary antibody at 4°C. The membranes were washed three times (3x10min)with TPBS (100 mM Na2HPO4, 100mM NaH2PO4, 0.5N NaCl, 0.1% Tween-20), and were incubated with a secondary horseradish peroxidase-conjugated-HRP antibody (1:10000) for 1 hour at room temperature. The membranes were washed three times (3x10min)with TPBS and were incubated with chemiluminescent solution (SuperSignal West Pico and Femto Substrate-ThermoFisher Scientific) for 2-3min.The primary antibodies used for western blotting included $p75^{NTR}$ extracellular domain (1:1000,Cell signaling), MAP LC3β (ATG8) mouse monoclonal (1:1000,Santa Cruz Biotechnology), beta-Tubulin (1:5000). The secondary antibodies used were horseradish peroxidase-conjugated anti-mouse, anti-goat and anti-rabbit (Abcam).

**Behavioral Tests**

***Contextual Fear Conditioning***

*Atg5* deficient mice have been subjected to fear conditioning chamber (MedAssociates, St. Albans, VT, USA) controlled by a custom-made interface computer. All animals were habituated to the experimental room for 1hour. On day one (training), the animals received one mild foot shock

(1000ms, 0.75mA) 7 min after being placed in the experimental chamber. 3 min after the shock, the mice were returned to their cages. Twenty-four hr later (testing), subjects were located again in the chamber for 10min without receiving any foot shock. It is worth to highlight that all contextual information, for instance odor, light, visual cues, should be absolutely the same with the first day. All recording videos were analyzed by an algorithm generated by Dr. Maria Pateraki (ICS-FORTH) and freezing behavior of animals of day 2 has been measured. The percentage of freezing behavior per session is calculated. All behavioral experiments were blind tests to the genotype of the mice.

***Hot plate***

Hot plate testing was used to identify nociceptive differences in autophagy deficient mice. Temperature was defined at 48°C (mild temperature). Mice were placed in the experimental chamber for 1minute. Withdrawal, licking of foot and hand, immobility and vocalization were recorded as well as the latency of response. The licking of paw and the latency of response were analyzed in order to detect nociceptive differences. All behavioral testing was conducted on blind. Leica HI1210 water path was used as hot plate.

**Statistical Analysis**

Student's t test was performed using the Graph Pad program (La Jolla, CA, USA). Mean, standard error of mean (±SEM) and statistical significance (pValue) were assessed by unpaired two-tailed Student's t-test. Statistical significance was established at pValue≤0.05.

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## G. List of abbreviations

Akt; or PKB; Protein kinase B
ATG; autophagy-related
ATP; Adenosine triphosphate
Bcl-2; B-cell lymphoma extra large
BDNF; Brain-delivered neurotrophic factor
bHLH; basic helix-loop-helix
BMP; Bone Morphogenetic Proteins
cAMP; cyclic Adenosine Monophosphate
CMA; Chaperone-Mediated Autophagy
CNS; Central Nervous System
DAG; Diacylglycerol
DFCP1; double FYVE-containing protein-1
DRGs; Dorsal Root Ganglia
ER; Endoplasmic Reticulum
FADD; FAS Associated Death Domain
FOXO3; Forkhead Box Protein O3
GDNF; Glial cell-derived neurotrophic factor
Hsc70; Heat shock protein cognate
IL1β; Insulin Growth Factor 1
IP3; Inositol trisphosphate
JNK; Jun-N-terminal kinase
Lamp-2A; Lysosome-associated membrane protein 2
LTP; Long term potentiation
MOM; Outer Mitochondrial Membrane
Mrgpr; class G protein coupled receptors
mTOR; mammalian target of rapamycin
NBR1; Neighbor of BRCA1 gene 1 protein
NF-kB; NF-kB essential modulator
NGF; Neurotrophic factor
Ngn1; Neurogenin 1; Ngn2
Neurogenin 2
NO; Nitric acid
NT3; Neurotrophic 3
NT4/5; Neurotrophic 4/5
p27kip1; or CDKN1B; cyclin-dependent kinase inhibitor
p53; tumor protein p53
p62; or SQSTM1; sequestosome
PBS; Phosphate-buffered saline
PE; Phosphatidylethanolamine
PI3K; Phosphatidyl Inositol 3 Kinase
PLCγ; Phosphoinositide phospholipase Cγ
PNS; Peripheral Nervous System
PtdIns3; Phosphatidylinositol 3,5-bisphosphate

PUMA; p53 upregulated modulator of apoptosis
RET; RETproto-oncogene