Study of Survival Motor Neuron protein regulation and the role of autophagy in Spinal Muscular Atrophy

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Abstract

Abstract

Spinal muscular atrophy (SMA) is a genetic disorder characterized by the degeneration of spinal cord motorneurons (MNs), resulting in muscular atrophy and weakness. SMA is caused by loss or mutation of the *Survival motor neuron 1* gene *(SMN1)*, lead to reduced SMN protein level and selective dysfunction of MNs. SMN is ubiquitously present in all cells and is essential for the assembly of small nuclear ribonucleoproteins (snRNPs), which is important for pre-mRNA splicing requirements. In the neuron, SMN is also essential for the transport of mRNA to axonal terminals for growth, pathfinding and elongation. SMN reduction causes neurite degeneration and cell death without classical apoptotic features, but the direct events leading to MN degeneration in SMA are still unknown. Autophagy is an important biological process that is essential for the removal of damaged organelles and toxic substances or proteins through lysosome degradation. It includes several sequential steps including nucleation, double membrane formation, and maturation of autophagosomes, and finally autophagosome fusion to the lysosomes for degradation. Autophagy is being a primary target for the treatment of many neurodegenerative diseases. Accumulation of autophagosomes in neurites observed in several neurite degenerative models suggests a close relationship between the autophagy and neurite collapse. An accumulation of autophagosomes causes axonal transport disruption, interference of intracellular space trafficking, and originates neurite degeneration. The objective of the present study is to analyze the role of autophagy in SMA pathology, the mechanisms that regulate SMN protein degradation and the origin of neurodegeneration in spinal cord MNs. To this end, we have reduced the Smn protein by using the lentivirus knockdown method. Smn decrease causes neurite degeneration and non-apoptotic cell death in cultured embryonic mouse MNs. Over-expression of Bcl-XL, an anti-apoptotic and autophagy inhibitor protein counteracts this effect. We have analyzed changes of autophagy markers in Smn-reduced MNs from lentivirus Smn knockdown and SMA type I transgenic mice models and we have observed that Smn reduction causes an increase of autophagy markers and autophagosome accumulation. Treatment with autophagy activators, autophagy inhibitors or proteasome inhibitors induce changes of Smn protein level in MNs suggesting the role of autophagy and proteasome in the regulation of Smn protein in these cells. Finally, the calcium dependent protease calpain is also involved in Smn stability, endogenous calpain reduction increases Smn protein level. Together all these results contribute to new insight about Smn protein regulation in MNs and the possible role of autophagy in SMA neurodegeneration.

Resum

L'atròfia muscular espinal (AME) és un trastorn genètic caracteritzat per la degeneració de les neurones motores (MN) de la medul·la espinal que resulta en atròfia i debilitat muscular. L'SMA està causada per la pèrdua o la mutació del gen de la supervivencia de les neurones motores 1 (*SMN1*), cosa que condueix a una reducció dels nivells de la proteïna SMN i una disfunció selectiva de les MN. La proteïna SMN és ubiqua en totes les cèl·lules i és essencial per a l'assemblatge de les ribonucleoproteïnes petites (snRNP) que són importants per a l'splicing dels pre-mRNA. A les neurones, l'SMN també és essencial per al transport dels mRNA als terminals, cosa que permet el creixement, el guidatge i l'elongació axonal. S'ha descrit que la reducció d'SMN causa la degeneració de les neurites i la mort cel·lular sense les característiques apoptòtiques clàssiques, però els esdeveniments directes que condueixen a la degeneració de les MN en l'SMA encara són desconeguts. L'autofàgia és un procés biològic essencial per a l'eliminació d'orgànuls danyats i de substàncies o proteïnes tòxiques mitjançant la degradació al lisosoma. L'autofàgia inclou diversos passos seqüencials com la nucleació, la formació de doble membrana, la maduració dels autofagosomes i la fusió dels autofagosomes amb els lisosomes per ser degradats. L'autofàgia és una diana principal per al tractament de moltes malalties neurodegeneratives. L'acumulació d'autofagosomes a les neurites que s'observa en diversos models de degeneració de neurites suggereix que existeix una relació estreta entre l'autofàgia i el col·lapse de les neurites. Una acumulació d'autofagosomes provoca la interrupció del transport axonal, la interferència del trànsit dins l'espai intracel·lular i provoca la degeneració dels axons. L'objectiu d'aquest estudi és analitzar el paper de l'autofàgia en la patologia de l'SMA, els mecanismes que regulen la degradació de la proteïna SMN i l'origen de la neurodegeneració en les MN de la medul·la espinal. Amb aquesta finalitat, hem reduït la proteïna SMN utilitzant el mètode de silenciament amb lentivirus. En cultius de MN embrionàries de ratolí, la disminució d'SMN provoca la degeneració dels axons i la mort no apoptòtica d'aquestes cèl·lules. La sobreexpressió de Bcl-XL, una proteïna anti-apoptòtica i inhibidora de l'autofàgia, contraresta aquest efecte. Hem analitzat els canvis en els marcadors d'autofàgia en les MN en cultiu amb l'SMN reduïda amb lentivirus i en cultius de MN de models de ratolins transgènics de SMA de tipus I. Hem observat que la reducció de l'SMN provoca un augment dels marcadors d'autofàgia i l'acumulació d'autofagosomes. A més, el tractament amb activadors de l'autofàgia, inhibidors de l'autofàgia o inhibidors del proteasoma indueix canvis en els nivells de la proteïna SMN en les MN, la qual cosa suggereix un paper de l'autofàgia i el proteasoma en la regulació de la proteïna SMN en aquestes cèl·lules. Finalment, la proteasa dependent de calci calpaïna també està involucrada en l'estabilitat de l'SMN, ja que la reducció de la calpaïna endògena augmenta els nivells de proteïna SMN. Conjuntament, aquests resultats contribueixen a una nova visió sobre la regulació de la proteïna SMN en les MN i sobre el possible paper de l'autofàgia en la neurodegeneració en l' SMA.

Resumen

La atrofia muscular espinal (AME) es un trastorno genético caracterizado por la degeneración de las neuronas motoras (MN) de la médula espinal que resulta en atrofia y debilidad muscular. La AME está causada por la pérdida o mutación del gen de la supervivencia de las neuronas motoras 1 (*SMN1*), cosa que conduce a una reducción de los niveles de la proteína SMN y una disfunción selectiva de las MN. La proteína SMN es ubicua en todas las células y es esencial para el ensamblaje de las ribonicleoproteínas pequeñas (snRNP) que son importantes para el *splicing* de los pre-mRNA. En las neuronas, SMN también es esencial para el transporte de los mRNA terminales, cosa que permite el crecimiento, la dirección y la elongación axonal. Se ha descrito que la reducción de SMN causa la degeneración de las neuritas y la muerte celular sin las características apoptóticas clásicas, pero los eventos directos que conducen a la degeneración de las NM en la AME son aún desconocidas. La autofagia es un proceso biológico esencial para la eliminación de orgánulos dañados y de substancias o proteínas tóxicas mediante la degradación en el lisosoma. La autofagia incluye diversos pasos secuenciales como la nucleación, la formación de la doble membrana, la maduración de los autofagosomas y la fusión de los autofagosomas con los lisosomas para ser degradados. La autofagia es una diana principal para el tratamiento de muchas enfermedades neurodegenerativas. La acumulación de autofagosomas en las neuritas que se observa en diferentes modelos de degeneración de neuritas sugiere que existe una relación estrecha entre la autofagia y el colapso de las neuritas. Una acumulación de autofagosomas provoca la interrupción del transporte axonal, la interferencia del tránsito dentro del espacio intracelular y provoca la degeneración de los axones. El objetivo de este estudio es analizar el papel de la autofagia en la patología de la AME, los mecanismos que regulan la degradación de la proteína SMN i el origen de la neurodegeneración en las NM de la médula espinal. Con este fin, hemos reducido la proteína SMN utilizando el método de silenciamiento con lentivirus. En cultivos de NM embrionarias de ratón, la disminución de SMN provoca la degeneración de los axones y la muerte no apoptótica de estas células. La sobreexpresión de Bcl-XL, una proteína anti-apoptótica e inhibidora de la autofagia, contrarresta este efecto. Hemos analizado los cambios en los marcadores de autofagia en las NM en cultivo con SMN reducida con lentivirus y en cultivos de NM de modelos de ratón transgénicos de AME tipo I. Hemos observado que la reducción de SMN provoca un aumento de los marcadores de autofagia y acumulación de autofagosomas. Además, el tratamiento con activadores de la autofagia, inhibidores de autofagia o inhibidores del proteasoma induce cambios en los niveles de la proteína SMN en las NM, la cual cosa sugiere un papel de la autofagia y el proteasoma en la regulación de la proteína SMN en estas células. Finalmente, la proteasa dependiente de calcio calpaina también está involucrada en la estabilidad de SMN, ya que la reducción de calpaina endógena aumenta los niveles de proteína SMN. Conjuntamente, estos resultados contribuyen a una nueva visión sobre la regulación de la proteína SMN en MN y sobre el posible papel de la autofagia en la neurodegeneración en AME.

Index

Abbreviations

3-MA	3-Methyladenin
AAV	Adeno-associated virus
AD	Alzheimer Disease
AIF	Apoptosis Inducing Factor
Akt	Protein kinase B, PKB
ALS	Amyotrophic lateral sclerosis
AMPK	AMP Activated Protein kinase (or)
	Adenosin Monophosphate activated Protein Kinase
Apaf-1	Apoptotic protease activating factor-1
APP	Amyloid Precursor Protein
ASO	Antisense oligonucleotides
Atg	Autophagy
ATP	Adenosine 5’-triphosphate
ATPase	Adenosine 5’trisphosphatase
Aβ	Amiloid beta
B27	B27 neuron supplement
Bad	BCL-2-associated death promoter protein
BafA1	Bafilomycin A1
Bak	BCL-2-antagonist/killer-1
Bax	BCL-2-associated X protein
Bcl-10	BCL-2-like-10-protein
Bcl-2	B-cell lymphoma-2-protein
Bcl-W	BCL-2-like-2-protein
Bcl-xL	BCL-2-like-extra large protein
BDNF	Brain-derived neurotrophic factor
Bid	BH3-interacting-domain death agonist

Bik	BCL-2-interacting killer
Bim	BCL-2-like-11 protein
BMP	Bone Morphogenetic Protein
BSA	Bovin Albumin Serum
CLC	Corticotrophin-like cytokine
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
CNTFRα	CNTF receptor alpha
CT-1	Cardiotrophin-1
Cyt C	Cytochrome C
DAG	Diacylglycerol
DD	Death Domain
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's Medium
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinases
ES	Embryonic stem cells
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
EV	Empty Vector
FADD	Fas-Associated protein with Death Domain
Fas	Apoptosis antigen
FasL	Fas Ligand
FasR	Fas Receptor
FDA	Food and Drug Administration
FKBP	FK506-binding protein

GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFP	Green Fluorescent Protein
GFRα	GDNT family receptor-α
GHEBS	Glucose-HEPES buffer saline
GP130	Glycoprotein 130
HAT	Histone acetylation
HD	Huntingtin Disease
HEK293	Human Embryonic Kidney 293
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HGF	Hepatocyte growth factor
HI-HS	Heat inactivated-Horse serum
hnRNP	heterogeneous nuclear ribonucleoprotein
Hsp	Heat shock protein
Htt	Hungtingtin
IAP	Inhibitor of apoptosis proteins
IL	Interleukin
JAK	Janus Kinase transcription factor
L15	Leibovitz's medium
L15c	L15 complete
LB	Lysis Buffer
LC3	Microtubule-associated protein 1 light chain 3
LIF	Leukemia inhibitory factor
LIFRβ	LIF Receptor beta
MAP	Mitogen acitated protein
MAPK	Mitogen activated protein kinase

Met	Mesenchymal epithelial transition factor HGF receptor
MN	Motoneuron
MPT	Mitochondrial permeability transition
mTOR	mechanistic Target of Rapamycin
mTORC	mTOR complex
mtSMA	SMA type I mice mutant
N2	N2 neuron supplement
NAIP	*Neural Apoptosis Inhibitory Protein*
NBM	Neuro basal medium
NBMc	Neurobasal medium complete
NCAM	Neural cell adhesion molecule
NT	Neural Tube
NF-kB	Nuclear Factor kappa B
NGF	Nerve Growth Factor
NMJ	Neuromuscular junction
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
NTF	Neurotrophic Factor
OSM	Oncostatin M
P/O	Poly-DL-ornithine
P/S	Penicillin and Streptomycin
PCD	Programmed cell-death
PD	Parkinson Disease
PDK1	Phosphoinositide-dependent kinase-1
PE	Phosphatidylethanolamine
PEI	Polyethylenamine
PFA	Paraformaldehyde

PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatiylinositol-3-phosphate
PIP	Phosphatidylinositol phosphate
PLC	Phospholipase C
PNS	Peripheral Nervous System
PS	Pluripotent stem cells
Puma	p53 upregulated modulator of apoptosis
RA	Retinoic acid
Ret	Receptor tyrosine kinase
RIP	Receptor interacting serine/threonine protein
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
SBMA	Spinobulbar muscular atrophy
SCA	Spinocerebellar ataxias
SERF1	*Small EDRK-Rich Factor*
Shh	Sonic hedgehog
shRNA	small hairpin RNA
siRNA	small interference RNA
SMA	Spinal muscular atrophy
SMN	Human Survival Motoneuron protein
Smn	mouse Survival Motoneuron protein
SMN	Survival Motoneuron gene
SMNΔ7	SMN protein lacking exon 7
snRNA	small nuclear RNA
snRNP	small nuclear Ribonuclear protein

SOD1	Superoxide dismutase Protein 1
SQSTM	Sequestosome
SR	serine/arginine
SRSF	serine/arginine splicing factor
STAT	Signal Transducer and Activator of Transcription
TAD	Trans activation domine
TF	Transcription Factor
Tg	Transgenic
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TNFR	TNF receptor
TRADD	TNF receptor type 1-associated death domain
TRAF	TNFR Associated Factor
TrK	Tyrosine Kinase receptor
TSC	Tuberous sclerosis complex
Ub	Ubiquitination
ULK1	UNC-51-like kinase 1
UNRIP	UNR interacting protein
UPS	Ubiquitin proteasome system
V-ATPas	Vacuolar H+ ATPase
WT	Wild type

Introduction

1. NERVOUS SYSTEM

1.1. Nervous system organization

The human body is made by trillions of cells, the cells of the nervous system are called neurons. Neurons are specialized to carry "messages" through an electrochemical process. They connected to each other to form neural networks. Neurons and glial cells form the core components of the nervous system in the Central Nervous System (CNS) and the Peripheral Nervous System (PNS). In CNS, brain is considered to be most complex organ that serves as the centre of the nervous system. The brain contains 15-33 billion neurons in humans (Pelvig et al. 2008). The spinal cord is a thin, long and tubular structure that supports nerve cells and it extent outside from brain (Figure 1). It serves as a central pathway for information by connecting the brain and the PNS. In CNS, nerve cells are arranged into nuclei, compact accumulations of nerve cells that are present all over the brain and the spinal cord. The cortex is a sheet of array of nerve cells arranged in cerebral hemispheres.

The PNS is made up of sensory neurons, which connect sensory receptor on the surface of the body to CNS. It comprises of two motor systems (i) somatic motor division that connect the brain and the spinal cord with the skeletal muscles (ii) autonomic or visceral motor unit, control cells and axons that innervate smooth muscles, cardiac muscles, and glands.

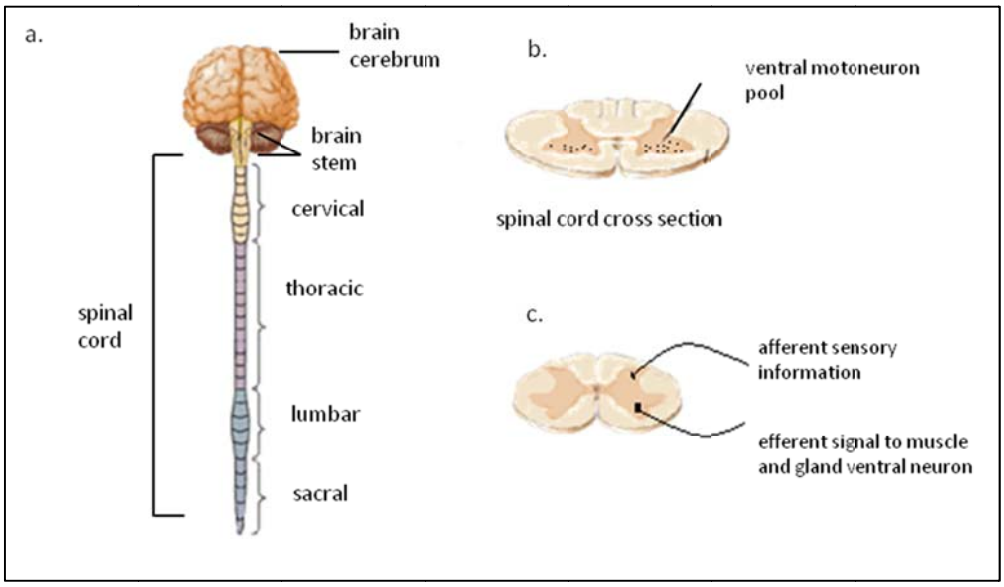

Figure 1. Nervous system organization: CNS division consists of the brain and the spinal cord. Spinal cord is divided into cervical, thoracic, lumbar and sacral. B. Cross section of the spinal cord shows the pool of moto neurons (MNs) present in the anterior horn of the ventral region. C. In spinal cord, ***afferent neuron*** *carries signal from sensory part to CNS, and* ***efferent neurons*** *carry signal away from CNS to muscle and glands*

1.2. Morphology of the neurons and classification

Neuronal function is related to the transfer of signals for long distances, and the glial cells protect and help them in their function during developmental and in the adult life. Nerve cell structural skeleton is made of cytoskeleton, that is critical for the stability of the neuronal processes and the synaptic junction functions (Vitriol & Zheng. 2012).

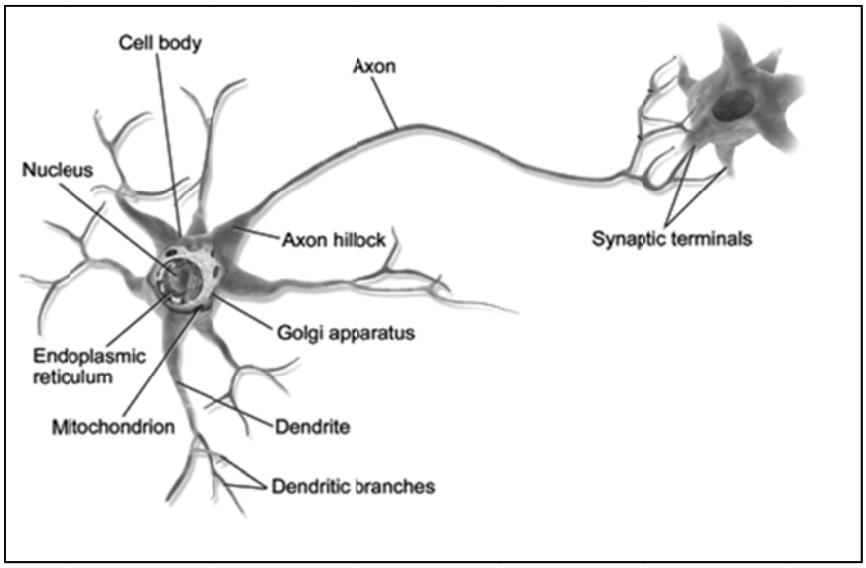

Figure 2. Morphology of the neuron. Neuron consists of the cell body containing nucleus, ER, Golgi apparatus, and mitochondria. Multiple dendrites branch out from the cell body. Axon originates from the axon hillock, carry the signal from the cell body to the synaptic terminals.

Neurons are distinguished from other cells by the mechanism of intercellular communication. The specialized structural organization of a neuron is for electrical signal transmission and synaptic contact between neurons (Shimizu & Stopfer. 2013). This communication transfer takes place through the unique morphological structures called axons and dendrites (Figure 2). Dendrites arise from the cell body, there are small cells with one or without dendrites, but there are neurons with a bunch of dendrites that branch like a tree in shape (Figure 3). Dendrites normally receive information from other neurons and they have a high content of ribosomes and specific cytoskeletal proteins that helps in its function. The signal transmits from one neuron to other between the space called synapsis (Eberwine et al. 2001). The presynaptic terminal is adjacent to the postsynaptic terminal of the target cell.

The level of synaptic inputs received by each neuron varies from 1 to 100,000 in human nervous system. The information conveys by synapses on the neuronal dendrites, integrates and transmits through axon to the other site of synaptic interaction. The axon is a

unique extension from the neuron cell body that may travel a few hundred micrometers, depending on the type of neuron and the size of the species. The neurons with shortest axon, known as interneurons, are present throughout the brain. The neurons with the longest axon are Motoneurons (MNs), they extent their axons from spinal cord to the big toe of each foot (Figure 3).

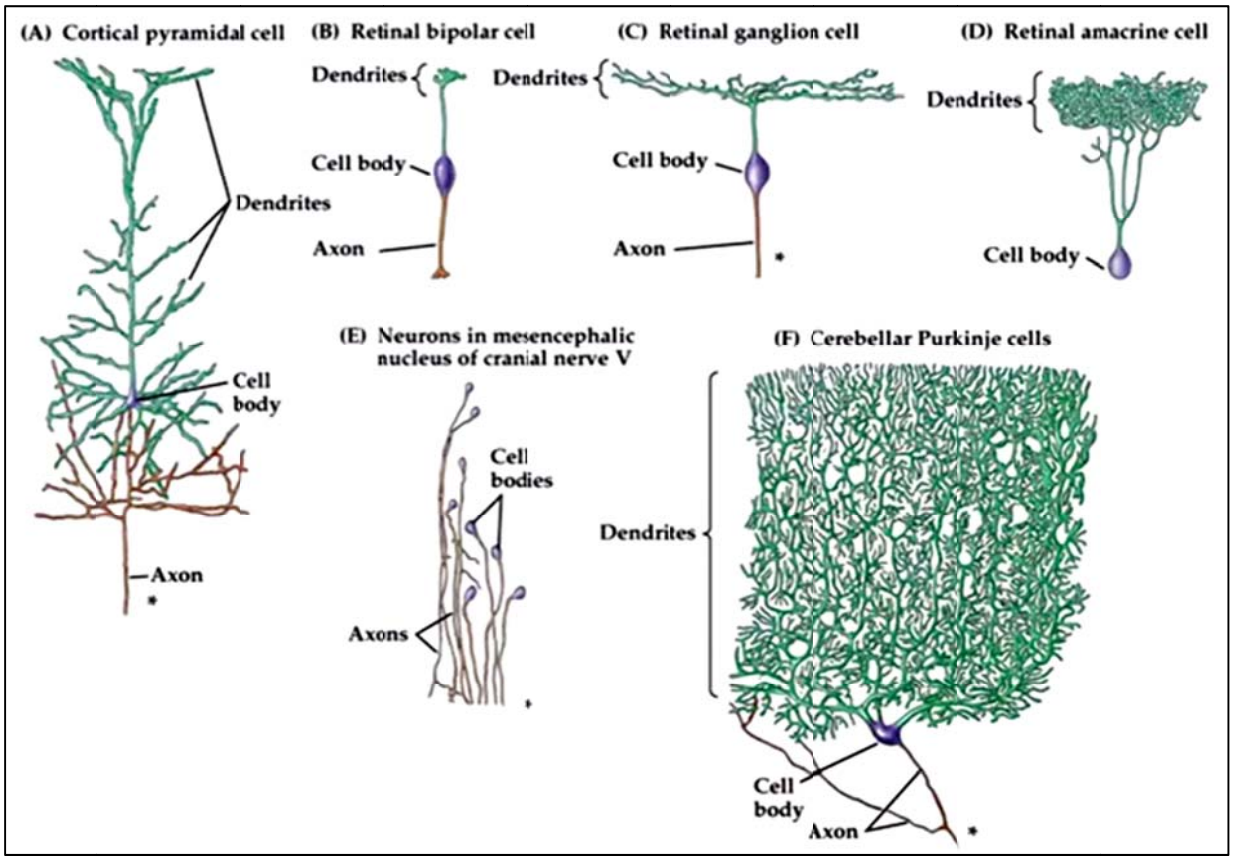

Figure 3. Types of neurons. Varieties of nerve cell morphology found in human nervous system. (a). Pyramidal like cells, that found in the cerebral cortex, (b). Retinal bipolar cells with very short axon (c). Retinal ganglion cells with multiple dendrite branching, (d) Retinal amacrine cells with no axon, (e). Neurons with no dendrites, (f). Cerebellar Purkinje cell, dendrite branches (obtained from Neuroscience Biology edition III by Purvesh).

1.3. Spinal cord organization

The spinal cord function consists in the transmission of the signal between the brain and the rest of the body. It also contains neural circuit that can independently control numerous reflexes. There are thirty-one spinal cord nerve segments in humans divided: 8 cervical segments, 12 thoracic segments, 5 lumbar segments, 5 sacral and 1 coccygeal segment. Neurons from spinal cord innervate different parts of muscle fiber for locomotion, movement, contraction and expansion (Fournier Le Ray & Fontaine-Perus 1991). The cervical and the lumbar part, where the spinal cord enlarges, innervate the upper limb and the lower limb respectively (Kobayashi et al. 1987).

1.4. Types of muscle fibers

Muscles are derived from the mesodermal layer of embryonic germ cells during development (Torres et al. 2012). There are three types of muscle fibers specified as skeletal, cardiac and smooth muscles. (i) The skeletal muscles are striated muscles attached to the skeleton. They facilitate contraction of voluntary muscle movement by applying force to bones and joints. (ii) The smooth muscle is involuntary muscle, found in walls of organs including stomach, intestine, esophagus, bronchi, uterus or blood vessels that control contraction and movement. (iii) The cardiac muscle is the striated and involuntary heart muscle (Table 1). These three muscle cells use the movement of actin against myosin to create contraction (Smith. 2014). In skeletal muscle its contraction is stimulated by electric impulse generated by the spinal cord MNs (Heckman & Enoka. 2012). MNs dysfunction or degeneration causes muscle degeneration or atrophy (D'Amico et al. 2011).

Type	function
Skeletal muscle	Control voluntary mudes movement that is connected to bones and joints.
Cardiac muscle	Help in movement of heart muscle contraction or expansion
Smooth muscle	Present in organ namely stomach, intestine, reproductive tracts, respiratory tracts, iris of eye ect.

Table 1. Types of muscle that control by nerve fiber connected to the spinal cord.

1.5. Types of neurons in the spinal cord

Neurons are primarily classified into three types: (i) the afferent neuron that responds to sensory organs, signals to the spinal cord and the brain called SENSORY NEURONS. (ii) The efferent neurons that carry signals from the spinal cord to the muscles and glandular tissues for contraction called MNs. (iii) The neuron that involve in connecting neurons to each other within the same region of the brain or the spinal cord called INTERNEURONS (Figure 4).

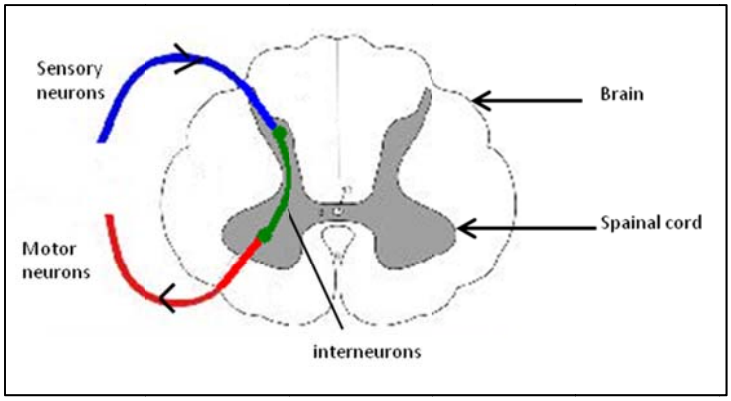

Figure 4. Types of neurons in the spinal cord. Sensory neurons (blue), MNs (red), interneurons (green).

1.6. Motoneurons

Motor pools comprise a mixture of fast and slow MNs that exhibit distinct profiles of activation and produce different degrees of force during the process of muscle contraction (Friese et al. 2009) (Figure 5a).

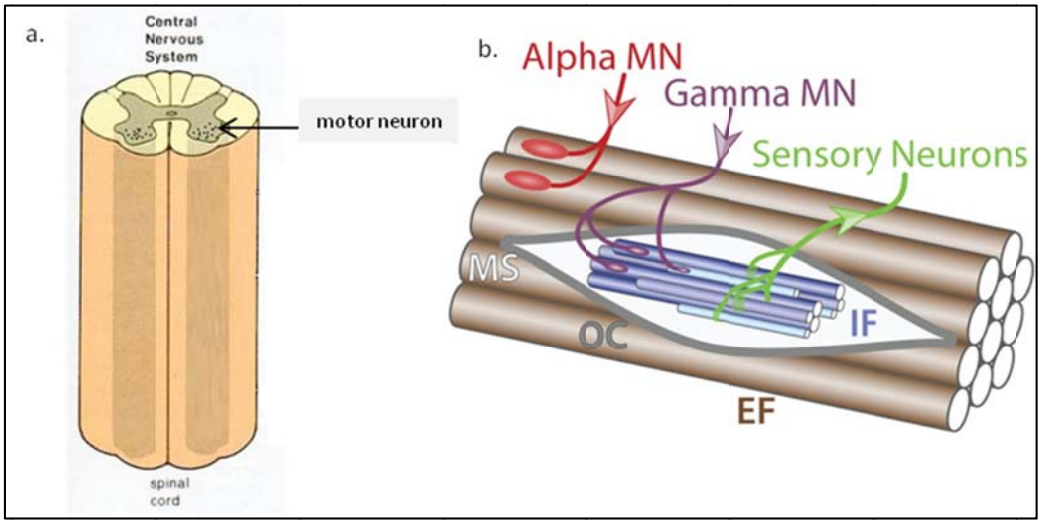

Figure 5. (a)MNs present in the ventral horn of spinal cord and innervate skeletal muscle fibers. (B). Types of MNs, alpha MN innervate extrafusal muscle fiber (EF), gamma MN innervate intrafusal muscle fiber (IF) within the muscle spindle (MS). Sensory neurons carry signal from IF to CNS. Obtained from (Stifani 2014)

There are two main types of MNs, upper and lower MNs. Upper MNs are located in the central cortex and lower MNs are located in brainstem and the spinal cord. The lower MN cell bodies are located in the brainstem nuclei and in the ventral horn of the spinal cord. The important feature of lower MNs are their axonal extension that connecting outside of the CNS. The lower MNs are subdivided into branchial, visceral, and somatic MNs. (i) Branchial MNs situated in the brainstem, and they innervate branchial arch to muscles of the face and neck. (ii) Visceral MNs located in PNS and they control the smooth muscles of

heart and arteries and glands, and (iii) Somatic MNs located in the brainstem and the spinal cord, they innervate skeletal muscles responsible for movements.

Somatic MNs are divided into three groups: alpha, beta and gamma according to the type of muscle fibers they innervate within the particular muscle target (Stifani 2014). Alpha MNs innervate extrafusal muscle fibers of skeletal muscles and are directly responsible for initiating their contraction. Alpha motor fibers have a longer diameter and higher conduction velocity. Beta and Gamma MNs, innervate intrafusal muscle fibers of muscle spindles (Figure 5b).

Alpha MNs further subdivide based on the type of muscle fiber it innervates and may vary in size. (i) Small or slow motor unit comprises small muscle fiber that contract slowly and generate relatively small force. It consists of rich myoglobin and high mitochondria content, and capillary beds, so they are resistant to fatigue. It is especially important for sustained muscle contraction activity such as the maintenance of an upright position. (ii) Larger or fast motor units innervate large, pale muscle fibers that generate more force. It consists of few mitochondrial distribution and hence quickly fatigue. Slow and fast fibers are present in different slow and fast fibers are present in different ratio in mammalian muscles (Heckman & Enoka. 2012).

1.7. Neuronal transport

Neurons are highly polarized cells in comparison with others. It consists of axon that can run up to a meter or more in some organisms. The transport of vital components like mitochondria, protein, mRNA, and growth factors along the long axons are complex and are mediated through microtubules (Roy et al. 2005). Microtubules work as a rail along the axon and dendrites for the transport. Microtubules are made of polymers of α, β-tubulins.

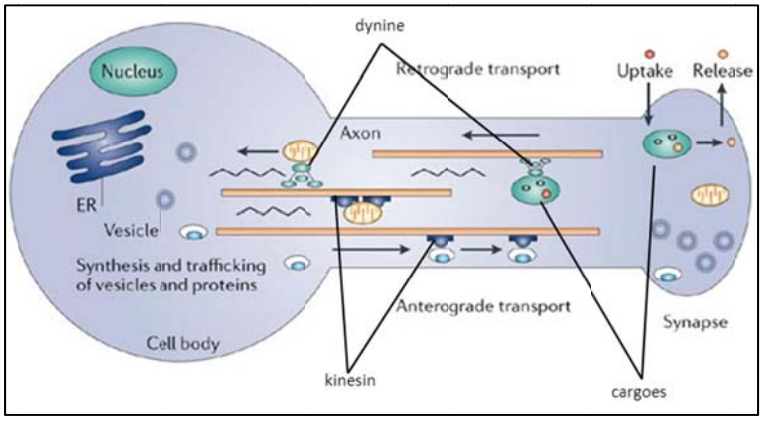

Figure 6. Kinesin and dynein mediated transport in neurons. The cargos can contain mitochondria, proteins, mRNA or growth factors.

kinesins and dyneins are small molecular motors that moves the cargos in specific directions. Kinesin, move towards the plus end of microtubules (plus-end directed motors) and participate in antrograde transport, which is selectively transporting molecules from the cell body to axon and dendrites. On the contrary, dyneins move towards minus end direction (minus-end directed motors) participate in retrograde transport along dendrites (Hirokawa & Takemura. 2005) (Figure 6). These motors utilize the energy of the ATP hydrolysis to move unidirectionally along the microtubules. Therefore, neuronal transport is considered as a critical cellular process for axonal and synaptic function. Defects in the transport along the neurons causes neurodegeneration (Dale et al. 2011; Marinkovic et al. 2012).

In the axon, many membranous organelles are transported from the cell body to the synaptic terminals. These include the components of synaptic vesicles and plasma membrane at synaptic terminals, ion channels, adhesion molecules and molecules that are present in growth cones. Mitochondria are also included in the transport. Molecules that are transported in dendrites include those associated to the postsynaptic structures such as neurotransmitter receptors, ion channels and specific mRNAs (El-Husseini et al. 2001). Therefore, neuronal transport is critical for proper functioning of neuron. For example in disease such as AD is caused by Tau and amyloid beta peptide, HD caused by huntingtin and PD caused by α-synuclein form the accumulation of aggregate prone protein causes defects in this transport and causes neurodegenration (Poças et al. 2015).

1.8. Nervous System Development

The development of multicellular organisms begins with a single-cell, zygote, and it undergoes rapid cell division termed cleavage that produces over 100 of cells to form the blastula. Gastrulation is defined as a single layer midline division of blastula into three layers. At the end of the gastrulation an elongate structure, called notochord, changes the embryo from a circular organization to an axial one—a critical step in the development to form the nervous system. Gastrulation forms three distinct germ layer called the endoderm, the mesoderm and the ectoderm (Figure 7). (i) Ectoderm gives rise to the nervous system and the epidermis, (ii) the mesoderm gives rise to the muscle cells and connective tissue in the body, (iii) the endoderm gives rise to columnar cells that present in the digestive system and many internal organs.

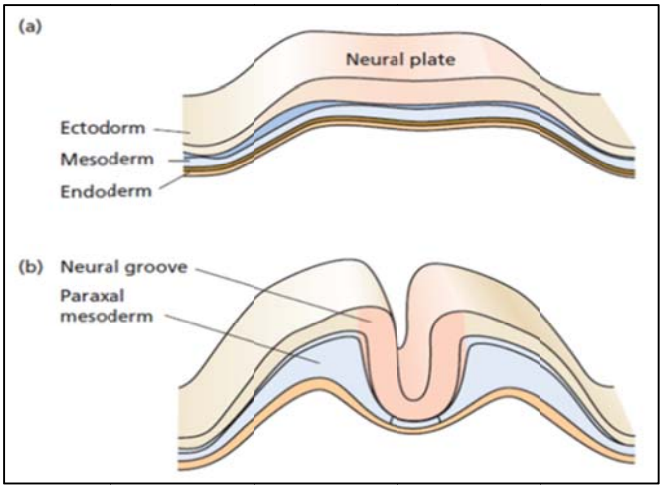

Figure 7. The embryonic germ layer form three distinct parts ectoderm, mesoderm, and endoderm. Ectoderm form the neural plate and folding of neural plate form the neural groove.

The ectoderm undergoes neurulation forming the neural plate in the dorsal part of the embryo. The neural plate folds inward and eventually transforms the neural plate into a tube. This neural tube subsequently gives rise to the brain and the spinal cord. The dorsal part of the neural tube forms the neural crest (Figure 8). The neural crest cells migrate away from the neural tube through loosely packed mesenchymal cells that fill the spaces between the neural tube, embryonic epidermis, and somites. Neural crest cells give rise to a variety of progeny, including the neurons, glia of sensory and visceral motor units, the neurosecretory cells of the adrenal gland and the neurons of the enteric nervous system. Neural crest cells also contribute in pigment cells, cartilage, bones of face and skulls.

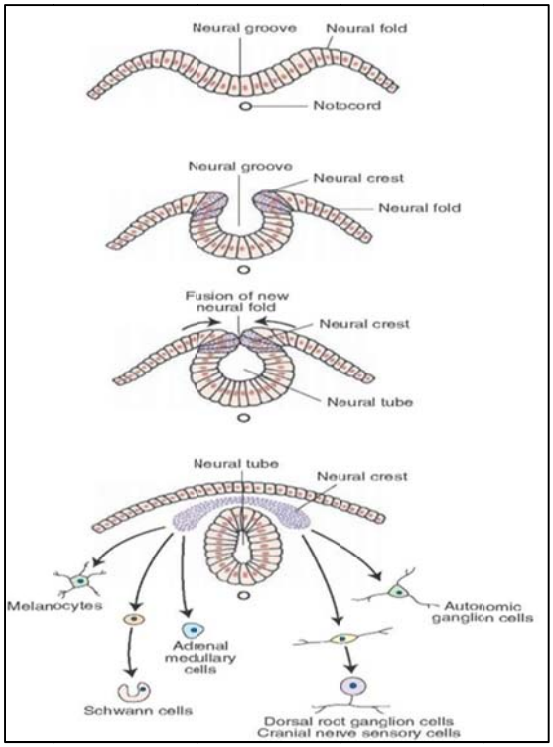

Figure 8. The neural plate folds inward to form neural tube and neural crest cells migrate to form several peripheral nervous system structures.

The cells at the ventral midline of the neural tube differentiate into a particular strip of epithelial-like cells to form the floor plate (Figure 9). The floor plate provides molecular signals to specify the neuroblast cells. The signal induction from both the notochord and floor plate lead to differentiation of cells in the ventral portion of the neural tube that eventually give rise to spinal and hindbrain MNs. MNs are formed at an early stage of embryonic development (E10-E11) (Chen & Chiu. 1992).

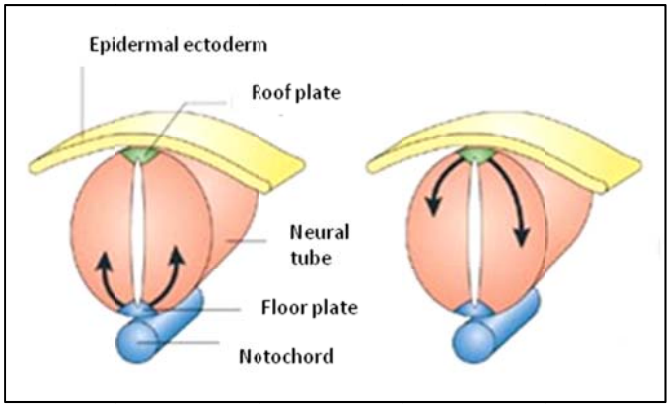

Figure 9. Ventral portion of neural tube differentiate to form the floor plate that signal to differentiate the neuroblast cells.

1.9. Development signaling molecules

The inductive signals from primitive pit and notochord molecules modulate the gene expression in the developing embryo. The signaling molecules include, (i) retinoic acid (RA),

a membrane of the steroid/thyroid superfamily hormone that modulate the expression of a number of target genes, (ii) the bone morphogenetic protein (BMP) and transforming growth factor (TGF) family of proteins are important for a variety of events in neural induction and differentiation, (iii) Sonic hedgehog (Shh) is important for the differentiation of MNs (Patten & Placzek 2000), (iv) Wnt family of proteins modulate several aspects of neural induction and differentiation of neural crest. Each of these molecules is secreted by embryonic tissues- including the notochord, the floor plate, and the neural ectoderm.

These signaling molecules induce changes in shape, motility and gene expression to the target cells for their differentiation (Turner et al. 2014). They produce their effects through binding to specific receptors that are essential to determine the consequence of the inductive signal. For example, the receptors for FGF and BMP families are protein kinases. Upon binding to them, FGF activates RAS/MAP kinase pathway among other intracellular pathways. This signal can modify cytoskeletal and cytoplasmic components and alter the shape or motility of a cell. BMP binds to a serine-threonine kinase receptor, phosphorylate a group of cytoplasmic proteins called SMAD. When SMAD phosphorylates, is translocated into the nucleus, interact with other DNA-binding proteins for transcription, and facilitate gene expression. Shh binds to its surface receptors Patched-1 followed by internalization of the receptor (Murone et al. 1999). This internalization leads to nuclear translocation of transcription factors and modulation of gene expression (Figure 10).

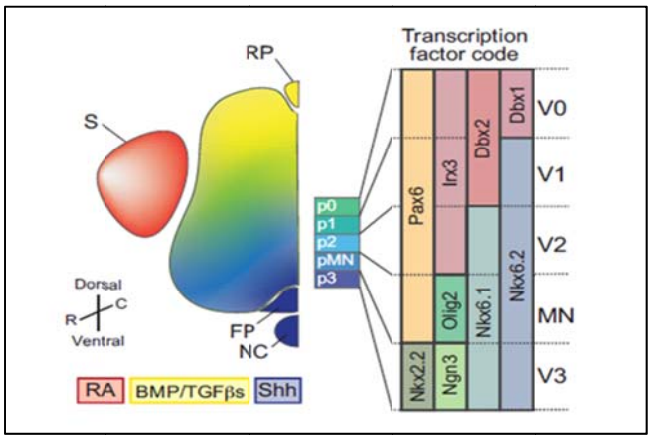

Figure 10. In spinal cord development, signaling molecules act in the ventral axis (Shh) and in the dorsal axis (BMP/TGF β). RA signaling involves in the elongation of rostral-caudal axis of spinal cord. Ventral portion of spinal cord can be divided in several areas p0-p3, where pMN gives rise interneurons and MN differentiation.

1.10. Neurotrophic factors (NTFs)

NTFs are the group of proteins responsible for the growth and survival of developing neurons in the CNS and the PNS. In addition, they are involved in the maintenance of

mature neurons. The target tissue of the neurons often releases the NTFs in order to guide the growth of developing axons. NTFs are capable of regulating several important physiological processes, including neuronal differentiation, maintenance of synapses, neuronal survival through the inhibition of apoptosis, neurogenesis and axonal outgrowth (Tovar-Y-Romo et al. 2014). NTF support is essential for neurons in the spinal cord and can be provided by different cellular sources including astrocytes, microglia, neurons and endothelial cells. In some models, they are capable to promote the re-growing of damaged neurons during injury or degeneration (Hollis & Tuszynski 2011). NTFs have been proposed as a promising therapeutic strategy for neurodegenerative diseases, in cellular therapy for re-innervation of lost neuromuscular synapses, and to improve MN survival (Casella et al. 2010; Grumbles et al. 2009).

There are several families of NTFs including: (i) Neurotrophins: Nerve Growth Factor (NGF), Brain-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin 4/5, (ii) Cytokines: Ciliary neurotrophic factor (CNTF), leukemia inhibitory factor, cardiotrophin-1 (CT-1), (iii) Glial cell line-derived neurotrophic factor family ligands (GFLs): GDNF, Neurturin, Persephin and Artemin, (iv) Fibroblast growth factor and, (v) Hepatocyte growth factor (HGF) (Henderson 1996; Oppenheim 1996; Yamamoto et al. 1997).

MN physiology and development depend on a continuous and tightly regulated NTF support by a variety of cell sources. It supports the generation and survival of MNs at every stage of the developmental process, axonal guidance and synapse formation. The NTFs exert their action by binding to their specific receptors, which in turn activate intracellular signaling pathways that promote cell survival and have protective action on MN both *in vivo* and *in vitro* (Figure 11) (Tovar-Y-Romo et al. 2014).

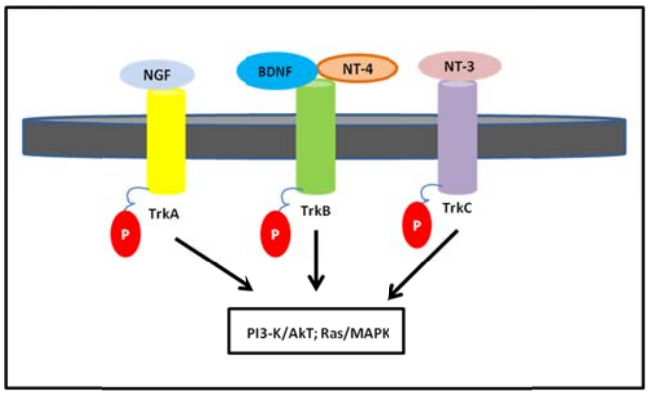

Figure 11. NTFs bind to the specific receptor and activates PI3K/AKT, MAPK/ERK signaling pathways, the response of the cell is survival and differentiation (modified from Segal 2003).

The following NTFs are involved in MN survival and development:

1.10.1. Brain-Derived Neurotrophic Factor (BDNF)

BDNF is a member of the neurotrophin family. BDNF binds to the tyrosine kinase TrkB receptor and induces activation of downstream signaling cascades such as MAPK/ERK and PI3K/AKT pathway responsible for cell survival (Kim 2014). Deficiency in BDNF signaling causes striatal degeneration in Huntington disease model (Maryna et al., 2004). In Alzheimer disease, reduction of BDNF expression increases GSK3β protein, which is responsible for neurodegeneration. Expression of BDNF inhibits the activation of caspase-3 and apoptotic cell death in neuroblastoma cells and protects for degeneration (Kim . 2014). BDNF and Neurotrophin-3 are mainly considered to be important agents for repairing the injured spinal cord (Boyce & Mendell. 2014).

1.10.2. Ciliary Neurotrophic Factor (CNTF)

CNTF is a member of the cytokine family of NTFs that contain interleukin 6 (IL-6), leukemia inhibitory factor (LIF), interleukin 11 (IL-11), oncostatin M (OSM), cardiotrophin-1 (CT-1) and corticotrophin-like cytokine (CLC) (Weisenhorn et al. 1999; Dutta et al. 2007). CNTF play a significant role in brain development, improve spinal cord injury and enhances survival of Dorsal Root Ganglia, hippocampal, striatal and retinal neurons (Stockli 1991; Winter et al. 1995; Peterson et al. 2000; de Almeida et al. 2001). CNTF promotes regeneration of the skeletal muscle and survival of MNs in a variety of models (Pun et al. 2006; Lee & Rydyznski et al. 2013). CNTF signals through the complex of CNTFRα, LIFRβ and GP130 receptor activating downstream cascades including JAK/STAT (Heinrich et al. 1998), PI3K/AKT, MAPK/ERK and NF-kB pathways (Frank & Greenberg 1996; Middleton 2000). Activation of STAT and NF-kB increases transcription of neuroprotective molecules and increases cell survival (Dutta et al. 2007). CNTF also contributes in adult and embryonic MN development (Liu et al. 2014) and it promotes axonal regeneration and functional recovery after peripheral nerve lesion. Its deficiency causes severe loss of MNs and reduces muscle strength (Masu et al. 1993), demonstrating a developmental role of CNTF receptor signaling (Lee & Rydyznski et al. 2013).

1.10.3. Cardiotrophin-1 (CT-1)

CT-1 is an interleukin-6 related cytokine that promotes survival of MNs *in vivo and in vitro* (Oppenheim et al. 2001). It is highly expressed in embryonic skeletal muscle

(Oppenheim et al. 2001). CT-1 signal through the gp130/LIFRβ receptor complex and activates several signaling pathways including JAK/STAT. By CT-1 binding to its receptor, JAK tyrosine kinases gets phosphorylated and activates STAT transcription factor, which activates the transcription of several genes (Stahl et al. 1995). CT-1 induced NF-kB activation promotes the survival of developing neurons (Middleton 2000). #

1.10.4. Glial cell line- derived Neurotrophic Factor (GDNF)

GDNF is a member of the family of NTF named GDNF-family ligands (GFLs) (Treanor et al. 1996). GDNF supports cell survival in a wide variety of neuronal populations, including MNs (Henderson et al. 1994). All GFLs signal through either a common Receptor tyrosine kinase (Ret) or through the neural cell adhesion molecule (NCAM) in cells lacking Ret (Durbec et al. 1996; Paratcha et al. 2003). Ret is activated when GFLs first bind to the specific glycosylphosphatidylinositol-linked co-receptor named GDNF family receptor-α (GFRα). RET activates several pathways, including the PI3K/AKT and MAPK/ERK pathways (Vega 1996; van Weering & Bos 1998). GDNF plays a critical role in entire nervous system development (Lin et al. 1993; Zhu et al. 2014). During brain injury, the presence of GDNF increases survival through activation of MAPK/ERK signaling pathway (Jezierski et al. 2014). GDNF increases survival or protects neuromuscular junction and MNs against neurodegeneration in ALS rat model (Krakora et al. 2013).

1.10.5. Hepatocyte Growth Factor (HGF)

HGF is a multifunctional cytokine that exerts a variety of effects on many cell types. It was first identified as a potent mitogen for hepatocytes (Nakamura et al. 1989). The effects of HGF are mediated via the receptor tyrosine kinase Met (Bladt et al. 1995). Binding of HGF to Met activates cytoplasmic effectors via multifunctional docking sites located in its intracellular domain. Met receptor activates PI3K/AKT pathway and promotes outgrowth of particular MN axons *in vitro* (Maina et al. 2001). HGF promotes the survival of a subset of MNs and also has a role guiding motor axons to their targets (Ebens et al. 1996). HGF promotes survival and axonal regeneration of MNs during spinal cord injury (Kitamura et al. 2007; Wong et al. 2014).

2. REGULATION OF NEURONAL SURVIVAL

Autophagy and apoptosis are the most important self-restrictive processes that are essential for neuronal survival. In the adult brain tissues, the process of replacement cannot occur since neurons cannot divide. Neurons have to adjust all their intracellular processes in order to survive for decades. Hence, the process of regulation of survival is crucial in neurons, and its malfunction leads to neurodegenerative disease. There are two essential cellular processes involved in the maintenance of neuronal cells: apoptosis and autophagy. In several CNS disorders defects in autophagy and apoptosis have been described (Taylor et al. 2008; Sigrist et al. 2014).

2.1. Apoptosis

Apoptosis is the process of Programmed cell-death (PCD) that takes place in the eukaryotic organism. Kerr introduced the term apoptosis in 1972, derived from the Greek word apo "outside" and ptosis "falling" (Kerr et al. 1972). Apoptosis is activated during embryonic development to correct the morphogenesis of cellular material in tissues or organs. It helps in cell homeostasis by maintain a balance between cell proliferation and cell death (Mondello & Scovassi 2010). It causes cell death after DNA damage due to virus attack or environmental factors in order to protect the organs from further damage (Thompson 1995). The apoptotic features observed by light and electron microscopy show morphologic changes that include blebbing, shrinking, nucleus fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Figure 12).

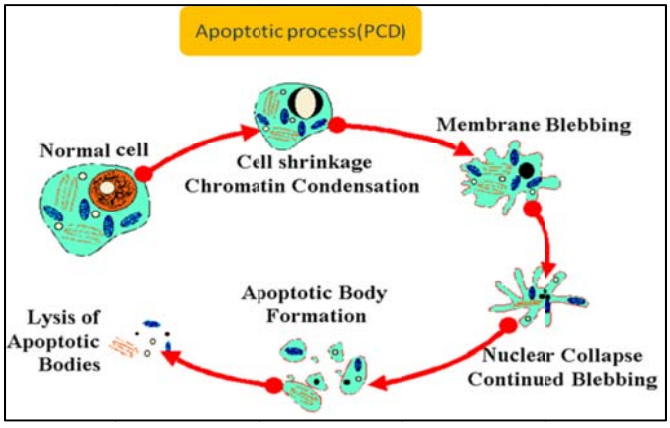

Figure 12. Morphologic characterization of apoptotic cell death and necrotic cell death by enzyme action.

Apoptosis can divide into two main pathways: (i) the extrinsic or death receptor pathway and (ii) the intrinsic or mitochondrial pathway. In both pathways, cysteine aspartyl-

specific proteases (caspases) get activate to cleave the cellular substrates, and lead to the biochemical, morphological changes that facilitate apoptotic death.

2.1.1. Extrinsic pathway

The extrinsic pathway initiates apoptosis through transmembrane receptor-mediated interactions. It consist of death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley et al. 2001). These receptors share similar cysteine-rich extracellular domains and have a cytoplasmic domain called death domain (Ashkenazi & Dixit 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. The best-characterized ligands and corresponding death receptors are FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Ashkenazi & Dixit 1998; Suliman et al. 2001).

The binding of ligand with receptors recruits the cytoplasmic adaptor proteins, which exhibit corresponding death domains that bind with the receptors. For example, binding of Fas ligand to Fas receptor results in the binding of the adaptor protein FADD or binding of TNF ligand to TNF receptor results in the binding of the adaptor protein TRADD and the recruitment of FADD and RIP. FADD associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed and results in the autocatalytic activation of procaspase-8. Once caspase-8 is activated, the execution phase of apoptosis is triggered.

2.1.2. Intrinsic pathway

The intrinsic signaling pathway initiates apoptosis through non-receptor mediated stimuli that produce intracellular signaling directly on targeting cells or through mitochondrial-initiated events. The absence of certain growth factors, hormones and cytokines lead to apoptosis due to the failure of suppression of cell death programs. In addition, other stimuli including radiation, toxins, hypoxia, hyperthermia, viral infections and free radicals, mediate cell death through mitochondrial-initiated apoptosis. All of these stimuli cause changes in the inner mitochondrial membrane.

This results in an opening of the mitochondrial permeability transition (MPT) pore, releases of cytochrome c (Cyt C) into the cytosol (Saelens et al. 2004). Cyt C binds and activate Apaf-1 as well as procaspase-9, forming an “apoptosome”(Chinnaiyan 1999). Clustering of procaspases-9 activates caspase-9 which in turn activates the effector caspase,

caspase-3. Caspase-3 activation leads to the condensation of chromatin and DNA fragmentation, as a characteristic feature of apoptotic cell death (Ulukaya et al. 2011).

Apoptosis is regulated by numerous modulators including changes in Ca2+ influx, Bcl-2 family proteins, p53, caspases or IAPs (Ruchaud et al. 2002; Mattson & Chan 2003; Hoetelmans et al. 2003; Dubrez-Daloz et al. 2008; Alsafadi et al. 2009), and some intracellular organelles such as mitochondria or endoplasmic reticulum (Breckenridge et al. 2003; Gershoni et al. 2009). The Bcl-2 protein family governs mitochondrial membrane permeability either by pro- or anti- apoptotic proteins (Cory & Adams 2002). The anti-apoptotic proteins are Bcl-2, Bcl-X, Bcl-XL, Bcl-XS, Bcl-w, and BAG. The pro-apoptotic proteins are Bcl-10, Bax, Bak, Bid, Bad, Bim and Bik (Elmore 2007). Puma and Noxa are two members of the Bcl-2 protein family that are also involved in pro-apoptotic action (Figure 13).

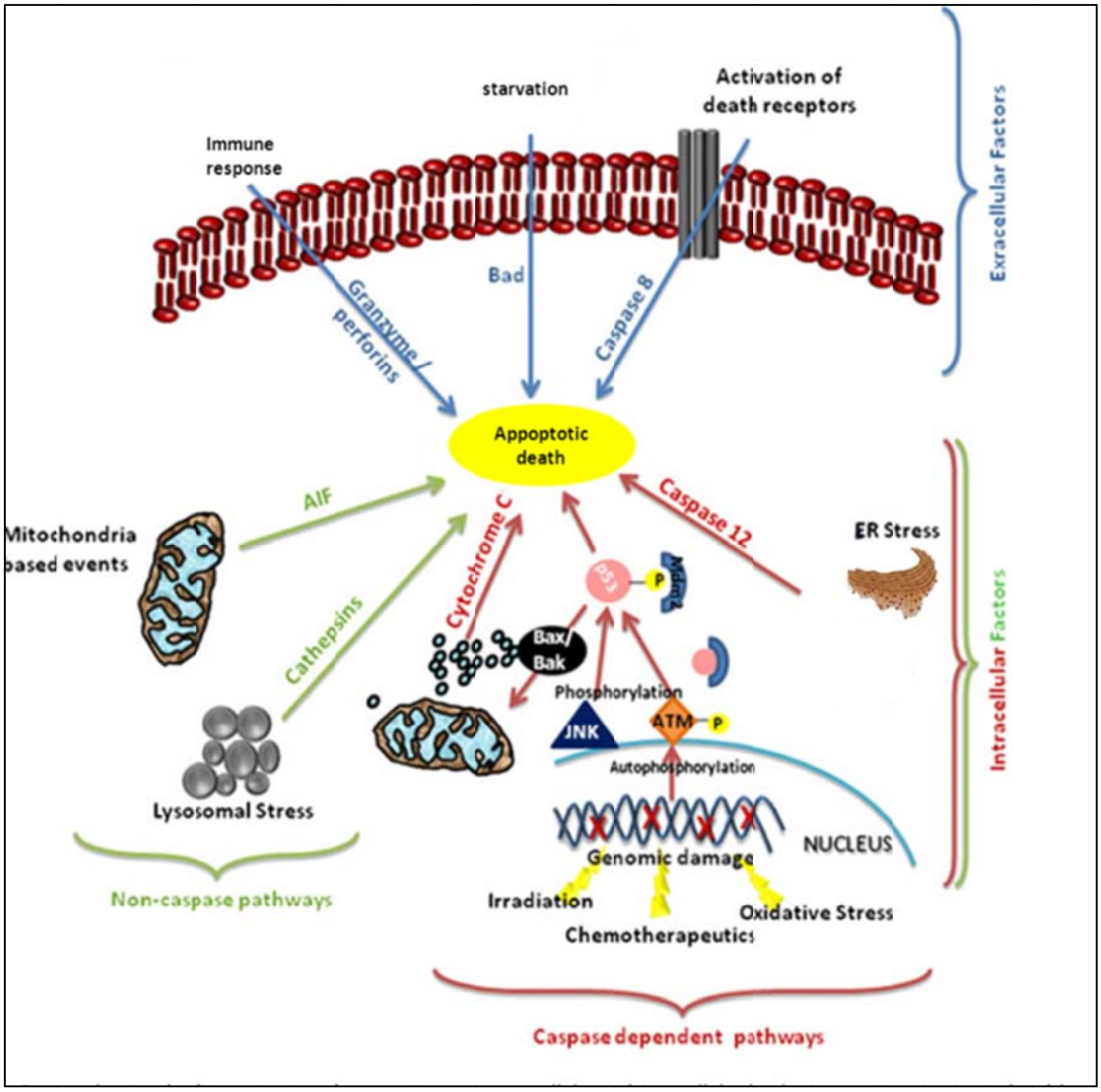

Figure 13. Factors involved in the activation of apoptosis acting at extracellular and intracellular levels (Ulukaya et al. 2011).

Abnormalities in cell death regulation can be a significant cause of diseases such as cancer, autoimmunity, and neurodegeneration. Activation of apoptosis in pathological conditions in neurons can contribute to neurodevelopmental abnormalities. In some pathological features including hypoxic-ischemic damage, trauma, gene mutation or in case of exposure to environmental stress can also alter the apoptosis resulting in neurodegeneration (Roth & D'Sa 2001). For example in ALS, mutation in SOD1 gene resembles apoptotic activated neurodegeneration identified by prolonged caspase-1 and caspase-3 activation in affected MNs (Li et al. 2000). In HD, the increased accumulation of huntingtin polyglutamine repeats causes the activation of apoptotic cell death. In AD, deposition of amyloid β in extracellular surface causes oxidative stress, or Fas ligand

activation induce apoptotic neurodegeneration in neurons and glia (Chen et al. 2000; Ethell & Buhler 2003).

2.2. Autophagy

Autophagy is a significant intracellular mechanism that removes damaged organelles or non-functional proteins in order to maintain cell homeostasis. It is a catabolic process that degrades the cellular components through the autophagosome fusion with the lysosome. It is an important process in the biological functions such as cell survival, cell death, cell metabolism, development, aging, infection, and immunity (Mizushima et al. 2008). Basal level of autophagy should be maintained in order to ensure the balanced cellular metabolism. The failure in autophagy may underlie individual cell dysfunction and causes cell death. Excessive or insufficient autophagic activity in neurons lead to altered homeostasis and influences their survival rate and may cause neuronal degeneration (Wong & Cuervo 2010).

There are three types of autophagy in eukaryotic cells: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). (i) Macroautophagy is the best characterized autophagy, it is characterized by the formation of double-membrane vesicles called autophagosomes, that originate from ER, Golgi complex, and mitochondria. Autophagosome wraps a portion of cytoplasm or organelle which are subsequently delivered to the lysosome. (ii) Microautopagy, the cytosolic components are directly taken up into the lysosome/vacuole lumen by itself. (iii) Chaperon-mediated autophagy involves the translocation of unfolded proteins across the limited membrane of the lysosome through a molecular chaperon, Heat shock protein (Hsp 70) for degradation (Figure 14). When the process is characterized by the selective degradation of peroxisomes or mitochondrias it is termed pexophagy (Sakai et al. 2006) or mitophagy, respectively (Tolkovsky 2009).

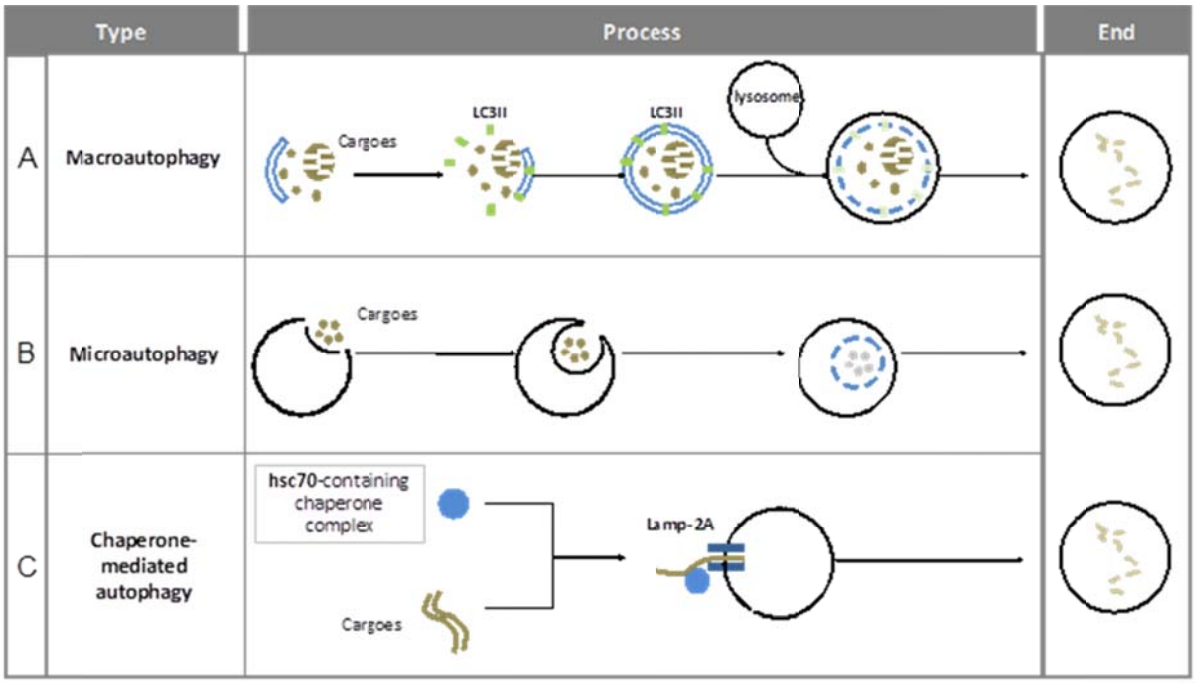

Figure 14. Types of autophagy that take place in mammalian cell. (a). Macroautophagy, recognizes cytoplasmic cargo, form double membrane sac called autophagosome and fuse with the lysosome for degradation. (b). Microautophagy, cargos directly engulfed by lysosome lumen for digestion. (c). Chaperon-mediated autophagy, cargo is recognized by Hsp70 protein and is directly taken to lysosome.

2.3. Macroautophagy

Macroautophagy is the best characterized type of autophagy and it is mentioned as autophagy by itself. Autophagy can be divided into several mechanistically distinct steps including initiation, cargo recognition, vesicle formation, autophagosome-vacuole fusion, and degradation. The breakdown of the cargos by the lysosomal enzymes, release back into the cytosol small peptides of amino acids for recycle (He & Klionsky 2009) (Figure 15).

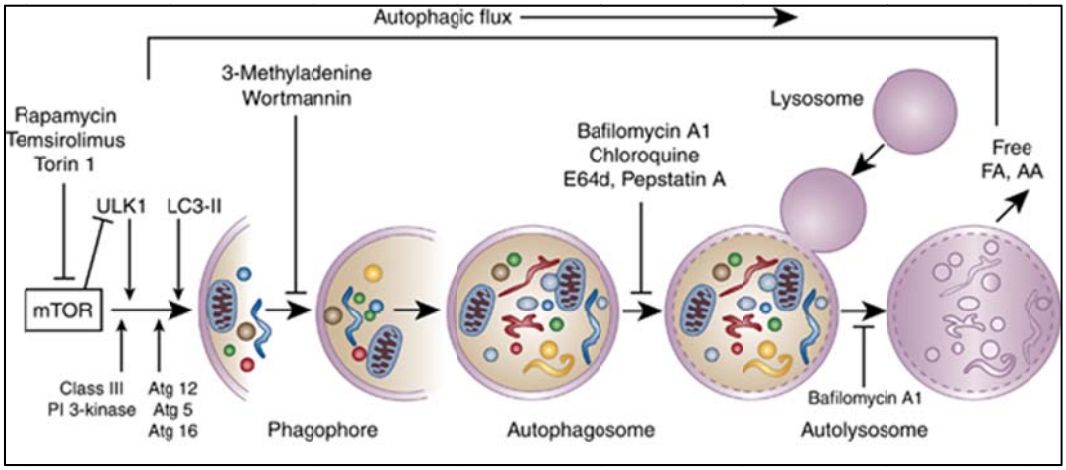

Figure 15. Autophagy is a multistep process that initiated by precursor protein PI3K III, ULK1, Beclin-1, in the formation of phagophore, autophagosome and lysosome fusion for degradation. mTOR control autophagy activation in nutrient rich condition or normal homeostasis of cells.

2.3.1. Autophagy Initiation

Autophagy initiation begins with the formation of isolated membranes in the cytoplasm called phagophore. The membrane source from where phagophore initiates is still under study, and there may be multiple sources. The membrane source can be generated de novo from preexisting intracellular precursor molecules, or can be originated from other intracellular membrane structures like ER, mitochondria, or plasma membrane (Hayashi-Nishino et al. 2009; Ylä-Anttila et al. 2009; Tooze & Yoshimori 2010; Ravikumar et al. 2010). The phagophore engulfs the cytoplasmic cargo by elongation and forms the autophagosome. The formation of new autophagosomes requires the activation of the class III phosphatidylinositol 3-kinase (PI3K). Phosphatidylinositol-3-phosphate (PI-3-P), the product of Vps34, plays a significant role in the activation of phagophore formation. Pharmacological inhibition of Vps34 activity by 3-methyladeine (3-MA) or PI3K inhibitors such as wortmannin and LY294002, inhibits autophagosome formation (Blommaart et al. 1997; Petiot et al. 2000).

PI3-K is a lipid kinase that phosphorylates phosphatidylinositol (PI) at the 3' –position of the inositol ring. In mammalian cells, there are three classes of PI3 K: (i) the class I PI3 K, mainly phosphorylates PI 4,5-bisphosphate to produce phosphatidylinositol 3,4,5-trisphosphate. (ii) the class III PI3 K/h Vps34 only phosphorylates PI to generate phosphatidylinositol 3-phosphate (PI3P). (iii) class II PI3 K, which appears to catalyze PI3P and PI 3,4-bisphosphate from PI (Backer 2008). The class I PI3- K is a heterodimer consisting of a p85 regulatory and a p110 catalytic subunit and is mainly activated via the insulin receptor. The binding of insulin receptors activates AKT by two kinases: PDK1 (Phosphoinositide-dependent kinase-1) and mTORC2. The fully activated AKT acts on the tuberous sclerosis complex (TSC1-TSC2) and Rheb, leads to activation of mTORC1 and subsequently suppress the autophagy (Reiling & Sabatini 2006). The class III PI3 K/h Vps34 is known to be a positive regulator of autophagy. It initiates autophagy via recruitment of other ATG proteins at the isolation membrane or by phagophore (Wu et al. 2010). Inhibition of autophagosome accumulation delays neuronal toxicity induced by a mutant protein (Lee & Gao 2009).

The activity of Vps34 is enhanced by its interaction with Beclin-1 (Furuya et al. 2005). Whereas, the binding of the anti-apoptotic proteins Bcl-XL or Bcl-2 to Beclin-1 inhibits autophagy (Pattingre et al. 2005) (Figure 16).

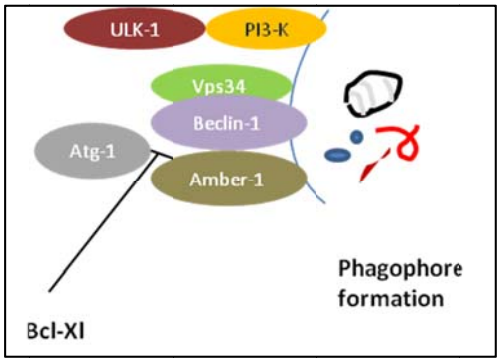

Figure 16. Autophagy initiation by phagophore formatio.

2.3.2. Elongation

Elongation of phagophores requires several Atg (autophagy proteins) such as transmembrane protein Atg9 and the ubiquitin-like protein Atg12 covalently bonded to Atg5 (Mizushima et al. 1998; Young et al. 2006). The complex Atg12-Atg5 forms a conjugate with Atg16L1, which is essential for the elongation of the pre-autophagosomal membrane formation (Figure 17). The second ubiquitin-like reaction requires the protein microtubule-associated protein 1 light chain 3 (MAP1-LC3 or LC3 or yeast Atg8). LC3 is synthesizing as a precursor form and is cleaved at its COOH terminus by the protease Atg4B (Hemelaar et al. 2003) resulting in the cytosolic isoform LC3-I. LC3-I is covanlently conjugated to phosphatidylethanolamine (PE) to form LC3-II (Kabeya et al. 2000). LC3-II is specifically present in both the lumenal and the cytosolic surfaces of the autophagosome until it fuses with the lysosomes. The luminal pool is degrades after fusion with lysosomes, while the LC3-II on the cytosolic side can be delipidated and recycled. This relatively specific association of LC3-II with autophagosomes makes it an excellent marker for studying autophagy (Klionsky et al. 2012).

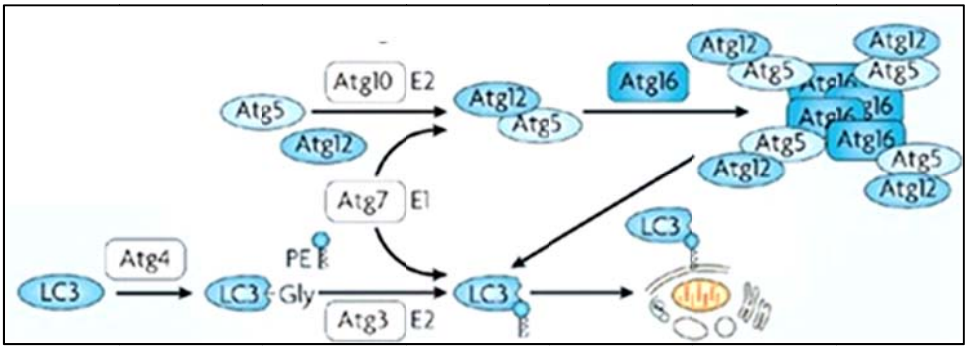

Figure 17. Elongation of phagophore through several Atg protein complexes in autophagy.

2.3.3. Lysosomal Fusion

Autophagosomes after maturation fuse with the lysosomes to form autolysosomes or autophagolysosomes (Mizushima 2007). The inner membrane of the autophagosome and the cytoplasm-derived materials contained in the autophagosome are degraded by lysosomal/vacuolar hydrolases. Yeast lipase Atg 15/Aut5/Cvt17 is one of the enzymes that has been involved in the intravacuolar lysis of autophagic bodies (Epple et al. 2001).

2.4. Mechanisms related to autophagy modulation

2.4.1. mTOR dependent autophagy

Different signaling mechanisms are known to modulate autophagy in mammalian cells (Mehrpour et al. 2010). The classical regulation of autophagy is maintained by the mTOR pathway (Efeyan et al. 2012). mTOR forms two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Laplante & Sabatini 2012). The serine/threonine protein kinase mTOR is important in the regulation of cellular function, such as translation and cell growth (Zoncu et al. 2011). During nutrient rich condition, mTOR inhibits autophagy maintaining its downstream effector ULK1 in an inactive state (Chan. 2009; Hosokawa et al. 2009). Alternatively, inhibition of mTORC1 activates autophagy through ULK1 and promotes autophagosome formation (Klionsky 2000; Shang et al. 2011; Sarkar 2013). mTOR promotes cell cycle and cell survival through PKA-induced PI3 K/Akt pathway (Cutler et al. 1999; Schmelzle et al. 2004). Nutrient deprivation inhibits mTORC1 activity and induces autophagy to recycle intracellular constituents to provide a source of energy.

Rapamycin is a potent inducer of autophagy by inhibiting mTORC1 and it is widely used as an autophagy inducer in therapeutic conditions (Kamada et al. 2004). In mammalian cells, rapamycin helps raptor-mTOR association and inhibits mTORC1 kinase activity (Kim et al. 2002). In neurodegenerative diseases autophagy induction favors the clearance of mutant protein aggregates preventing its accumulation into the cell (Heras-Sandoval et al. 2014).

2.4.2. mTOR independent autophagy

The mTOR independent autophagy is regulated by inositol signaling pathway (Sarkar et al. 2005). This pathway stimulates G protein-coupled receptor-mediated activation of phospholipase C (PLC) that hydrolyses PtdIns(4,5)P2 to form Ins(1,4,5)P3 and diacylglycerol (DAG). Ins(1,4,5)P3 function as a second messenger and binds to its receptor (IP3R) on the

ER. The binding releases Ca^{2+} into the cytoplasm that elicits a range of cellular responses (Berridge 1993). Thus, Ca^{2+} induction suppress the inositol pathway that activates autophagy. An elevated level of intracellular Ins(1,4,5)P3 decreases autophagosome formation. Drugs like lithium, carbamazepine or valproic acid can lower inositol action, induce autophagy and facilitates the clearance of cellular components without inhibiting mTOR activity (Sarkar 2013).

2.5. Control of autophagy by the Ca^{2+} pathway

Autophagy can be regulated by changes in intracellular Ca^{2+} levels (Gordon et al. 1993). Increase of intracellular Ca^{2+} inhibits the autophagosome formation (Williams et al. 2008a). Ca^{2+} influx induces the activity of the calpain proteases, calpain 1 and calpain 2 (Goll et al. 2003; Williams et al. 2008). The activation of calpain by Ca^{2+} channel openers or by overexpression of calpain 2 inhibits autophagy (Williams et al. 2008). Pharmacological inhibitors of calpains (calpeptin) or its genetic knockdown increases autophagic flux through mTOR independent pathway. Calpain modulation of autophagy is mediated by the cleavage-dependent activation of the α-subunit of heterotrimeric G-proteins that increases the activity of adenylate cyclase generating cAMP (Williams et al. 2008). Many cellular stimuli function through Phospholipase C (PLC) activation and generate Ins(1,4,5)P3 that induces the release of Ca^{2+} from intracellular stores. These intracellular events create a link between the Ca^{2+}/calpain and the cAMP pathways, and modulate autophagy.

2.6. Autophagy and neurodegenerative diseases

Autophagy is an essential component in maintaining cellular and tissue homeostasis and neuroprotection (Mariño et al. 2011). In neurons, cellular division is mostly limited in developmental stages, and mature neurons cannot proliferate unlike other cells. It is important to remove damaged organelles and misfolded proteins that forming aggregates inside neuron to maintain healthy survival. Hence, the maintenance of a proper intracellular quality control is one of the most important processes that neurons should facilitate.

Autophagy has been implicated in several human diseases, including neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), Huntington's diseases (HD), Spinobulbar muscular atrophy (SBMA), Spinocerebellar ataxias (SCAs) (Zoghbi & Orr 2000; Ross & Poirier 2004). Autophagy modulation can be considered as a therapeutic strategy for several

neurodegenerative diseases (Sarkar 2013). In this context, cytoplasmic aggregation-prone protein causes neurodegeneration and the autophagy inducers facilitate their clearance. On the contrary, inhibiting autophagy process can lead to the accumulation of the toxic substances (Rubinsztein et al. 2012). Aggregates formed by autophagy dysfunction blocks axonal transport, interfere with intracellular space trafficking and transcriptional dysregulation that is toxic to neurons In HD, accumulation of huntingtin bodies increases and form aggregates causes neurodegeneration. Therefore, presence of autophagy inducer, decrease the aggregates and increases the survival in the cells (Sapp et al. 1997; Tanaka et al. 2004).

2.7. Autophagy modulators

2.7.1. Autophagy inhibitors

2.7.1.a. Bafilomycin A1 (Baf A1)

BafA1 is a macrolide antibiotic isolated from Streptomyces species which is an extremely specific inhibitor of the V-ATPase. Treatment of Baf A1, prevents maturation of autophagosome into autolysosome by inhibiting fusion between autophagosomes and lysosomes, thereby it blocks the autophagy degradation (Bowman et al. 1988). Vacuolar H+ ATPase (V-ATPase) is localized in organelles of the central vacuolar system such as coated vesicles, endosomes, lysosomes, Golgi apparatus and maintains the acidic environment in these compartments. Lysosome has an extremely acidic pH and during the autophagy process the fusion of the autophagosome with the lysosome causes the degradation of its cargo. BafA1 treatment induces an increase of autophagosomes into the cytoplasm and can also be used for the measurement of the autophagy flux (Figure 18).

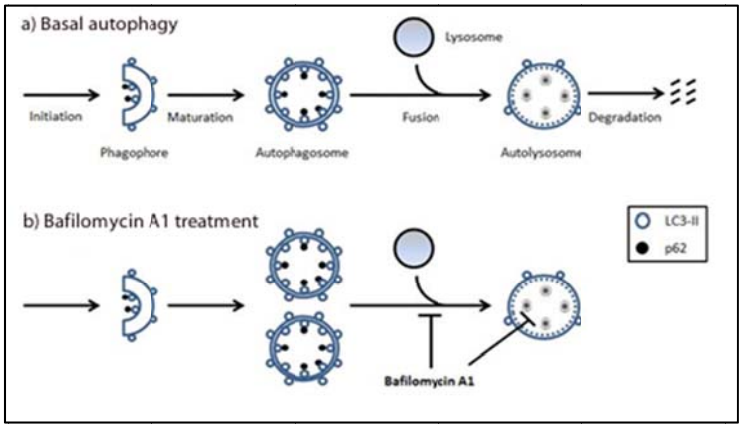

Figure 18. (a). Basal level of autophagy in normal condition of equilibrium. (b). Treatment of BafA1, interfere with fusion of autophagosome with lysosome causes increased accumulation of autophagosome in the cell cytoplasm.

2.7.1.b. Ammonia

Treatment of ammonia inhibits autophagosome-lysosome fusion by alkalizing lysosomal pH (Lu et al. 2013). Each organelle maintains a characteristic internal pH, which is essential for facilitating proper functioning. The most extreme example of an organelle acidification in mammalian cells is the lysosome. It is maintaining an extremely acidic pH (less than pH 5.0) in its lumen to successfully perform its digestive function and to drive efflux of digested materials (Mindell 2012). Therefore, Ammonia treatment increases the accumulation of autophagosome formation by decreasing lysosomal fusion degradation.

2.7.1.c.3-Methyladenine (3-MA)

3-MA blocks autophagy activity through inhibition of class III phosphatidylinositol 3-kinases (PI3K) (Seglen & Gordon. 1982; Blommaart et al. 1997) (Figure 19). Class III PI3K inhibition and reduces the number of autophagosomes.

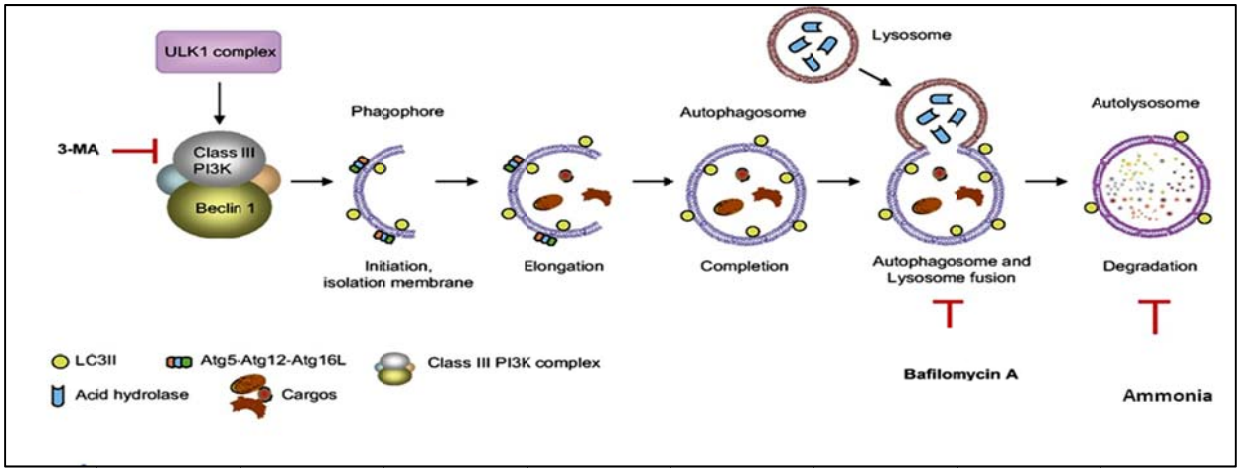

Figure 19. 3-methyladenine (3-MA) inhibits class III PI3K and phagophore initiation process. BafA1, blocks the fusion of autophagosome and lysosome; and Ammonia blocks autophagy by inhibiting autophagosome with lysosome fusion.

2.8. Autophagy inducers

2.8.1. mTOR dependent

2.8.1.a. Rapamycin

Rapamycin, also termed sirolimus, is a natural antibiotic isolated from *Streptomyces hygroscopicus.* It is widely used as an autophagy inducer through the inhibition of mTOR. Rapamycin binds to mTOR by forming a complex with the immunophilin FK506-binding protein (FKBP12) that induce autophagy (Berger et al. 2006). Rapamycin is considered as a neuroprotective factor after spinal injury and ROS condition, which help in clearance of accumulated cargos from cytosol (Song et al. 2014). Rapamycin promotes the clearance of

other intracytoplasmic disease-associated or aggregate-prone proteins associated with neurodegeneration (Berger et al. 2006; Williams et al. 2006; Menzies et al. 2015).

2.8.1.b. Curcumin

Curcumin is a natural compound that is present in plant *Curcuma longa* (turmeric). It is used in dietary or medicinal purposes for thousands of years among many regions (Gupta et al. 2012). Curcumin enhances autophagy proteins LC3II and SQSTM1 through the activity of AMPK signaling pathway (Xiao et al. 2013).

2.8.2. mTOR independent

2.8.2.a. Trehalose

Trehalose is a non-reducing disaccharide present in many organisms including yeast, fungi, bacteria, invertebrates, plants and insects. Its function is to protect protein denaturation and the integrity of cells against various environmental stress like heat, cold, desiccation, dehydration and oxidation (Chen & Haddad 2004). It is not clear how Trehalose protects protein from toxic effects, but it has been suggested the involvement of its its action as chemical chaperone that help in proper functioning of mutant proteins (Brown et al. 1996). Trehalose induces autophagy through the mTOR independent pathway and helps in the clearance of protein aggregates (Arora et al. 2004; Tanaka et al. 2004; Liu et al. 2005).

2.8.2.b. Resveratrol

Resveratrol is a plant product commonly found in the root of Japanese Knotweed and the skin of red grapes. Resveratrol helps in plant to produce phytoalexin, a chemical protection response against harmful stimuli like UV radiation, infection or other pathogenic threats (Jeandet et al. 2013). Resveratrol induces autophagy through cAMP signaling pathway in mTOR independent process (Chen et al., 2013). It increases intracellular Ca^{2+} that results in the phosphorylation and activation of AMPK (Zhang et al., 2014). Resveratrol induced autophagy helps in neuroprotection, reduce oxidative stress and prevents mitochondrial damage (Wang et al., 2014).

3. SPINAL MUSCULAR ATROPHY (SMA)

Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disease described for the first time by Werdnig-Hoffmann in 1890s. It causes muscle weakness and atrophy associated with progressive degeneration of alpha MNs in lower lumbar and bulbar region of spinal cord (Markowitz et al. 2012). It is considered as one of the most common genetic diseases that affects 1 in 6000 to 10000 newborn (Pearn 1978). SMA is caused by mutation or deletion of the telomeric gene copy, *Survival Motor Neuron 1* (*SMN1*) located on human chromosome 5q13 (Lefebvre et al. 1995). Humans have two identical *SMN* genes one copy of *SMN1* in the telomeric position and multiple copies of *SMN2* gene in centromeric position. Both genes are essential for the synthesis of Survival Motor Neuron (SMN) protein. SMA affected individual have at least one copy of *SMN2* gene and the number of copies of *SMN2* is variable between patients. SMN is present in all type of cells and its particular role in MNs is still unknown.

3.1. SMN gene

In 1990 the gene responsible for the SMA was discovered as *SMN* that is present on chromosome 5 in the region between q11.2-13.3 (Melki et al. 1990). There are four genes present in this area: *SMN*, *Neural apoptosis Inhibitory Protein (NAIP)*, *p44 encodes a basal subunit of transcription factor TFIIH*, *Small EDRK-Rich Factor (SERF1*) (Lefebvre et al. 1995; Roy et al. 1995). These four genes are present as duplicates in telomeric and centromeric regions of human and primates.

The sequences of *SMN1* and *SMN2* gene consist of nine exons. *SMN1* gene differs from *SMN2* at C to T transition at sixth position of exon 7. This change causes alternative splicing of exon 7 during pre-mRNA transcription and produces an unstable or truncated SMN protein. The transcripts lacking exon 7 have impaired their ability to oligomerize and associate with their binding partners and may be rapidly degraded. Therefore, only 10% of SMN protein synthesis by *SMN2* gene is functional with exon 7 included (Figure 20).

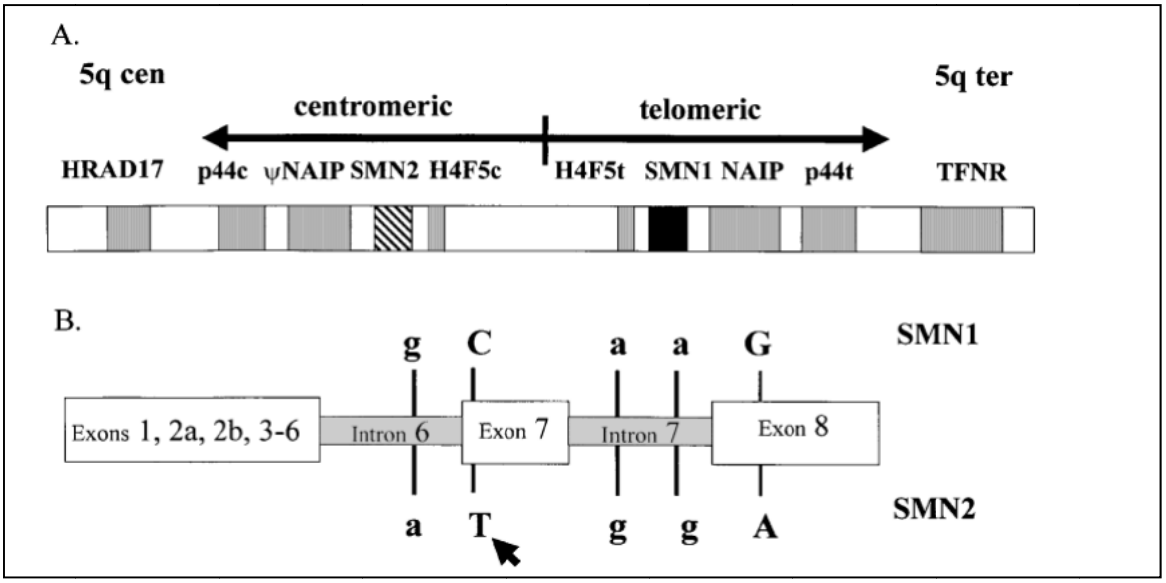

Figure 20. (A) Localization of SMN gene in chromosome 5, one copy of SMN1 and multiple copies of SMN2. (B) SMN1 and SMN2 differ because of a C to T transition in sixth position of exon 7. (obtained from (Wirth 2000).

Two competing models have been proposed to explain the mechanisms by which C to T transition disturbs the natural splicing of *SMN2*.

(i) The C to T alteration disrupts the binding of the exonic splicing enhancer SF2/ASF (ESE model). Disrupting the binding of an active regulator of splicing present in *SMN2* leads to inefficient definition of exon 7 and results in predominantly skipped spliced product (Figure 21).

(ii) The C to T alteration creates an exonic splicing silencer (ESS model) which binds hnRNP A1 (ESS model). hnRPN A1 binds specifically to the *SMN2* pre-mRNA transcript and disrupts the binding of other splicing factors leading to increased skipping of exon 7 (Bebee et al. 2010). Likewise, serine/arginine (SR) splicing factor protein family plays a role in the influence in exon 7 inclusion. SRSF1 binds and promote exon 7 inclusion in SMN1. However, the C to T transition in exon 7 of *SMN2* disrupts this exonic splicing enhancer motif, contributing to the low level of *SMN2* exon 7 inclusion. Hence some of these SR and hnRNP found to be involved in the inhibition of exon 7 splicing inclusion in *SMN 2* (Wee et al. 2014).

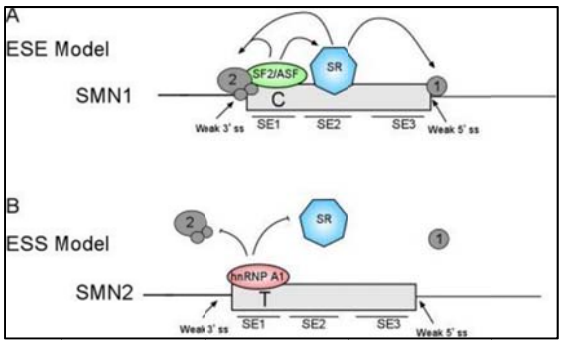

Figure 21. Exon 7 ESE and ESS model of pre-mRNA splicing. (Bebee et al. 2010).

In general, this C to T transition during pre-mRNA transcription exon 7 spliced alternatively. This results in the synthesis of 80–90% of *SMN2* mRNAs lacking exon 7 (SMNΔ7). SMNΔ7 is not fully functional and rapidly degraded (Lorson & Androphy 2000; Cho & Dreyfuss 2010). However, *SMN2* can also provide some fully functional SMN protein; consequently the copy number of *SMN2* modifies the severity of SMA phenotype in humans (Figure 22).

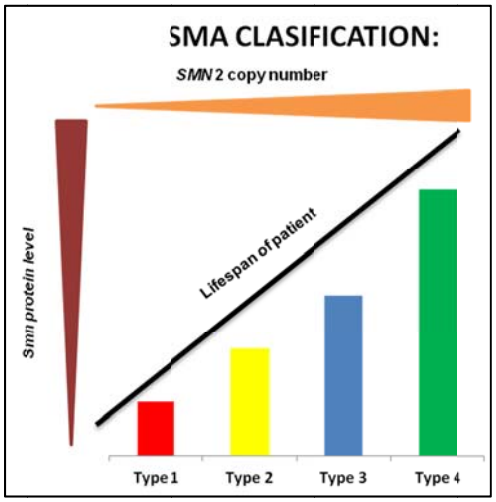

Figure 22. Classification of SMA depends on copy number of SMN2 gene.

3.2. Classification of SMA

SMA is classified into four types depending upon the onset of the disease and the *SMN2* copy number (Russman 2007)

1. Type I SMA or Werdnig-Hoffmann disease is the most common and severe form, representing 45% of cases. It associates with the onset after birth but before the age of 6 months. Infants with type I SMA usually have 1 or 2 copies of *SMN2*. Symptoms are characterized by a severe muscle weakness and hypotonia and respiratory problems occur early during the disease progression. Because of severe hypotonic weakness in the lower limbs, affected babies develop a splayed leg or frog-leg. A bell-shaped chest results in reduced diaphragm strength and breathing problem during inspiration. Tongue fasciculation is also common. Facial and eye muscles spared. Patient cannot sit independently. In the most of cases, the natural history includes death prior to the age of two (Thomas & Dubowitz. 1994).
2. Type II SMA or chronic SMA, represents about 20% of cases, the onset of the disease is between 6 and 18 months. Type II SMA usually associates with 3 copies of *SMN2*. Type II children never stand or walk independently, but some patients will be able to

stand with the assistance of supportive equipments. Lower limbs are prone to severe weakness. Facial and eye muscle spared and tongue fasciculation is similar to Type I SMA. Impaired swallowing and ventilator insufficiency are frequent in Type II patients. The majority of patients with SMA type II survives until age of four and sometimes reaches adulthood stage.

3. Type III SMA or juvenile SMA or Kugelberg-Welander disease occurs in the 30% of cases. Type III SMA usually associates with 3 or 4 copies of *SMN2.* Age of onset is between 18 months and adulthood. Standing or walking without support is achieved later. Type III symptoms include falls, difficulty in climbing stairs, and other features of proximal weakness. Survive until adulthood.
4. Type IV SMA represents less than 5% of patients. They usually have five to six copies of *SMN2* gene. It is the milder form of SMA. The symptoms are similar to type III and are distinguished by later onset during adulthood. Age of onset is not explicitly defined but is often considered to be at age of 30 or later (Castro & Iannaccone 2014).

Types	Onset	Causes	Lifespan
Type I SMA (Werdnig-Hoffmann)	Six weeks from birth	Severe muscle weakness and hypotonia in few months. Fatal respiratory failure usually occurs before the age of 2 years.	Less than two years.
Type II SMA (intermediate)	Before 18 months	Ability to sit but not to walk unaided	Survival beyond four years of age and sometimes they reach the adolescence stage.
Type III SMA (Kugelberg-Welander)	After two years	An ability to walk independently for some distance and less severe respect to SMA type I and II.	Adulthood
Type IV SMA	After five years	The disease evolves slowly and does not affect lifespan.	Normal lifespan

Table 2. Classification of SMA and its phenotypes

3.3. Survival Motor Neuron (SMN) Protein

SMN is a ubiquitously expressed protein with a molecular weight of 38 kDa. It consists of 294–amino acid polypeptide expressed in both the cytoplasm and the nucleus (Liu & Dreyfuss 1996). In the nucleus, it forms complex structures called gems that overlap with or are closely opposed to Cajal bodies (Fischer et al. 1997; Liu et al. 1997). Cajal bodies contain high levels of factors involved in the transcription and the processing of many types of nuclear RNAs. SMN binds or associates with many binding partner proteins and form complexes involved in many cellular functions. For example, snRNP biogenesis, axonal transport in neurons or pancreas development and the regulation of glucose metabolism (Bowerman et al. 2014).

3.3.1. Assembly of snRNP biogenesis

The best characterized SMN function is to regulate the assembly of the small nuclear ribonuclear proteins (snRNPs) (Yong et al. 2004). SMN protein forms complexes with other proteins in the nucleus where they accumulate in structures called Cajal bodies. These complexes play an essential role in the assembly of spliceosomal snRNPs and biogenesis during mRNA processing (Fischer et al. 1997; Meister et al. 2002; Paushkin et al. 2002). The SMN (biogenesis) complex is composed by the SMN protein and seven additional proteins including Gemin2-8 and Unrip (Baccon et al. 2002; Carissimi et al. 2006).

It has been proposed that the interaction of SMN with Gemin complex is a functional requirement for MN function (Borg & Cauchi 2013). Decreased levels of Gemin2 in a *Smn +/-* background induce MN degeneration and correlates with disturbed snRNP assembly. When the interaction of Gemin with SMN protein is disrupted the snRNP biogenesis decreases (Borg & Cauchi 2014) (Figure 23).

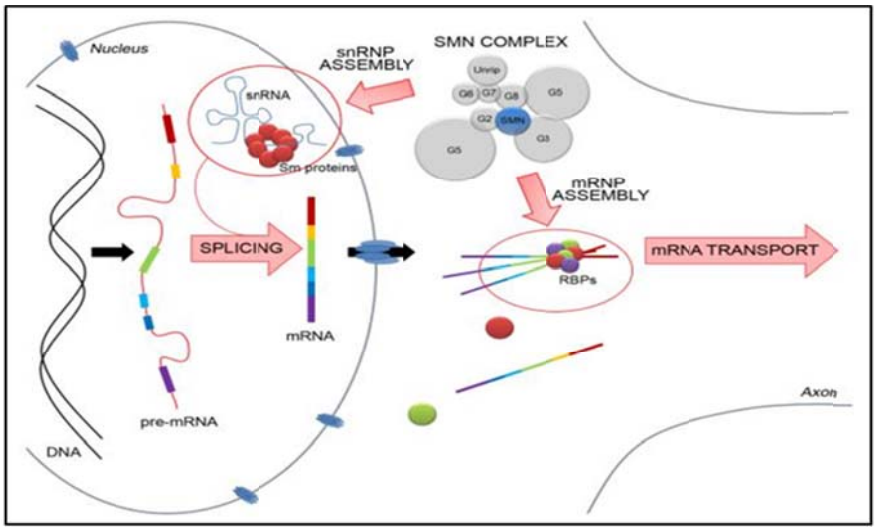

Figure 23. SMN protein, the primary role in the assembly of snRNP biogenesis and pre-mRNA splicing requirement. SMN protein forms complexes with other co-proteins (Unrip, Gemin2-7) in the cytoplasm and inside nucleus it forms Cajal bodies that involve in splicing of intron during pre-mRNA transcription process.

3.3.2. Embryonic development

SMN is necessary for the development during the blastocyst stage (Hsieh-Li et al. 2000). In contrast to the human genome, mouse genome contains only one copy of *Smn* gene, and its homozygous disruption leads to massive cell death during early embryonic development (Schrank et al. 1997). This particular mutation in *Smn* highlights the essential housekeeping function of SMN during development. In *zebrafish* MNs mutation in *Smn* also shows abnormal development. Deletion of *Smn* in mouse shows decrease in the number of progeny and locomotive defects. In addition knockdown of *SMN* leads to sterility caused by a defect in germ cell maturation. This unusual behavior in the germline, indicate that *SMN* may be also involved in particular gene expression events at very early developmental stage (Miguel-Aliaga et al. 1999).

3.3.3. Axon transport and growth cone

In addition snRNP assembly SMN may have other functions in MNs. The SMN protein can form granules that are actively transported in neuron processes and growth cones (Zhang et al. 2003). In axons and neurites SMN binds to the protein hnRNP that in turn binds to the β-actin mRNA (Rossoll et al. 2003). This interaction is essential for the transport of β-actin mRNA to the growth cones of MNs. The presence of β-actin mRNA and protein in the growth cone facilitates axonal outgrowth, where the actin is the driving force for growth cone mobility. Deficiences in SMN protein level fails to bind with β-actin and its delivery in growth cone decreases. In this context MNs show shortened axons and small growth cones

(Figure 24). Therefore, SMN protein is important in the transport of mRNPs and mRNAs for normal outgrowth and maintenance of MN axon and growth cone.

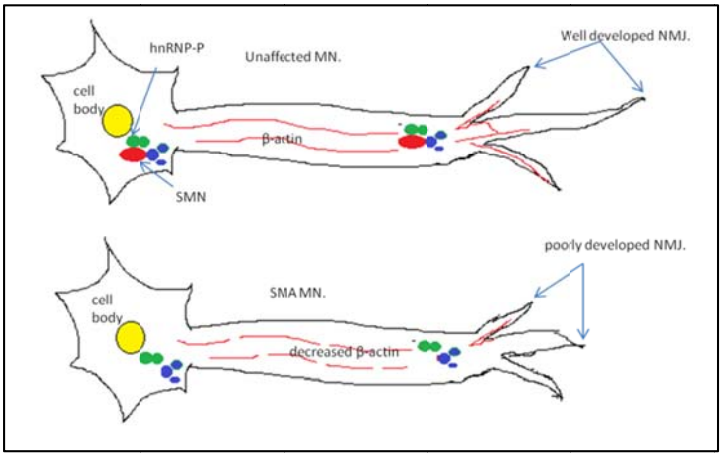

Figure 24. SMN interacts with hnRNP-P and complex with β-actin mRNA. Absence of SMN decreased β-actin delivery in the growth cone and results in reduced size.

SMN protein is also essential in the neuromuscular junction (NMJ) synaptic function. SMN reduction causes decrease in the amplitude of evoked endplate current, abnormalities in the neurotransmission, NMJs weakness and cardiac defects in SMA mouse model (Kariya et al. 2008; Kong et al. 2009; Heier et al. 2010; Shababi et al. 2010). Smn knockout causes axonal outgrowth and pathfinding defects in the *zebrafish* model (McWhorter et al. 2003).

3.4. SMN protein degradation process

SMA causes reduced synthesis of SMN protein due to *SMN1* mutation. Because SMN disease severity correlates with SMN protein levels, a major goal of SMA therapeutics development is to identify compounds that increase SMN protein levels. Drug treatments that lead to increased SMN expression could be not sufficient without a basic understanding of SMN protein dynamics since the protein may be degraded as quickly as it is syntethized. Therefore, studies of the regulation of SMN protein stability are essential for SMA therapeutics.

There are two major cellular clearance pathways involved in protein degradation – the proteasome system and the autophagy/lysosomal process (Cortes & La Spada 2014). Recent studies show that the degradation of intracellular proteins occurs predominantly through the proteasome pathway (Ciechanover et al. 2000). Therefore, regulation of SMN protein degradation by protease process can prevent further loss of SMN protein from endogenous *SMN2* synthesis.

3.4.1.Proteasome

Proteasome is a large, barrel-shaped protease complex that removes the intracellular misfolded, oxidized or aggregated proteins in the presence of ubiquitin (Ub) and ATP. The UPS (ubiquitin proteasome system) is one of the most significant degradation mechanisms that remove the aggregated or misfolded proteins. This pathway composes of the Ub-conjugating system and the 26S proteasome, whereas the latter degrades polyubiquitin-conjugated proteins via a multicatalytic proteinase complex. The conjucation of Ub to the protein substrate proceeds by step wise process. It has been described that proteasome has a direct effect on SMN degradation in SMA derived cell lines (Chang et al. 2004). The proteasome and the autophagy systems play a dependent role in protein degradation, for example proteasome inhibition activates the autophagy in order to prevent protein accumulation (Shaid et al. 2013).

3.4.2. Ca^{2+} activated calpain protease

Neurons depend on Ca2+ level to maintain many functions including cell survival. Ca^{2+} activates cysteinyl/thiol proteases consist of several tissue-specific isoforms (n-calpains) and two ubiquitous isozymes (μ-calpain and m-calpain). The ubiquitous μ-calpain (calpain I) binds calcium with relatively high affinity, whereas m-calpain (calpain II) bind with low affinity. Calpain I and II are found specifically in distinct subcellular localization and thus help in particular physiological function (Hamakubo et al. 1986). Calpain I, present higher levels in the dendrites and cell bodies of neurons whereas calpain II exist relatively higher level in axons and glia (Nixon 2003). Calpain is important for certain cell functions including: cell development, cell growth cone motility and guidance, memory and learning (Arthur et al. 2000; Glading et al. 2002; Robles et al. 2003).

During pathological condition, the ability of neuron to control Ca^{2+} fluxes and recover from a Ca^{2+} level is compromised. This excessive influx of Ca^{2+} can result in the activation of several enzymes, such as proteases that lead to neuronal degeneration and cell death (Das et al. 2005). For example, elevation of Ca^{2+} activates calpain and it degrades essential proteins MNs survival (Mattson 2007; Gou-Fabregas et al. 2009). MNs are particularly more vulnerable to Ca^{2+} influx than other neuronal cells. .

Recently it was proposed that SMN can be a direct target of calpain cleavage. It has been observed that SMN is a sarcomeric protein present in both skeletal and cardiac myofibrils. This sarcomeric position of the protein is a cleavage site for calcium and calpains

(Walker et al. 2008). The truncated cytosolic protein are more sensitive to calpain cleavage and level of calpain action is more in this condition (Fuentes et al. 2010).

3.5. SMA Models

Due to limited availability of suitable human material they are many animal models available to study the SMA disease. The development of the animal models helps in the identification and validation of the efficacy of potential therapeutics for the disease.

Table 3. Percentage of SMN genome identity (similarity) within species

	Human	Mouse	*Zebrafish*	*C.elegans*
Human	100	83 (88)	52 (66)	36 (56)
Mouse	-	100	52(66)	38 (58)
Zebrafish	-	-	-	37 (58)
C.elegans	-	-	-	100

3.5.1. Caenorhabditis elegans

The nematode *C. elegans* is used as an efficient model for studying several disease-related gene expression and function. Genetic mutation in *C. elegans* can be induced by exposing worms to mutagens, like ethylmethanesulfonate (EMS) and gamma irradiation. RNA interference (RNAi)-mediated knockdown of gene expression is also useful by feeding worms with small interfering RNA (siRNA) libraries. The *C. elegans* genome contains a single SMN ortholog, Smn-1, that encodes an Smn protein 36% identical to the human ortholog (Bertrandy et al. 1999).

To study the developmental defects caused by SMA it was constructed a *C.elegans* with a deletion in *CeSMN* in egl-32 promoter region. Reducing the expression of *Smn-1* by RNAi causes larval lethality suggesting that *Smn-1* is essential for survival of *C. elegans* animals (Miguel-Aliaga et al. 1999). The developmental defects and the embryonic lethality limited further investigation on postembryonic roles of SMN. In *Smn1* (ok355) deletion allele, in which the *Smn1* translational start codon is removed. Therefore, the deletion of Smn1 in maternal shows late larva arrest, reduced lifespan as well as impared locomotion and loss of MNs (Briese et al. 2009).

3.5.2. Drosophila melanogaster

The *Drosophila* genome contains a single copy of SMN ortholog, *Smn*, with 41% sequence homolog to human *SMN1*. A SMA Drosophila model was established when several missense mutations in the Drosophila Smn gene resulted in a failure of the complex

formation in snRNP biogenesis (Will & Lührmann 2001). These mutant embryos survive until the late larval stage due to the presence of maternal wild-type Smn. The resulting larvae develop severe motor abnormalities related to disorganization of the neuromuscular junction and impaired clustering of postsynaptic neurotransmitter receptor. This phenotype was counteracted by expression of wild-type Smn protein in both MN and muscle but not in either tissue alone. This phenomenon suggested that Smn deficiency causes defects in both pre- and post-synaptic sides of neuromuscular junction (Chan et al. 2003).

The RNAi-mediated knockdown of Drosophila Gemin 3 leads to disruption in developing muscle, flightless in adults and muscle degeneration. The Gemin interaction with Smn protein is important in larval motor function (Cauchi et al. 2008).

Drosophila with Hypomorphic mutations in *Smn* causes reduced Smn protein level in the adult thorax, causing flightlessness and acute muscular atrophy. Mutant flights display pronounced axon routing and arborization defects (Rajendra et al. 2007). Presence of maternal wild-type Smn protein helps in survival of mutant Smn zygotic, and mutant larvae show severe motor abnormality and lead to paralysis in the later stages. This delayed lethality helps in understanding the effect of decreased activity of Smn in the neuromuscular system from Drosophila model organism (Chan et al. 2003).

3.5.3. *Zebrafish*

The *Zebrafish (Danio rerio)* is an excellent model organism for neurodegenerative research because of its conserved simplified vertebrate nervous system. It is much easier to produce transgenic organisms and to analyze genetic studies using this model (Grunwald & Eisen 2002). Due to its well-characterized MN circuits and relatively simple neuromuscular organization, this model is well suitable for the study of neuromuscular defects and MN diseases (Ramesh et al. 2010). The optical transparency of *zebrafish* embryos offers MN's accessibility , which can be manipulated *in vivo* for imaging, electrophysiology, and motor behavioral studies (McLean & Fetcho 2011).

A zebrafish model of SMA was created by morpholino antisense oligonucleotide knockdown technique to reduce the endogenous Smn protein level. The results show that fishes with defects in MN axonal outgrowth and pathfinding during development, without affecting other neurons or muscles, indicating that Smn has an essential role in MN development (McWhorter et al. 2003).

The knockdown of Smn binding partner Gemin2 also resembles the same phenotype of Smn reduction. Whereas injection of purified U snRNP into Smn or Gemin2-deficinet embryo rescues the phenotype. Therefore, the study indicates that the effects caused by Smn or Gemin2 reduction in MNs can be originated from insufficient levels of snRNP synthesis (Winkler et al. 2005).

Reducing Smn levels in single MNs in zebrafish, it has been found that defects are due to a cell-autonomous function of Smn in MNs and constitutes the primary discovery of morphological abnormalities during MN development in response to low levels of Smn. These data reveal that early reduction of Smn protein severely compromises motor axon outgrowth showing the essential role of Smn in MN development (McWhorter et al. 2003).

Expressing a human SMN2 transgene in *zebrafish*, carrying endogenous smn mutations closely resemble the genetic characteristics of human SMA (Hao et al. 2011). The feature of this model showed that SMN2 is similarly spliced in fish as in humans, producing decreased levels of full-length SMN protein and comparatively high levels of exon 7 spliced protein. The manipulation of SMN2 transcripts via an antisense oligonucleotide sequence directed against an intronic splicing silencer site in intron 7 was sufficient to promote exon 7 inclusion. Thus, increase in full-length SMN levels (Hao et al. 2011).

3.5.4. Murine

Similar to other subprimates, mouse contains only one *Smn* gene. Therefore, complete *Smn* gene knockout *Smn(-/-)* null mice leads to massive cell death during early blastocyst formation and to embryonic lethality (Schrank et al. 1997). To determine *Smn* gene defect in skeletal muscle, deletion of murine SMN exon 7, the most frequent mutation found in SMA, has been restricted to skeletal muscle by using the Cre-loxP system. Mutant mice display muscle necrosis with a dystrophic phenotype leading to muscle paralysis and death (Cifuentes-Diaz et al. 2001).

The type I SMA mouse model was generated by introducing one normal copy of human *SMN2* transgene to Smn +/- mouse, generating a *Smn-/-; SMN2tg/tg*. These mice have normal number of embryos with correct number of MNs at birth. However, the amount of MNs is vastly reduced by postnatal day 5 and the animals death by postnatal days 4 to 6 (Monani et al. 2000) (Figure 25).

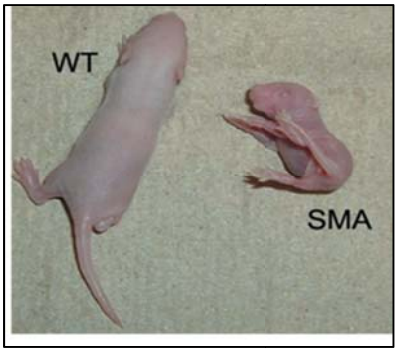

Figure 25. Images of wild typer and SMA mouse embryo Smn(-/-); SMN2+/+.

It has also been generated a triple mutant mouse (SMNΔ7) that harbors two transgenic alleles and a single targeted mutant. The Tg(SMN2*delta7)4299Ahmb allele consists of a SMA cDNA lacking exon 7 whereas the Tg(SMN2)89Ahmb allele consists of the entire human *SMN2* gene. Mice those are homozygous for the targeted mutant *Smn* allele and homozygous for the two transgenic alleles exhibit symptoms of type II SMA. This model shows accumulation of neurofilaments at motor endplates, defects of axonal growth and plasticity. The lack of axonal sprouting in MN lead to complete paralysis and death at age of four weeks (Cifuentes-Diaz et al. 2002). In addition, SMNΔ7 crossed with severe type I SMA mice extends the survival of mice from 5.2 to 13.3 days and it is associated with full-length SMN protein synthesis (Le et al. 2005).

Mice that are homozygous for the human Tg(SMN2)89 transgene, homozygous for the Smn1 null mutation and hemizygous for the Tg(SMN1*A2G)2023 transgene exhibit symptoms and neuropathology similar to patients afflicted with type III (mild) SMA.Those mice show reduced loss of MN and milder severity of SMA disease progression (Monani et al. 2003). Taiwanese-SMA mouse model was initially generated by the Hsieh-Li group (Hsieh-Li et al. 2000). The phenotype contains heterozygous Smn knockout mice (Smn+/-) and homozygous SMN2 transgene (Smn-/-;SMN2 tg/tg).. This model shows longer survival and it is usefull for disease progression and drug analysis (Riessland et al. 2010).

3.6. SMA therapy

There is no effective treatment for SMA, but advanced findings in research discovered more information about genetic and molecular mechanisms in SMA. Transcriptome profile comparison of RNA-sequence revealed differences in neuronal development and RNA transcript expression in SMA MNs (Maeda et al. 2014). Also based on

the data from transgenic animal models, it was clear that increased SMN protein levels would correct the SMA before symptoms are seen. Application of some of the strategies can actively help in increase full-length SMN protein level by activating SMN2 gene expression, or preventing SMN2 exon 7 skipping, or stabilizing SMN protein.

There are some techniques that can be used for SMA therapeutics, those techniques include to develop target drugs that can be neuroprotective for MN with low SMN levels, to replace the SMN1 gene using gene therapy, and to substute MNs or muscle cells using embryonic stem cells.

3.6.1. Antisense oligonucleotides (ASO)

Using ASO therapy the inclusion of exon7 was enhanced and the amount of full-length SMN protein level was increased (Castro & Iannaccone 2014). SMN2 splicing inclusion improves the life span of severe Δ7 SMA mouse model, increases Smn protein and improves motor function (Naryshkin et al. 2014). Gene therapy appears to be efficient in improving survival in a severe mouse model of SMA. It is necessary to find a better route for the administration and the safety profile of the viral vectors before clinical administration. Currently, ISIS pharmaceutical is conducting phase III clinical trials of intrathecal injections of their ASO and is expecting to be ready for the treatment of SMA patient soon.

3.6.2. Gene therapy

The target of gene therapy is to replace the missing SMN1 gene using virus vectors. An adenovirus vector, AAV9 is being used in gene therapy that is ability to cross the blood-brain barriers (Zanetta et al. 2014). AAV9 injection into SMAΔ7 mice showed increased levels of SMN protein. The most important result is that it improves the survival of treated mice for more than 250 days (Valori et al. 2010). Phase I trial was already started with SMA type I infant. However, the optimal route of delivery for the vector is still under study (Zanetta et al. 2014).

3.6.3. Drug treatment

In order to increase the transcriptional level of SMN protein, SMN promoters activatiors have been used. The SMN gene has a reproducible pattern of histone acetylation that is highly conserved among different tissues and species. The transcriptional start site consist of relatively high level of histone acetylation (HAT), whereas region further upstream or downstream have lower levels. Histone acetyltransferases (HATs), acting opposite to

HAT, a post-translational modification of nucleosomal histones that influences gene expression. HATs promotes gene transcription by relaxing chromatin structure and facilitates access to DNA by the transcriptional machinery, whereas HDAC promotes a condensation of chromatin state and transcriptional repressor (Kernochan et al. 2005). Thus, HDAC inhibitors, increases full-length SMN2 transcription level and protein level in SMA patient-derived lymphoblastoid cell lines. Administration of sodium butyrate, one of the HDAC inhibitor to the pregnant mothers of SMA transgenic mice improves the survival of its off-springs (Chang et al. 2001). Other HDAC inhibitors such as phenulbutyrate, valproic acid, and suberoyl anilide hydroxamic acid increased the SMN2 promoter activation, full length SMN mRNA synthesis and protein level increase in SMA patient-derived cell lines (Sumner et al. 2003; Andreassi et al. 2004; Kernochan et al. 2005).

Another drug that actively increases SMN2 gene expression is hydroxyurea. Treatment with hydroxyurea increases the amount of full-length SMN transcriptionally functional protein in SMA patient cells and currently it is studing in a clinical trial (Grzeschik et al. 2005). #

3.6.4. Stem cell therapy

Stem cell therapy in SMA is currently receiving considerable attention (D'Amico et al. 2011). Primary neural stem cells derived from spinal cord demonstrated improvement of the spinal muscular atrophy phenotype in mice, but this major source has limited translational application (Corti et al. 2010). Embryonic stem cells can differentiated into neural stem cells and then functional MNs by using RA, shh and NTF (Wichterle et al. 2002; Shin et al. 2005). Investigation in use of genetically engineered induced pluripotent stem (PS) cells for the utilization of the disease model and as cell source for cell transplantation (Corti et al. 2012). For this, they generated SMA human PS by non-viral or non-integrating episomal vectors and by editing oligonucleotides to modify SMN2 to produce a functional SMN1 like protein. The transplantation showed an improvement in the disease phenotypes, increased muscle connections and slight extension of lifespan. Pluripotent stem cells derived from embryonic stem (ES) cells showed the same potential therapeutic effects by injecting ES cell-derived neural cell precursors, into the spinal cord of a relatively severe SMA mouse model (Corti et al. 2009; Chang et al. 2011).

3.6.5. Small molecule treatment

Olesoxime (TRO19622) is a molecule, cholesterol-like structure that displays strong neuroprotective and promotes survival of MNs in culture. In 2010, efficacy and safety study of olesoxime was conducted in 150 SMA patients (Trophos. 2010). FDA accepts it for treatment of SMA and in the European Union, olesoxime has been granted Orphan Medicinal product designation for SMA by the European Commission (Zanetta, Nizzardo, et al. 2014). Similarly, Gabapentin and riluzole have also been studied for their neuroprotective properties in SMA treatments (Wadman et al. 2012).

Hypothesis and Objectives

HYPOTHESIS and OBJECTIVES

Hypothesis: Autophagy is a cellular regulatory process that has been recently related to neurodegenerative disorders. Accumulation of autophagosomes can originate neurite collapse or accumulation of toxic proteins into the neurons. Given the importance of autophagy homeostasis in the nervous system it is relevant to demonstrate the contribution of autophagy malfunctions to the development of several neurological diseases. SMA is a genetic disease associated to the reduction of SMN protein and to the perturbation of the axonal transport and MN degeneration. It is possible that increased autophagosome accumulation perturbs axonal transport, leading to progressive neuronal dysfunction. Moreover, the analysis of SMN protein regulation is one of the major goals of SMA therapeutics in order to find compounds that increase SMN protein level. The main objectives of the present thesis are:

Objective 1. To measure changes in the autophagy markers in cultured Smn-reduced motoneurons and in spinal cords from SMA type I mouse model.

Objective 2. To analyze the role of proteasome activity, autophagy level and calpain in Smn protein level regulation: effect of proteasome inhibitor, autophagy inductors and inhibitors or calpain protein reduction.

Materials and Methods

1. Cell culture#

1.1. Preparation of culture plates with cell adhesion substrates

1.1.1. Poly-DL-ornithine

To prepare the culture dishes stock solution of 10 mg/ ml poly-DL-ornithine (Sigma) was dissolved in boric-borate buffer to a working concentration of 35 µg/ml. The culture plates were covered with a suitable volume and kept at room temperature for 4 hours. After incubation, the plates were washed with sterile water for three times and air-dried at room temperature for 30 mints. The plates were ready to use or can be stored at 4˚C during 15 days.

1.1.2. Laminin coating

Once the poly-DL-ornithine treated plates were completely dry, 3.8 µg/ml of laminin (Sigma) diluted in L15 medium was added to the plates with a suitable volume. These plates were kept in the CO_2 incubator at 37˚ C for 2 hours. Laminin coated plates could be used during one week. Laminin solution is removed immediately before plating.

1.1.3. Preparation of collagen plates

For virus production, Collagen (Sigma) was used at working concentration of 100 µg/ml with acetic acid 200 µM. Adequate volume of solution was added in each p100 plate and left overnight at room temperature to dry. Once the plates were dry, they were stored at 4 ˚ C (up to 15 days).

1.2.Culture media and solutions

Solution/Medium	Preparation
GHEBS: pH 7.4	**137 mM NaCl** **2.6 mM KCl** **25 mM glucose** **25 mM Hepes** **20 mg/ml streptomycin.** **20 IU/ml of penicillin.**
Boric-borate pH 8.3	**150 mM Sodium tetraborate** **150 mM boric acid**
L15 complete (L15c)	**L15 medium (Gibco)** **18 mM D-glucose** **1% N2 solution (Gibco)** **20 mg/ml streptomycin** **20 IU/ml of penicillin**
NBMc	**Neurobasal medium (Gibco)** **2% B27 (Gibco)** **2% HI-HS (Heat Inactivated Horse Serum) (Gibco)** **125 nM L-glutamine (Gibco)** **50 µM β-mercaptoethanol**
BSA (Bovine Serum Albumin) 4%	**BSA 7.5% (Sigma)** **L15 medium**
NBMc+NTFs **(cocktail of Neurotrophic factors)**	**NBMc** **1 ng/ml BDNF and 10 ng/ml CNTF, GDNF, and HGF (Peprotech)**

Table 4. Culture media and solutions

1.3. Primary culture of spinal cord MNs

1.3.1. Dissection

The primary MN cultures were obtained from mouse embryos at 12.5 embryonic day (E12.5). At this stage of development MNs are large and less dense than the remaining cells of the spinal cord and can be easily isolated.

The first step is to remove the embryos from the uterus and transferred to a plate with the saline solution GHEBS. Next to extract the head of each embryo by holding the trunk with the help of mini-needles on a silicon support in the presence of GHEBS. To obtain the MNs from the ventral side of the spinal cord, the dorsal parts were removed and discarded. After dissecting the spinal cord, the meninges were removed to prevent the presence of other cell types in the purified culture. Each spinal cord was cut into 3-4 pieces of 2-3mm and transferred into a tube with conical bottom in the presence of GHEBS (Figure 26).

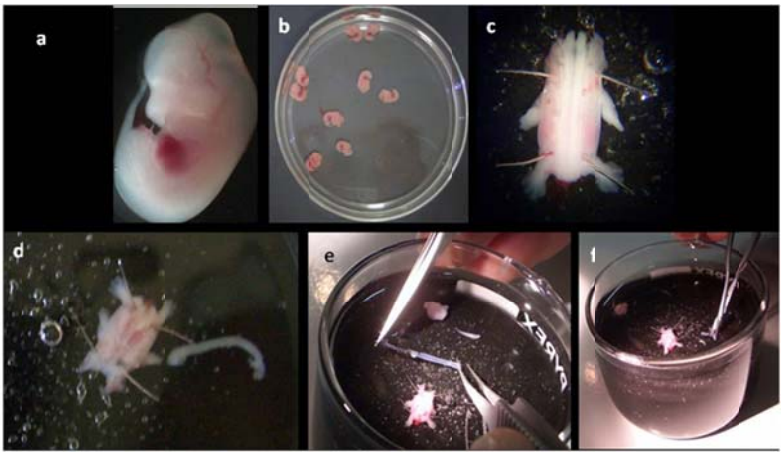

Figure 26. Extraction of spinal cords from E12.5 mouse embryos. (a and b) Embryos were removed from the uterus of CD1 pregnant female; they were kept in a plate with GHEBS and (c) heads were removed by placing on silicon support by fixing the four corners with mini-needles. (d, e, f) Removing the dorsal part of the spinal cord carefully, meninges detached, and the spinal cord cut into pieces as shown.

1.3.2. Purification

1. The spinal cord pieces obtained were washed twice with GHEBS.
2. Enzymatic dissociation: 0.025% trypsin (Sigma) for 9 min in GHEBS at 37 ˚C, shaking frequently.
3. Using a Pasteur pipette the spinal cord pieces were collected and placed into another tube with 100 µl of 4% BSA, 800 µl of L15c medium and 150 µl DNase (Boerhinger Mannheim, 1 mg/ml) shaking gently for 2 min.
4. First mechanical dissociation of the spinal cord pieces using 1 ml pipette tip for four times. The pieces were incubated for 2 min at room temperature to precipitate, the supernatant was collected and placed in another tube.
5. The pieces remaining in the bottom of the tube were incubated with 100 µl of 4% BSA, 900 µl of L15c medium, and 40 µl DNase to perform a second mechanical dissociation by passing-through the 1ml pipette tip for nine times. The supernatant

was collected and placed into the same tube containing the previously collected supernatant.

BSA Gradient

6. In the tube were the dissociated cells were placed, 4% BSA solution was slowly added forming two phases and centrifuged for 5 mints at 140 g.
7. The pellets were resuspended in 1 ml of L15c, passing through 5-6 times with 1ml pipette tip.

Optiprep Gradient

8. Optiprep (Axis-Shield) 12.5% solution was prepared in GHEBS to perform the density gradient. Cells were settle carefully on the Optiprep solution and centrifuged at 520g for 10 min. The interface band was collected and kept in a new tube (Figure 27).

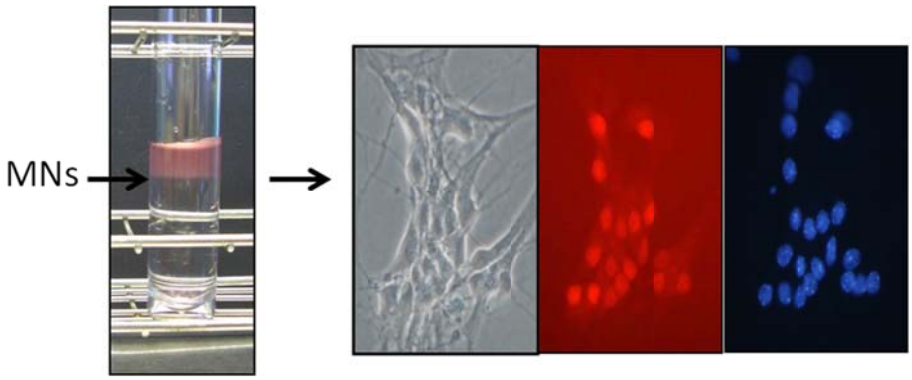

Figure 27. MNs collected from Optiprep density gradient. Image showing phase contrast, immunofluorescence microscope image stained with (Islet 1/2 and Hoechst dye).

1.3.3. Cell culture plating

MNs were seeded into culture plates pre-treated with poly-DL-ornithine and laminin. Cells were plated in medium containing NBMc with the cocktail of NTFs and were kept at CO_2 incubator for approximately 15 days in culture. To remove mitotic cell division of non-neuronal cells aphidicolin (2 µg/ml; Sigma) was added to culture medium. In order to evaluate the purification efficiency we used the anti-Islet 1/2 antibody (transcription factor expressed in MNs), and Hoechst dye for nuclear localization (Figure 27).

1.4. Primary culture of SMA mouse MNs

SMA type I mice FVB.Cg-Tg(SMN2)89AhmbSmn1tm1Msd/J were kindly provided by Dr. Josep E Esquerda (IRBLLEIDA-Universitat de Lleida). Heterozygous animals (*Smn +/-*

;SMN2+/+) were crossed to obtain homozygous *Smn -/-;SMN2+/+* (mtSMA). The littermates *Smn+/+;SMN2+/+* (WT) were used as controls.

1.4.1. Genotyping

For MN purification E13 embryos were removed from the uterus and a piece snipped from the head was used for genotyping. The RED Extract-N-Amp Tissue PCR Kit (Sigma) was used for genomic DNA extraction.

DNA Extraction

1. DNA extraction mix: add 3 volumes (75 µl) of extraction buffer with 1 volume (25 µl) of Tissue Prep.
2. Add 50 µl of the DNA extraction mix to each sample and incubate it for 10 minutes at room temperature.
3. Incubate the samples for 3 minutes at 95˚C (using thermo mixer)
4. Add 50 µl of neutralization buffer to each sample and centrifuged at 13000 rpm for 10 minutes.
5. Collect the supernatant to a new eppendorf and the samples were proceeded to PCR analysis. The supernatants can be stored at 4˚C and are stable up to 6 months.

PCR DNA amplification

The reaction mixture for PCR was prepared following the protocol.

Mix	Volume (µl)
Buffer red extraction	4.65
Sterile H20 MQ	2.55
Forward primer (20 µM)	0.6
Reverse primer SMN (20 µM)	0.6
Reverse primer Cassette (20 µM)	0.6
DNA sample	1

Table 5. PCR mix preparation.

Final volume of 10 µl was added to each PCR tube.

The primers used for PCR amplification were designed by The Jackson Laboratory.

Common 5'-CTCCGGATATTGGGATTG-3'

Mutant reverse 5'-GGTAACGCCAGGGTTTTCC-3'

Wild type reverse 5'-TTTCTTCTGGCTGTGCCTTT-3'.

PCR cycles

94 ˚C 5'// 94˚C 1'/ 62˚C 1'30'' / 72˚C 1'30'' // 72˚C 7' // 4˚C α

35 cycles

1.4.2. Genotype analysis

At the end of PCR amplification, the amplified DNA was analyzed by DNA electrophoresis using 1.2 % agarose gel. In wild type animals (+/+) the band was observed as a result of amplified DNA fragment at 800 Kb corresponding to mouse Smn, in the mutant mouse (-/-) the band was at 500 Kb corresponding to the neomycin resistance cassette and in heterozygotes (+/-) have both of the bands (Figure 28).

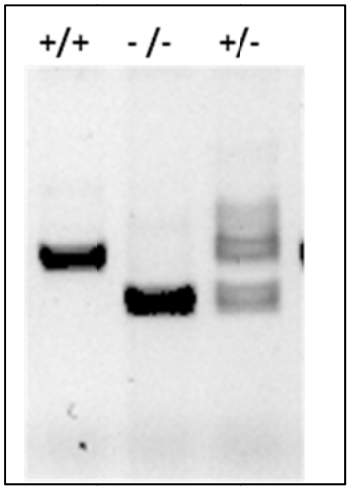

Figure 28. Agarose gel electrophoresis.

After genotyping WT and mtSMA animals were submitted to spinal cord dissection. MNs were isolated and cultured as described before.

2. Generation of lentivirus for RNA interference or protein over-expression

2.1. shRNA Interference Technique

RNA interference is a technique used to reduce mRNA expression thereby inactivating expression of target gene. There are several types of small RNA molecules described for silencing a target gene, the most widely used are the small interfering RNA (siRNA) sequences. These molecules are sized 21 to 25 nucleotides and are produced from precursors of double stranded RNA that may vary in size and origin. These precursors are processed by the members of the family of enzymes that degrade RNA, type III RNase family. In particular, Dicer is the enzyme that degrades the double stranded RNA precursors to siRNA. These siRNAs are then incorporated into a silencing complex called RISC (RNA-induced silencing complex). The incorporation of siRNA to the RISC complex is associated to the separation of the double strands into single strands. The guide strand remains associated to the Dicer complex, it identifies and binds to the complementary mRNA which interaction results in the cleavage of mRNA and its degradation (Hannon & Rossi 2004) (Figure 29). In the present study, we have used lentiviral vectors containing shRNA (small hairpin RNA) sequences to reduce the levels of Smn in cultured spinal cord MNs.

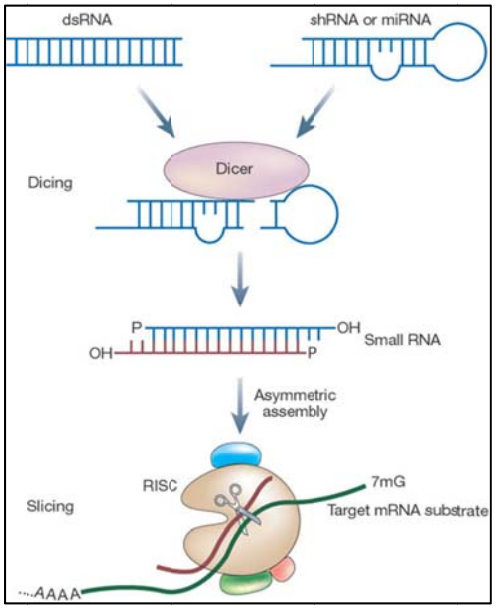

Figure 29. RNA silencing method using siRNA. (Hannon & Rossi 2004)

2.2. HEK 293T cell line (Human Embryonic kidney)

Human embryonic kidney cell line HEK 293T is a good model for over-expressing and to obtain intracellular, extracellular or membrane proteins. It is a version of the human kidney epithelial cell line 293 transformed with the E1A gene of adenovirus type 5 which expresses the SV40 T antigen.

The cells were maintained in 100 mm diameter culture plates (p100) (Falcon Labware Discovery, BD Bioscience). When they reached 80-90% confluence, they were detached mechanically from the plate (without using tripsinization), centrifuged approximately 130 g for 5 minutes and divided 1:10 or 1:20. The cells were incubated at 37˚C in controlled atmosphere (5 % CO2) incubator. The culture medium was changed every 2-3 days. To produce lentivirus HEK 293T cells were grown in culture plates previously treated with collagen.

To thaw cell lines the vials were removed from the N2 container and placed at 37˚C. Once thawed, the cells were collected into a conical bottom centrifuge tube. DMEM medium was added to reach a final volume of 10 ml, and centrifuged 5 minutes at 130 g. The pellets were resuspended in the appropriate medium and grown in culture dishes in the incubator with controlled CO2 atmosphere.

To freeze the cell lines, cells were detached using 1-2 ml of the culture medium and collected into a conical bottom centrifuge tube. DMEM medium was added to a final volume of 10 ml and centrifuged at 130 g for 5 minutes. The medium was aspirated and the cell pellet was resuspended in freezing medium (90% HI-FBS and 10% DMSO) at the ratio of $2x10^6$ cells/ ml in a 1 ml cryogenic vial. Vials were kept at -80˚C for 24 hrs to get a slow freezing process and then frozen in liquid N2.

2.3. Lentivirus production

For RNA interference experiments, constructs were generated in pSUPER.retro.puro vector (OligoEnine, Seattle, WA, USA) using specific oligonucleotide targeting *Smn or calpain* sequence indicated below.

Vector	RNA interference	sequences	Procedure
pSUPER pLTVHM	SMN	5'-TGACAAGTGTTCTGCTGTT-3'	Dr.A. Garcera
pSUPER pLTVHM	Calpain	5'-GCGCCAAGCAGGTAACTTA-3'	Dr. A. Garcera

Table 6. Lentivirus vector, the sequence of RNA interference cloned and procedure.

Oligonucleotides were obtained from Invitrogen.

shRNA sequences were cloned into the BgI II/HindIII sites of pSUPER.retro.puro. Lentiviral constructs were generated by digesting pSUPER.retro.puro and pLVTHM vectors with EcoR1 and Cla1 to replace the H1 promoter of pLVTHM with the H1-shRNA cassette from pSUPER.retro.puro. pLVTHM vector contains the Green Fluorescent Protein (GFP) under the control of an EF-1 alpha promoter for monitoring transduction efficiency.

psPAX2 vector is 2nd generation lentiviral packaging plasmid which include all the important packaging components Gag, Pol, Rev and Tat for producing viral particle. pMD2G is a VSV-G envelope expressing plamid. pLVTHM, psPAX2, and pMD2G were kindly provided by Dr. Trono (Université de Genève, Geneva, Switzerland). Lentiviruses were propagated in HEK293 cells using polyethylenimine (PEI) (Sigma) cell transfection method. Twenty micrograms of pLVTHM containing target gene (SMN or calpain or EV), 13 µg of pSPAX2, and 7 µg of pM2G were transfected to HEK293T cultures. Cells were allowed to produce the lentivirus for 4 days. The medium centrifuged at 1200 g for 5 min, and the supernatant were filtered using a 0.22 µm filter. The medium containing the lentiviruses were stored at 4 °C.

List of lentivirus vectors and their promoters.

vector	Promoter	Origin
pLVTHM	H1	Trono lab
psPAX2	CMV	Trono lab
pMD2G	CMV	Trono lab

Table 7. lentiviral vector and promoters

Table of restriction enzymes used in each plasmid.

vector	Restriction enzymes	
pLVTHM	**Cla1**	**EcoR1**
pSUPER	**Hind III**	**Bgl II**

Table 8. lentiviral vectors and specific restriction enzymes.

2.4. Virus titration

Biological titers of the viral preparations were expressed as the number of transducing units per ml (TU/ml). The titer was determined by transducing 20,000 HEK293T cells with different dilutions of lentivirus preparation. After 48 h the percentage of green fluorescent protein (GFP) positive cells was measured and viruses at 2×10^5–1×10^6 TU/ml were used for the experiments.

For lentiviral transduction, MNs were plated in 4-well dishes and 3 h later the medium containing lentivirus (2 TU/ cell) were added. The medium was changed 20 h later and transduction efficiency monitored in each experiment by direct observation of GFP positive cells.

3. Immunohistochemistry

Spinal cords were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 overnight. The samples were cryoprotected with 30% sucrose in PB, embedded in Tissue Freezing Medium (TFM, Triangle Biomedical Sciences, Durham, NC, USA), frozen and stored at 80˚C. Serial cryostat sections (16-µm thick) were obtained. For immunocytochemistry, sections were sequentially rinsed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100, blocked in normal goat serum and incubated with the appropriate primary antibody overnight at 4˚C. The primary antibody used was rabbit polyclonal anti-LC3 (Cell Signalling, dilution 1:50). The samples were rinsed with PBS for 10 minutes, repeated for three times and incubated with the secondary antibody (Alexa Fluor 555, diluted 1:300) diluted in PBS for 60 minutes at room temperature. Cellular nuclei were stained with Hoechst dye for 30 minutes and rinsed with PBS. Nissl staining were also performed to identify MNs. Cells were incubated for 60 minutes with NeuroTrace 530/615 Red Fluorescent Nissl stain (1:300), washed and mounted using Mowiol (Sigma). Mounted cells were examined with an Olympus BX51 epifluorescence microscope or an FV10i Olympus confocal microscope.

3.1. Image and morphometric analysis

Image analysis of spinal cord immunolabeling was performed using fluorescence micrographs taken from every 30th sections of the entire lumbar region, and processing them in the ImageJ software (National institutes of Health, USA). Slides from WT or mutSMA animals were processed in parallel for immunocytochemistry and subsequent fluorescence imaging. The same microscopy settings were used for the acquisition of digital images. The quantitative analysis of LC3 immunostaining was assessed by measuring the area occupied by the respective immunolabeled profiles in the ventral horn of the spinal cord. MN cell bodies in the ventral horn, identified by their characteristic shape and size and Nissl positive staining were examined. At least 60-90 MNs of the same condition were analyzed from three independent experiments.

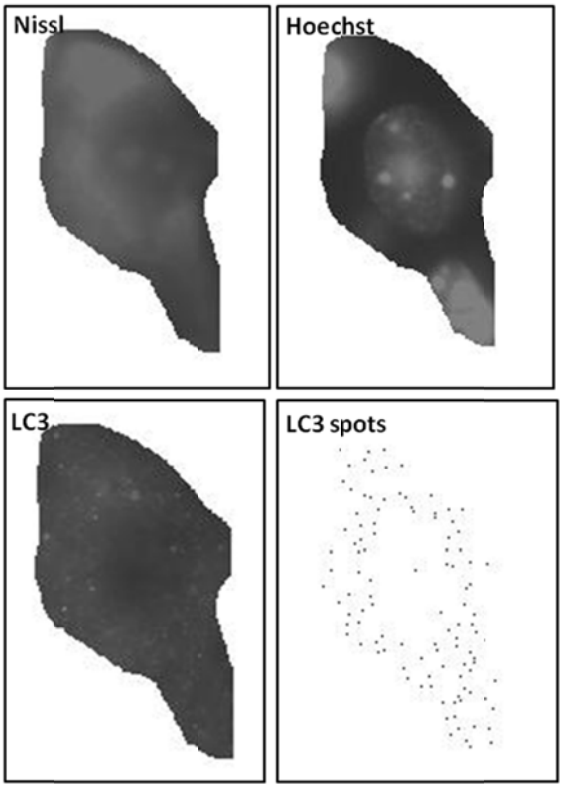

Figure 30. MNs were identified using Nissl, Hoechst and LC3. The number of LC3 positive spots was counted using the Image J software.

4. Electron microscopy

4.1. Sample fixation and analysis

Electron microscopy analysis of autophagosomes was performed in MNs from ventral horn fragments of P1 WT or mutSMA spinal cords or in Smn knockdown MN cultures. Samples

were fixed for 1hr at 4 ˚C in 1.6% glutaraldehyde in 0.5 M phosphate buffer (pH 7.3), washed, fixed again in aqueous 2% osmium tetroxide, stained with 2% uranyl acetate in 30% methanol and finally embedded in Eppon. Ultrathin sections were taken from selected area containing MN cell bodies and neurites previously identified in toluidine blue-stained 1-mm-thick semithin section. Ultrathin section were stained with lead citrate and uranyl acetate and observed by electron microscopy (Zeiss EM910, Oberkochen, Germany). To analize the number of autophagosomes one researcher loaded the section and another counted the organelles, blinded to which condition was loaded.

8. List of drugs used in MN treatment

Autophagy modulator	**Inhibitor**	**Doses**	**Treatment hours**	**Commercials**
	BafA1	50nM	4hrs	Sigma
	NH4CL	10mM	4hrs	Sigma
	3-MA	5mM	12hrs	Calbiochem
Autophagy modulator	**Inducer**			**Commercials**
	Rapamycin	100mM	6hrs	Sigma
	Curcumin	500nM	12hrs	Sigma
	Resveratrol	5µM	12hrs	Sigma
	Trehalose	100mM	6hrs	Sigma
Proteasome inhibitor	MG-132	2.5µM	12hrs	Sigma

Table 9. List of drugs used to analyze SMN protein level in MNs.

9. Western blot analysis

Western blot is an important technique used in cell and molecular biology. This technique used to identify specific proteins from a complex mixture of protein extracted from the cells. Western blot consists of step wise process (i) separation of protein by size using

electrophoresis (ii) transfer of protein from gel to membrane and (iii) marking target protein using a proper primary and secondary antibody to visualize.

Cell lysate

The cells were rinsed in ice-cold PBS buffer to remove the excess of the medium in the plates and they were collected using lysis buffer (Tris-HCl 125 mM pH 6.8 + 2% SDS; 27 µl). The cells were scraped from bottom of plates using pipette tips and samples were collected in an Eppendorf tube. Samples were heated at 95˚C for 5 minutes to denature the protein. The samples were stored at -20˚C for later use or submitted to electrophoresis.

Protein separation by electrophoresis

Five µl of loading buffer (LB 5X) (10% SDS, 50% glycerol, 250 mM Tris pH 6.8, 720 mM β mercaptoethanol and Bromophenol Blue) was added to 20 µl of cell lysates (10-50 µÆ#of protein). The samples were heated at 95˚C for 5 minutes and centrifuged (10000 rpm for 5 minutes). Samples were loaded at equal volume of protein into the wells of SDS-PAGE gel. The gels were run at 15 A for 20 minutes and then increased to 20 A to finish the run in about 1 hour.

Transferring the protein from the gel to the membrane

The PVDF membranes were activated in methanol for 5 minutes before to the transfer of protein from the gel to the membrane. The membrane, blot papers and gel were kept in transfer buffer for few minutes in shaker. The sandwich of transfer was made using blotting paper, membrane, gel and blotting paper (Figure 31). The transfer was run at 60 A for 60 minutes using an Amersham Biosciences semidry Trans-Blot.

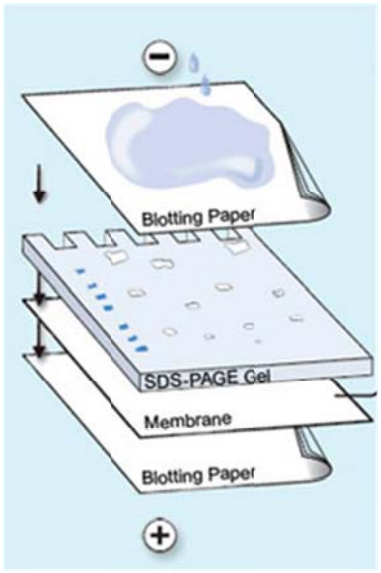

Figure 31. Transfer of protein from gel to PVDF membrane.

Antibody incubation

The membranes were rinsed with TBST buffer and blocked in 5% fat free milk powder at room temperature for two hours. Then they were rinsed for three times with TBST to remove the excess of blocking solution. The primary antibody was prepared in TBST with sodium azide and incubated overnight at 4˚C. The membranes were rinsed well to remove unspecific binding of antibodies. The secondary antibody was prepared with 5% milk powder and incubated for 1 hour at room temperature. The membranes were finally rinsed several times with TBST to eliminate the excess of secondary antibody.

To control the specific protein content per lane, membranes were reprobed with a monoclonal anti-α-tubulin antibody (Sigma, St Louis, MO, USA).

Antibody	Dilution	Commercial trade
anti-SMN	1:5000	BD Transduction laboratory
anti-LC3-II	1:1000	Cell signaling
anti-Beclin1	1:1000	Cell signaling
anti-calpain	1:1000	Biomol (Quimigen)
anti-Bcl-XL	1:2000	Sigma
anti-tubulin	1:50000	Sigma

Table 10. Primary antibodies

Antibody	Dilution	Commercial trade
Anti-mouse IgG-HRT	1:2000	Sigma
Anti-rabbit IgG-HRT	1:2000	Amersham

Table 11. Secondary antibodies.

Imaging and data analysis

Blots were developed using the Super Signal chemiluminescent substrate (Pierce) or the ECL Advance Western Blotting Detection kit (Amersham Biosciences) according to the manufacture's recommendation. The band intensity of target proteins were analysed using Image J software.

10. Statistics#

All experiments were performed a minimum of three times. Data were processed with the Graph Pad Prism program and are expressed as mean ± SEM, unless otherwise indicated. To determine significant differences we have used two statistical analyses.

(i) Student-t test when we compared two groups. The data presented in the Figures were expressed as the mean ± SEM of one representative experiment. All the experiments were repeated 3 or more times and p values <0.05 were considered significantly relevant.

(ii) One-way ANOVA when we compared three or more groups followed by post-hoc multicomparison Bonferroni's test. p-values < 0.05 were considered significant.

Results

To investigate the role of Smn protein in MN function and to identify mechanisms to protect Smn-deficient MNs from degeneration, we decided to establish an *in vitro* model of SMA. Interference of RNA represents a powerful method to inhibit gene expression at posttranscriptional level, and is an appropriate tool to generate an *in vitro* model of Smn loss-of-function (Dykxhoorn et al. 2003). In our work, we used shRNA lentivirus construction to reduce the expression of Smn in cultured MNs. MNs were isolated from mouse 12.5 embryonic day (E12.5) and maintained in the presence of a cocktail of NTFs (1ng/ml BDNF; 10ng/ml CNTF; 10ng/ml GNTF; 10ng/ml HGF) (Gou-Fabregas et al. 2009). Three hours after plating, the lentivirus construction of shSMN (shRNA SMN) or EV (empty vector) were added. Twenty hours later the medium was washed and substituted by fresh medium supplemented with NTFs. The lentivirus transduction was checked after three days by green fluorescent protein (GFP) (measured using fluorescence microscopy), were most of the cells present in the culture dish (90%) were showing positive fluorescence. We checked the Smn protein expression level by Western blot analysis.

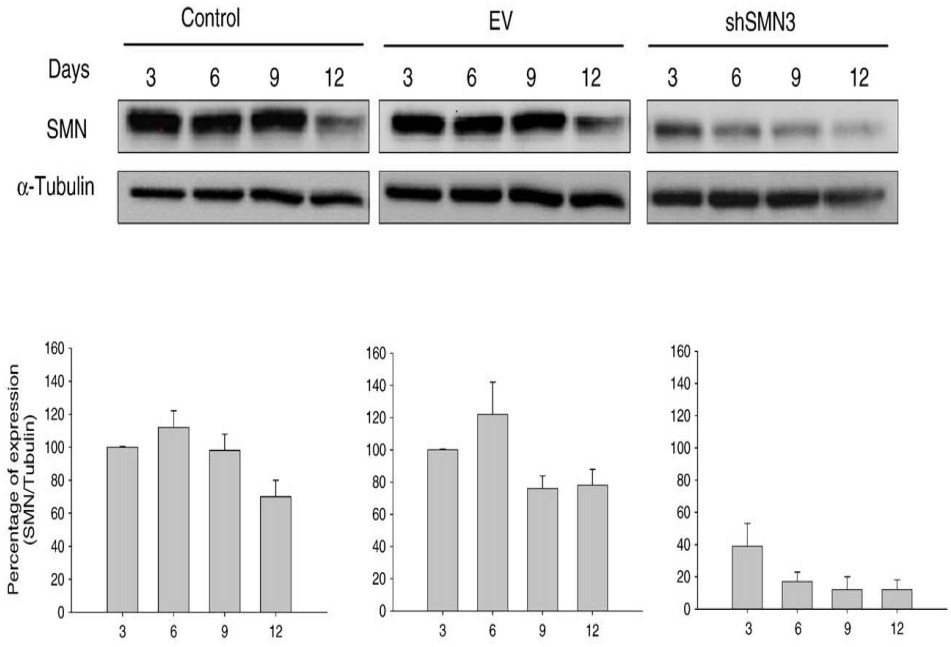

Figure 32. Western blot analysis showing the level of Smn protein expression in non-treated control, EV (lentivirus control) and Smn reduced condition in different culture Days -3, -6, -9, -12. Membranes were blotted with anti-Smn and the same membrane were re-bloted against α-tubulin considered as a loading control. Graph represents the percentage of Smn protein expression compared with tubulin (Garcera et al. 2011).

Smn knockdown conditions show the gradual decrease of Smn protein level after several days in culture (3, 6, 9, and 12) (Figure 32). In these cells, it was also observed neurodegeneration with disturbed soma and neurite degeneration on day 6, and at day 12,

non-apoptotic cell death occurred. To confirm that the cellular effect of neurite degeneration was due to endogenous reduction of Smn, we over-expressed Smn to prevent the cell death and as expected, Smn overexpression prevented neurite degeneration and cell death and established that the cellular effect observed were caused by Smn knockdown and not by other effects. We decided to analyse the effect of Bcl-xL over-expression because of its ability to prevent cell death in MNs. shSMN or EV MN cultures were co-transduced with lentivirus carrying an expression vector containing the human Bcl-xL gene (shSMN3-Bcl-xL; EV-Bcl-xL). To determine the effect of Bcl-xL over-expression in MNs, neurite degeneration experiments were performed after 6 and 12 days.

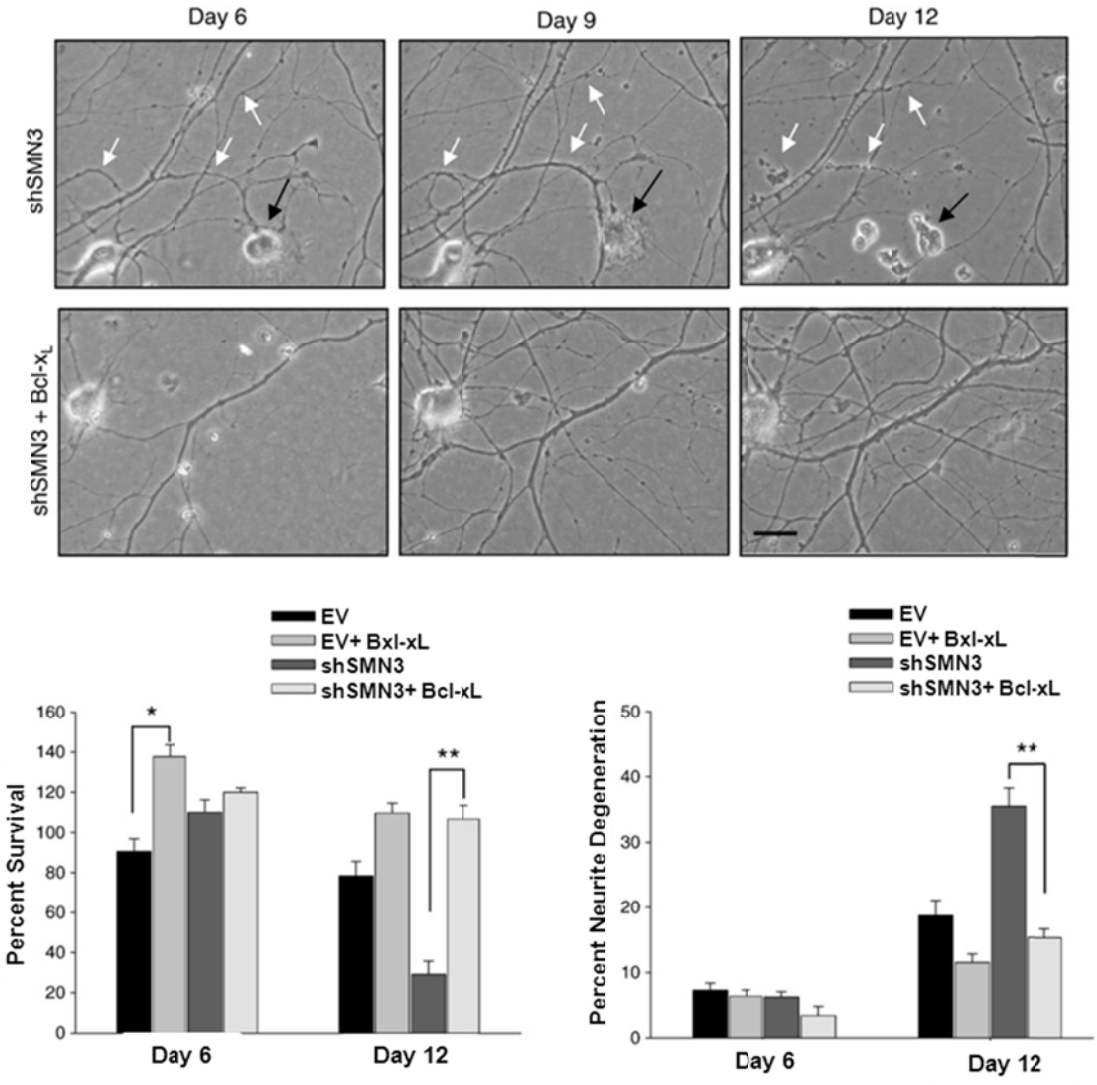

*Figure 33. A new model to study spinal muscular atrophy: neurodegeneration and cell death is counteracted by Bcl-xL overexpression in motoneurons. MN culture at different day 6, 9, 12 transduced with EV or shSmn virus knockdown. Phase contrast microscope pictures show neurodegeneration at Day 6 (white arrows) and cell death at Day 12 (black arrows). Over-expression of Bcl-xL in Smn reduced condition recover neurodegeneration and cell death. Graph represents the percentage of survival at different condition and percentage of neurite degeneration.*p<0.05 and **p<0.0001 one-way ANOVA test.*

As expected, Bcl-xL prevented neurite degeneration caused by Smn reduction. When we analyzed MN survival after 12 days, Bcl-xL over-expression suppressed the cell death caused by Smn reduction. Both results indicated that Bcl-xL was able to block the effect of Smn reduction on MN neurites and survival.

1. Smn reduction causes an increase in autophagy markers

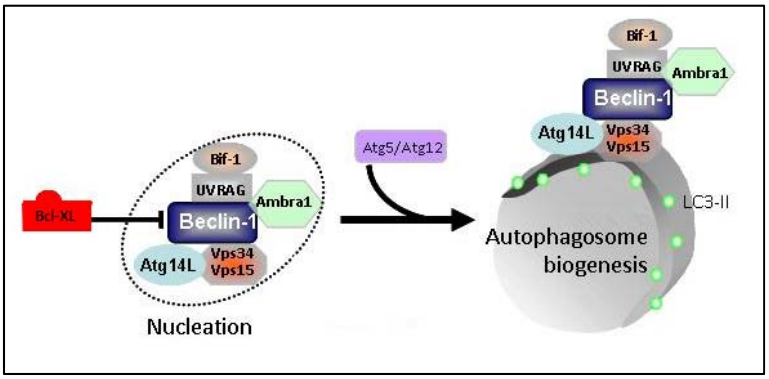

Figure 34. Beclin-1 involves in the formation of autophagosome. Binding of Bcl-xL, an anti-autophagy protein prevents beclin1 to initiate the autophagy.

1.1. Effect of Smn knockdown on LC3-II protein level

To measure autophagy level in Smn reduced cells, LC3-II protein expression level was used as a marker for autophagosome formation. After autophagy activation, free form of cytosolic LC3-I is converted to lipid conjugated membrane-bound LC3-II that binds within and outside of the autophagosome membrane. This LC3-II present all over autophagy process until it fuses with the lysosome for digestion (Kabeya et al. 2000). Embryonic MNs were cultured, transduced with a lentivirus containing EV or shSmn and maintained in the presence of the NTFs. After several days of culture, cells were collected at day 6 and day 12. LC3-II protein expression level was measured using Western blot technique. The analysis of LC3-II protein in Smn-reduced MNs showed increased level of LC3-II in both 6 and 12 days conditions compared to control (Garcera et al. 2013).

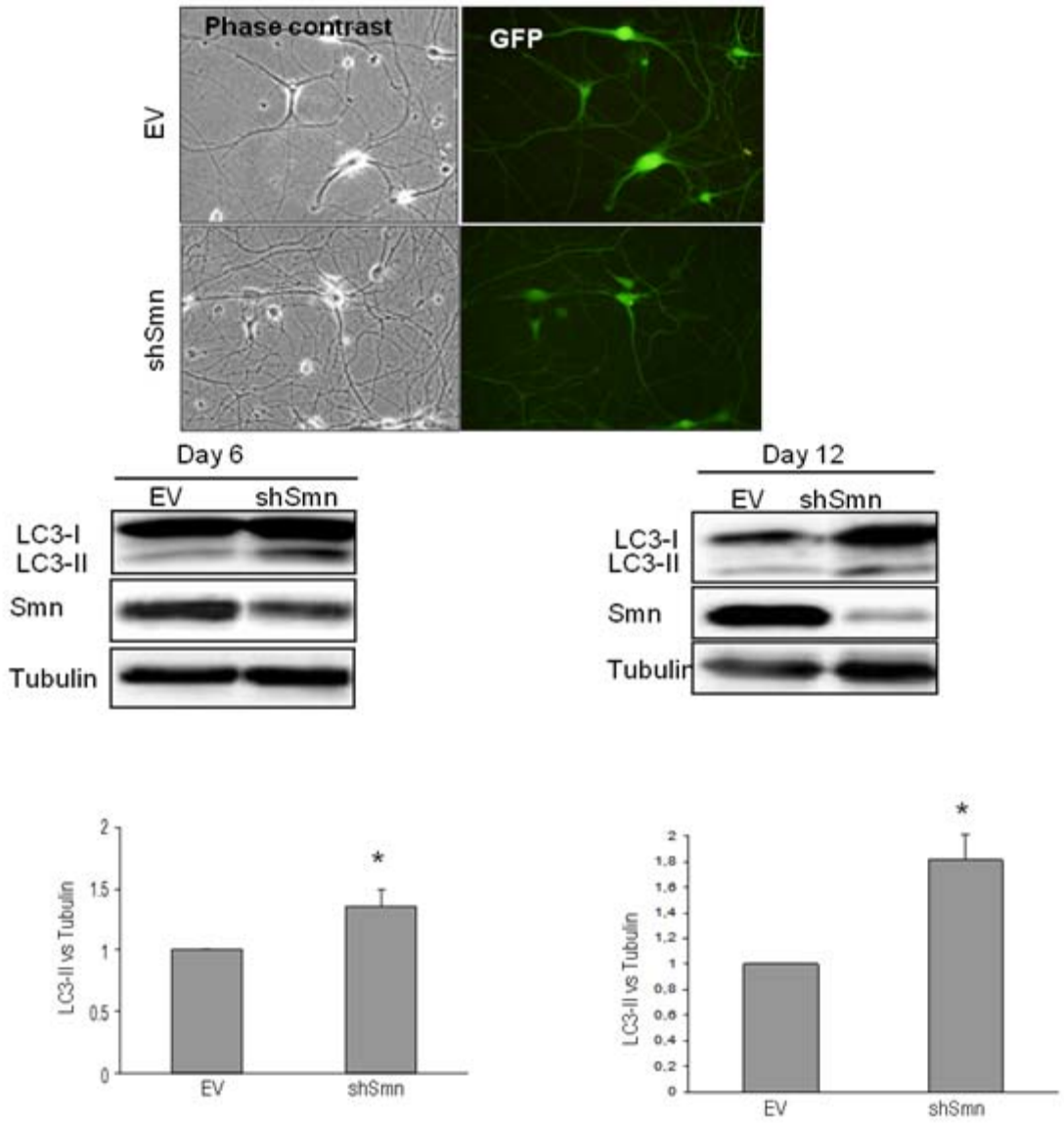

*Figure 35. Survival motor neuron protein reduction deregulates autophagy in spinal cord MNs in vitro. Phase contrast microscopy images of MN transduced with EV and shSmn, green fluorescence showing the effective transduction of lentivirus into MNs. Western blot analysis showing protein extracted EV and shSmn on day 6 and day 12 probed with anti-LC3-II antibody, anti-Smn antibody and the same membrane were re-probed with anti-tubulin antibody as a loading control. Graph represents the level of LC3-II measure compared with tubulin level, and the values correspond to the quantification of at least three independent experiments and the bar indicating a significant increase of Smn level. *p<0.05 one-way ANOVA test.*

1.2. Effect of Smn reduction on Beclin-1 protein level

Beclin1 is the mammalian ortholog of the yeast Atg6/Vps30 protein. It is part of the Class III PI3K complex that participate in autophagosome formation (Liang et al. 1998). Beclin1 is also a tumor-suppressor gene that is frequently deleted in the human sporadic breast, ovarian and prostate cancer (Levine & Klionsky 2004). Binding of Bcl-2 with Beclin1 disturbs the autophagy function (Pattingre et al. 2005) (Figure 36).

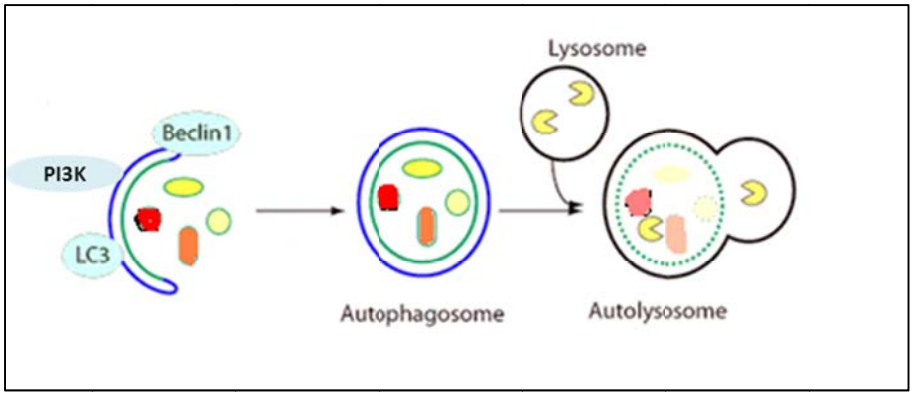

Figure 36. Autophagy initiation mediated by class III PI3K Beclin 1. Binding of Bcl-2 with Beclin 1 results in autophagy interruption (Pattingre et al. 2005).

To measure the autophagy activation in Smn-reduced cells, cultured MNs were transduced with lentivirus of EV or shSmn in the presence of NTFs. Protein extracts were obtained 6 days after plating and submitted to Western blotting. Increased Beclin 1 protein expression level in shSmn condition indicates induction of autophagy activation in shSmn MNs. The result suggests that reduction of Smn protein induce autophagy through Beclin1 dependent pathway in MNs (Garcera et al. 2013).

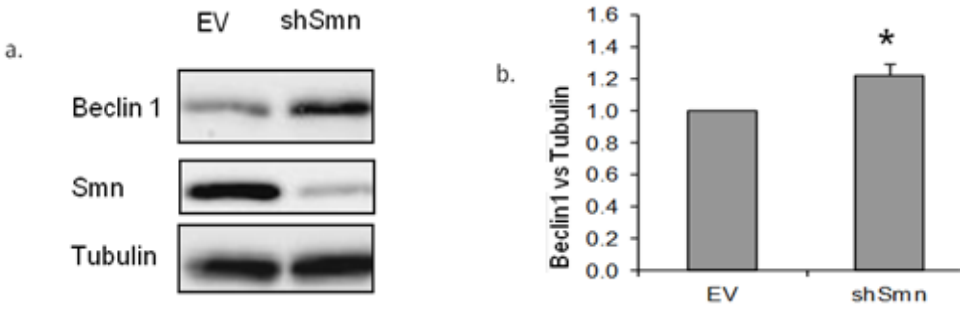

*Figure 37. Smn protein reduction deregulates autophagy in spinal cord MNs in vitro. a. Western blot analysis of protein extracts from EV or shSmn transduced. MNs Membranes were probed with anti-Beclin 1 or anti-Smn antibodies, and the same membrane were rebloted with an antibody against tubulin as a loading control. b. Graph represents the level of Beclin 1 compared with tubulin, and the values correspond the quantification of at least three independent experiments (*p<0.05 one way ANOVA test).*

1.3. LC3-II expression level in SMA mutant motor neurons

To study the expression of autophagy level in SMA *in vivo* model, we used the (*Smn(-/-); SMN2(+/+)* mtSMA) Smn null mouse with human SMN2 transgene that resembles human SMA type 1 . E13 SMA mouse embryos were submitted for genotype. After genotype, MNs were purified separately from WT and mtSMA spinal cord. After 12 days, protein extracts were obtained and submitted to Western blot. LC3-II protein was significantly increased in

mtSMA when compared to WT MNs indicating changes of the autophagy markers in mtSMA MNs. Together with the previous results, reduction of Smn causes an increase of LC3-II and Beclin-1 protein level in MNs, suggesting that Smn reduction causes alteration in the autophagy process (Garcera et al. 2013).

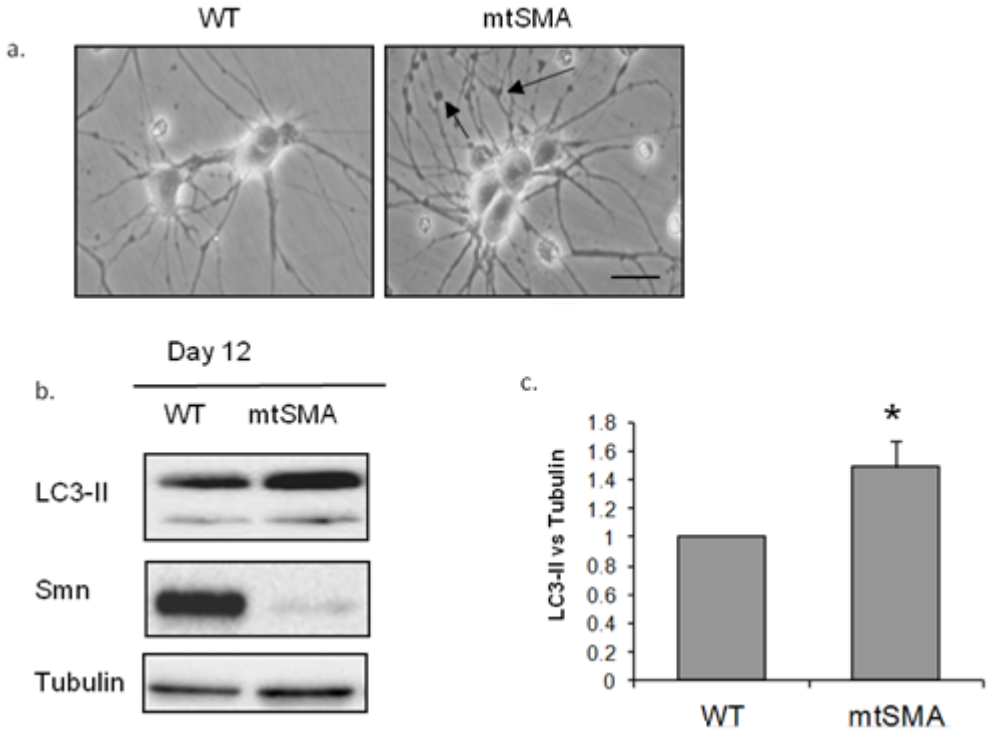

*Figure 38. Smn protein reduction deregulates autophagy in spinal cord MN in vitro. a. Representative image shows the MN culture from Day 8 WT and mtSMA. Arrows indicate neurite degeneration. b. Western blot analysis of Day 12 protein extracts from WT and mtSMA cultured MNs were probed with anti-LC3-II or anti-Smn antibody. Membranes were re-probed with an anti-tubulin antibody as a loading control. C. Graph represents the expression of LC3-II protein against tubulin and values are corresponding to the quantification of three independent experiments (*p<0.05 one way ANOVA test).*

1.4. Smn reduction does not alter autophagy flux

Alteration in autophagy can be either by increased autophagosome synthesis or reduced lysosomal degradation. To measure whether increased autophagosome synthesis or reduced autophagic flux causes the LC3-II increase in Smn-reduced MNs, cultures were treated with the autophagy inhibitor Bafilomycin A1 (Yamamoto et al. 1998). This treatment causes LC3-II increase when the autophagic flux is not altered (Klionsky et al. 2012) (Figure 39).

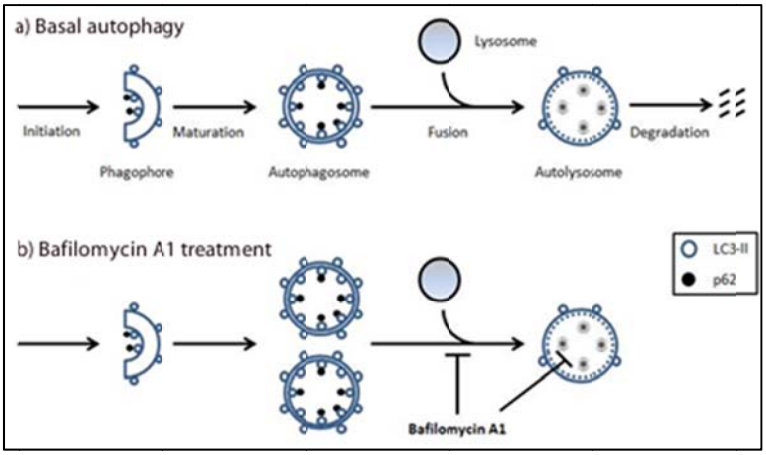

Figure 39. BafA1 interfere autophagosome and lysosome fusion. BafA1, treatment causes accumulation of autophagosomes into the cytoplasm.

MNs were transduced with EV or shSmn lentivirus constructions. After six days of plating, cells were treated or non-treated with BafA1 (50 nM during 4 hrs). Protein extracts were collected and submitted to Western blot analysis using the anti-LC3 antibody. BafA1 treated cells show an accumulation of autophagosome in both conditions (i.e., LC3-II is increased in shSmn condition compared with EV, and the addition of BafA1 increases LC3-II in both conditions). The result indicates that Smn reduction increases autophagosome synthesis without affecting the flux in MN culture.

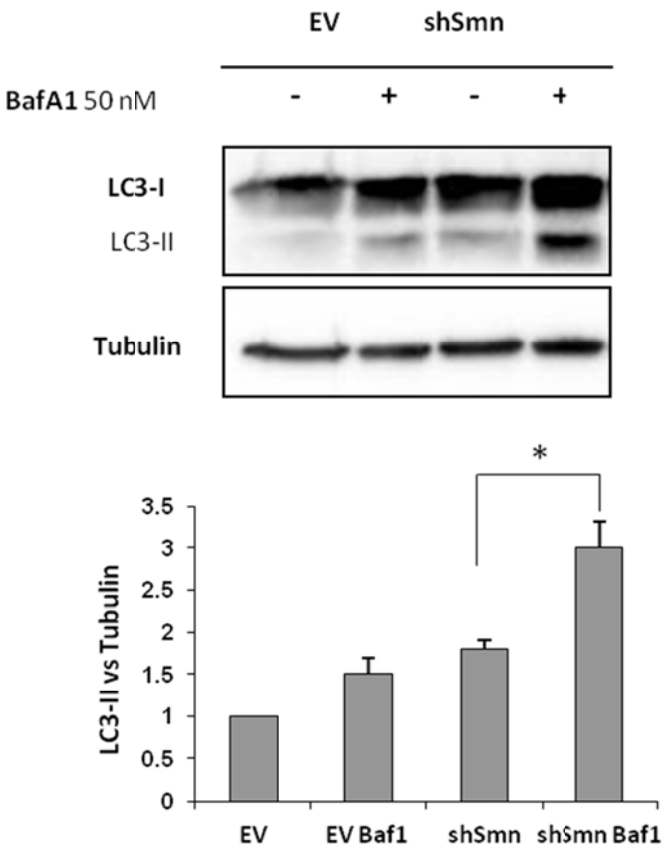

*Figure 40. Smn reduction does not alter Autophagy flux: MNs protein extracts were obtained and submitted to western blot. Membranes were probed with an antibody against LC3, and the same membrane was reprobed with an anti-α tubulin antibody as a loading control. Graph represents the expression of LC3-II versus α-tubulin and corresponds to the quantification of three independent experiments (*p<0.05 one way ANOVA test).*

1.5. Smn reduction causes an increase in autophagosome number

Reduction of Smn increases LC3-II protein expression level indicating changes of autophagy in MN cultures. The electron microscopy experiments and the measures of LC3 II by Immunofluorescence were used to analyze the presence of autophagosomes into the MNs and to analyze the endogenous localization of LC3 protein.

1.5.1. Smn reduction form LC3 aggregates in MN soma and neurites

Immunofluorescence can be used to measure intracellular localization of protein expression using a fluorescence tagged antibody against target protein. To localize LC3-positive structures in cell somas and/or neurites, we analyzed the endogenous LC3 protein by immunofluorescence. MNs were plated on glass coverslips and transduced with EV or shSmn lentivirus constructs. Eight days after transduction, cells were fixed, and LC3 immunostaining was performed. Measurement of LC3-positive spots shows increased endogenous spots in both MN soma and neurites (Figure 41 b).

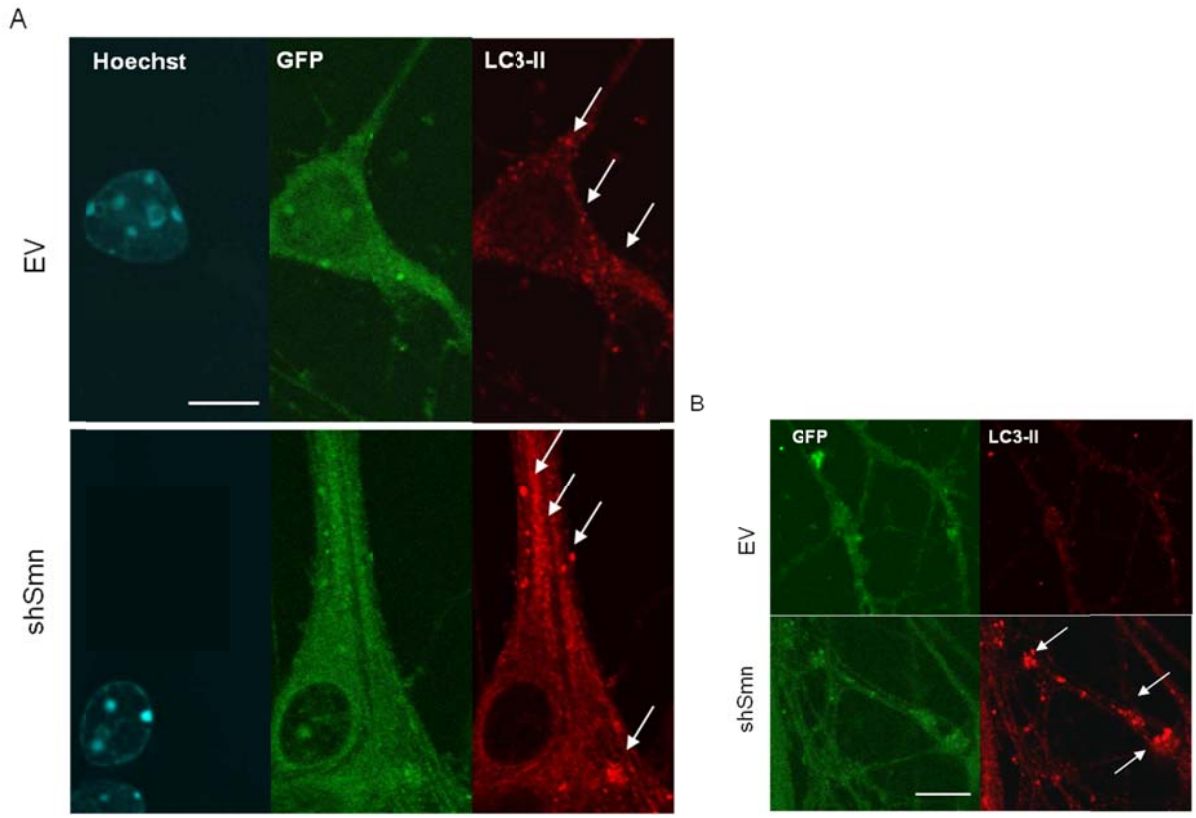

Figure 41. LC3-II localization in MNs transduced with EV or shSmn in culture. (a) Representative images are showing cell soma and neurites (b). Images are showing neurites of transduced MNs. Arrows in (a) (b) images indicate the presence of LC3-II positive aggregates. In the images, blue indicates Hoechst and green indicates GFP. Scale bar in (a) 6 µm and in (b) 12 µm. Images were acquired with an FV10i confocal microscope (Olympus), using the X 60 objective and the same microscopic settings.

1.5.2. Measurement of autophagosomes in SMA spinal cord MNs using electron microscopy:

a. MN culture from E12.5

The presence of autophagosomes indicates the level of autophagy in pathological conditions. Transmission electron microscopy is an important tool for detecting autophagosomes and can provide significant insight to the extent of on-going autophagy (Klionsky et al. 2012). We used this approach to analyse the presence of autophagosomes and autolysosomes in the cytoplasm and neurites of Smn-reduced MNs. MNs were transduced with lentivirus containing EV or shSmn, and 8 days after plating, MNs were processed and analysed. Non-transduced neurons were analysed as a control of the lentiviral transduction process (data not shown). Smn reduction produced a marked increase in the autophagosomes and autolysosome number in soma and neurites (EV n=4; shSmn n=16; $p<0.0005$) (Figure 42). Normal endoplasmic reticulum, Golgi complex and

mitochondria were found in EV and shSmn-transduced neurons. These results suggest an increase of the number of autophagy profiles in Smn-reduced spinal cord MNs when compared with non-transduced and EV controls.

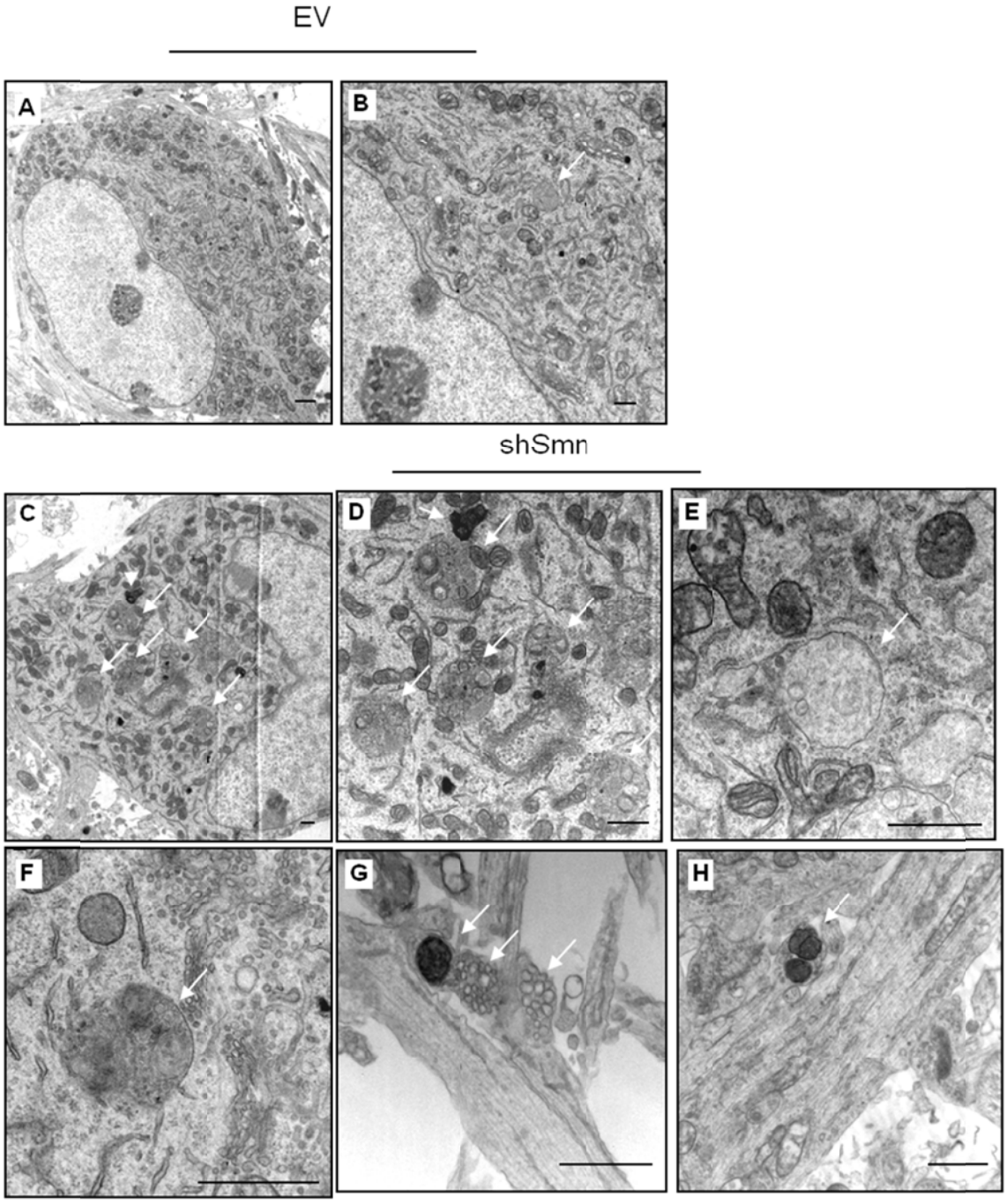

Figure 42. Electron microscopy images of MNs transduced with EV (a and b) or shSMN (c-h). Eight days after plating, cells were fixed and submitted to electron microscopy. Arrows indicate autophagic profiles present in the cell soma (b-f) and neurites (g and h). Scale bar: (a) 5 µm and (b-h) 1 µm.

b. SMA Postnatal Spinal cord MNs

The process of autophagy was first discovered by ultrastructure analysis of the presence of autophagosomes and autolysosomes by electron microscopy (Eskelinen et al. 2014). We used the same approach to analyse the presence of these structures in the soma and neurites of SMA spinal cord MNs. To measure the autophagy profile in mtSMA spinal cord, we used postnatal day 5 (P5) mouse spinal cords. P5 animals obtained from crossing two Smn(+/-); SMN2 mutants were submitted to genotyping. WT and mtSMA were selected and lumbar region of spinal cords were processed for sample preparation. MNs were selected by their localization in the ventral region of the spinal cord and by their cell-specific bigger nucleus.

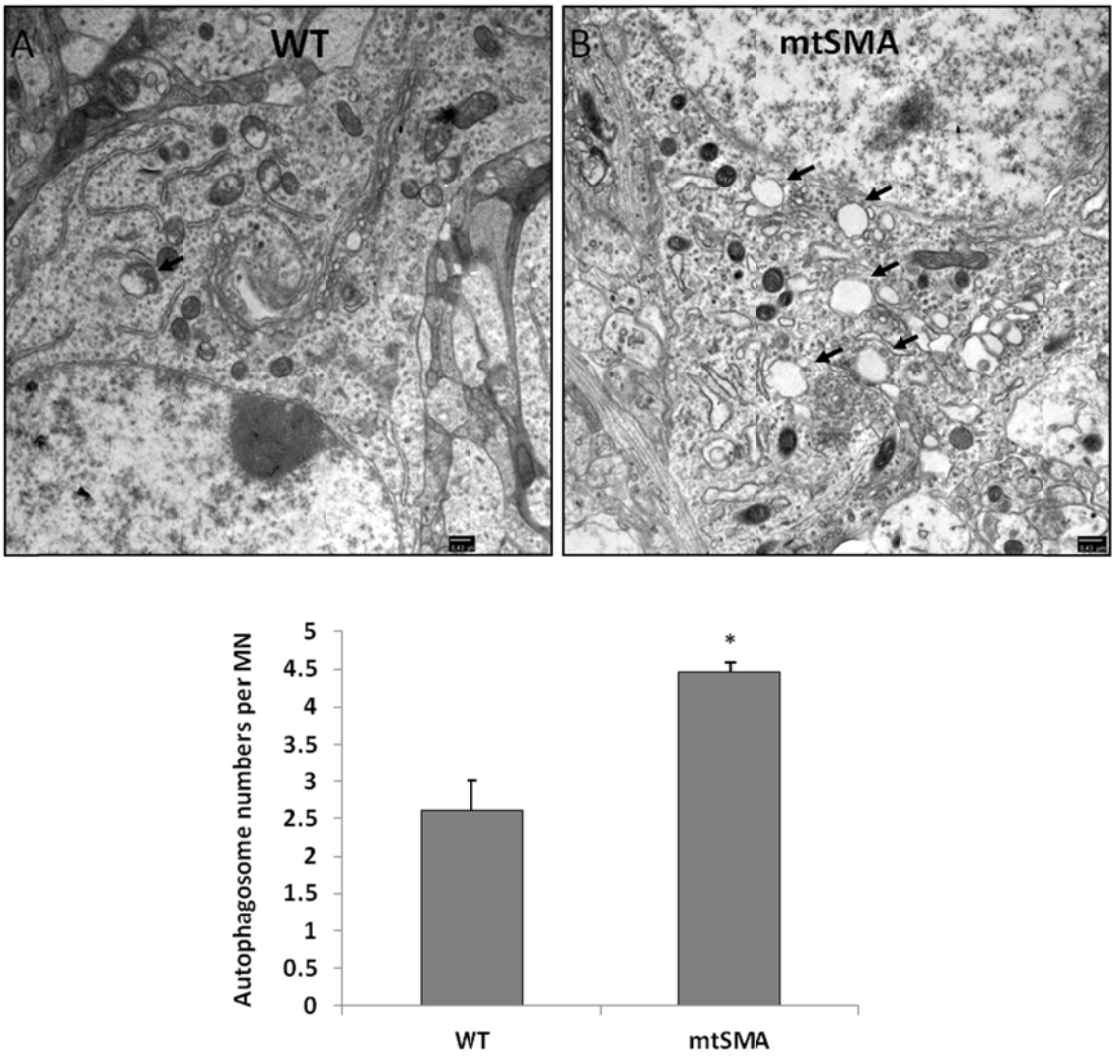

Figure 43. Analysis of autophagosome number by electron microscopy. Representative images from WT (a) or mtSMA (b) spinal cord MNs. Arrows indicate autophagosomes in both WT and mtSMA MNs. Graph represents the average of autophagosome number in mtSMA MNs compared to WT. (*WT n=30; mtSMA n=30; *p<0.05 Student's t test).*

Autophagosome can be recognised by double membrane formation that include cytoplasmic material like mitochondria, RER, ribosomes and some fragments of non-functional proteins (Figure 43 a/b, arrows). In mtSMA spinal cord, MNs shows increased a number of autophagosomes in soma and neurites (WT n=30; mtSMA n=30; $p<0.05$ Student's t test).

1.6. Immunofluorescence detection of autophagy in SMA model

1.6.1. Immunofluorescence analysis of LC3-II in embryonic SMA spinal cord MNs

Fluorescence microscopy gives a clear idea of specific protein expression inside the cellular compartment. Autophagy related proteins can be measured by probing an antibody against target protein that can be enhanced by fluorescence dye (Nagy et al. 2014).

To analyse changes in autophagy level between WT and mtSMA, we have used embryonic spinal cords (E15 and E18) from SMA *Smn (-/-); SMN2* mouse. Tails were submitted to genotyping and WT and mtSMA spinal cords were dissected and fixed for sample preparation. Immunodetection was performed using an antibody against LC3 protein. Cells were also stained using Nissl and Hoestch dyes. MNs were identified by evaluating location, size, morphology and Nissl staining in the ventral horn of spinal cords. The use of MN-specific markers is crucial for proper identification since not all large neurons within the ventral spinal cord are MNs. Large neurons have abundant rough endoplasmic reticulum (RER) which forms a bunch of aggregates, the Nissl granules. Nissl staining is used to label RER in neurons. Hoechst dye is membrane permeable and stains live cells through its binding to an adenine-thymine-rich region of DNA in the minor grooves. To analyze the level of LC3-II in WT (n=20) and mtSMA (n=20) MNs, we quantified the number of fluorescent spots in approximately 20-25 neurons of each condition using Image J program. MN specific areas were selected and the images were submitted to Image J to count the number of fluorescent spots bigger than 0,5 µm. The level of fluorescent spots in E15 and E18 was increased in mtSMA MNs compared to WT MNs. This result indicates that in embryonic mtSMA MNs the level of autophagosome generation and/or accumulation is higher than in non-mutant MNs and indicates an autophagy deregulation in these cells.

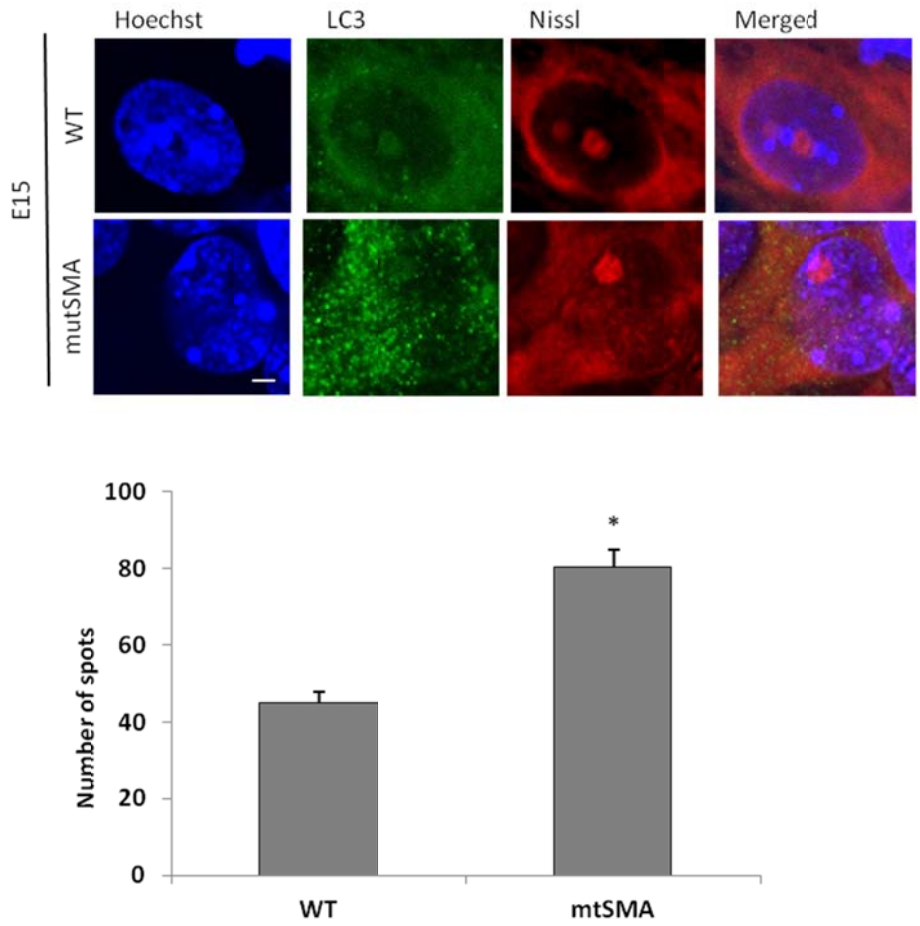

*Figure 44(a). Measure of Autophagy level using immunofluorescence. Fluorescence microscopy detection shows the number of LC3 spots in WT and mtSMA spinal cord MNs. E15 WT and mtSMA samples were processed for immunofluorescence detection using an antibody against LC3 II protein. Same slides were again stained with Nissl staining for MN specific detection and Hoesct staining to identify the nucleus. Graph represents the average number of spots WT and mtSMA and the normalized level of LC3-II protein expression in WT, and mtSMA condition. Scale bar 10µm.(*p<0.005 Student's t test)*

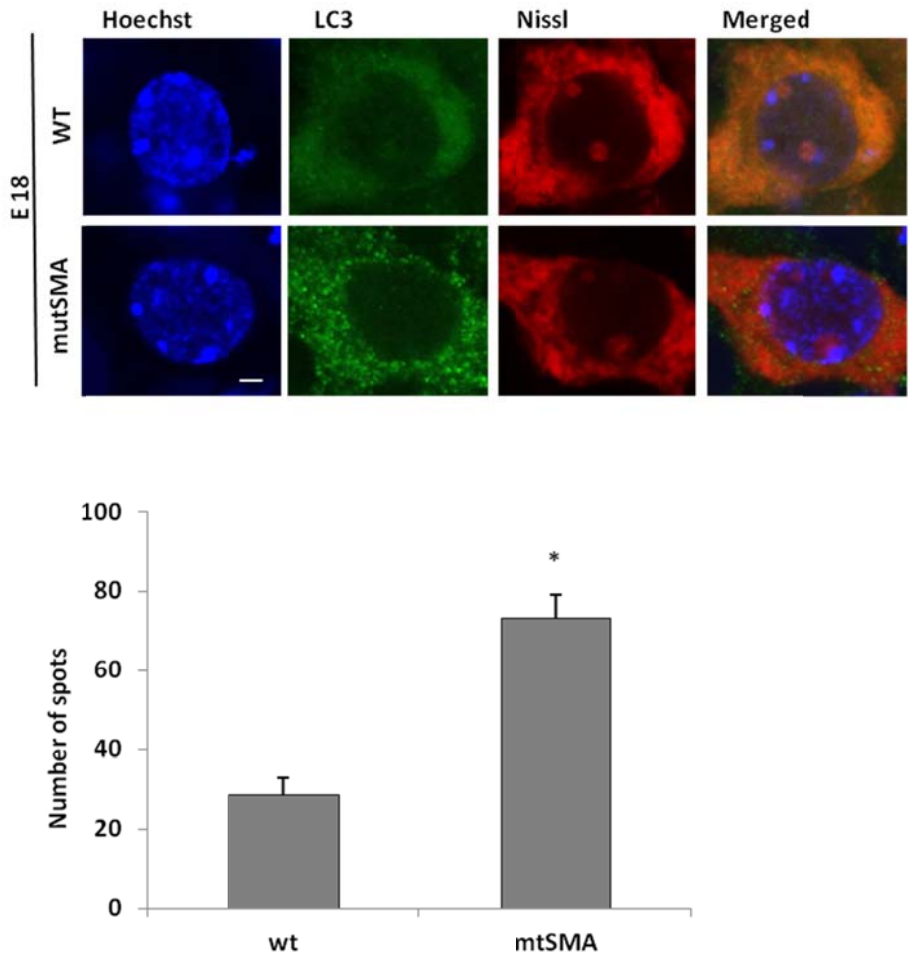

*Figure 44(b). Measure of Autophagy level using immunofluorescence. Fluorescence microscopy detection shows the number of LC3 spots in WT and mtSMA spinal cord MNs. E18 WT and mtSMA samples were processed for immunofluorescence detection using an antibody against LC3 II protein. Same slides were again stained with Nissl staining for MN specific detection and Hoesct staining to identify the nucleus. Graph represents the average number of spots WT and mtSMA and the normalized level of LC3-II protein expression in WT, and mtSMA condition. Scale bar 6µm.(*p<0.001 students t test)*

1.6.2. Immunofluorescence detection of LC3-II in postnatal SMA spinal cord MNs

Postnatal embryos P1, P3 and P5 were submitted to genotype. Wt and mtSMA spinal cords were fixed and submitted to immune detection. Approximately 20-25 MNs were processed for fluorescence quantification. As observed in figure 45 in postnatal spinal cords, LC3 fluorescent spots were increased in mtSMA MNs compared to WT. LC3 spots measured were three times higher in mtSMA indicating an increase of autophagosome formation in these cells. In P3, the number of LC3 spots was higher in both WT and mtSMA MNs compared to P1 and P5 measures. This observation suggests that during the postnatal period there is an increase of the autophagy process showing its maximum value at P3.

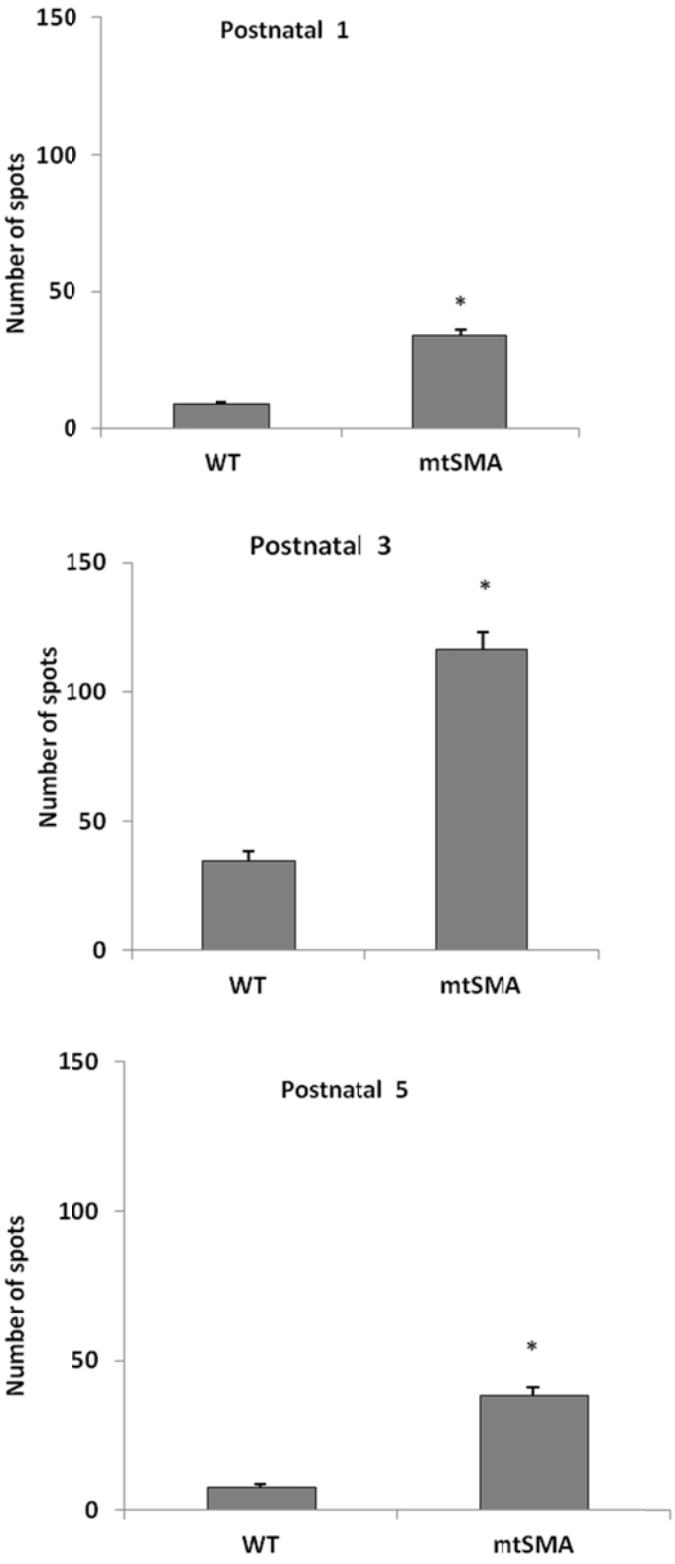

*Figure 45. Immunofluorescence detection of LC3 spots in WT and mtSMA MNs. WT or mtSMA spinal cords from P1, P3, P5 were processed for immune detection. Image showing immune staining, green fluorescence indicate LC3, blue indicates nuclei or Hoechst staining, and red indicates Nissl staining. Image also shows merged result of all three fluorescence markers. Graph shows the number of LC3 spots measured in MNs and the average level of LC3 expression in P1, P3, P5 stage of WT and mtSMA MNs. Scale bar measured at 6µm. (*p<0.001 Student's t test).*

2. Smn protein level is regulated by the proteasome

Intracellular proteins can be degraded or cleaved by several different proteolytic systems such as proteasome, the calcium-activated protease calpain system, lysosomal proteases, and autophagy (Vicencio et al. 2010). Proteasome system is a proteolytic pathway involved in the selective degradation of proteins that could potentially form toxic aggregates (Goldberg 2003; Malkus et al. 2009). MG-132 is a proteasome inhibitor used to prevent the protein degradation by proteasome.

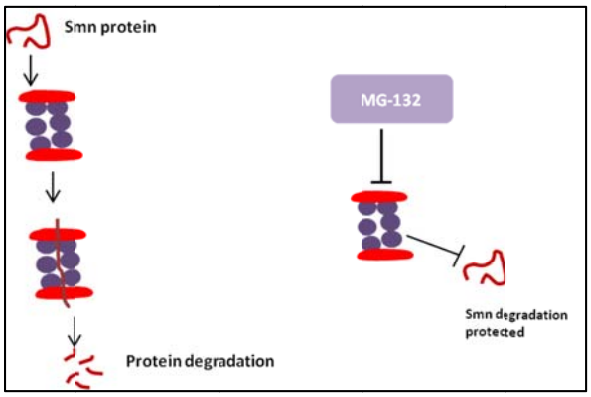

Figure 46. Proteasome is involved in the degradation of protein in the intracellular environment. MG-132 is a proteasome inhibitor.

To investigate whether SMN protein is degraded by proteasome pathway, we treated the cells with 2.5 µM of MG-132 for 12 hrs, 6 days after platting. Protein extracts were obtained and submitted to Western blot analysis. The result shows increased Smn protein expression level on MG-132 treated cultures. We also measured LC3-II protein level to analyse autophagy, and we observed increased LC3-II protein level in MG 132 treated condition. Our result suggests that Smn level is regulated through proteasome in MNs. Treatment with MG132 increases Smn protein and also increases autophagy (Figure 47).

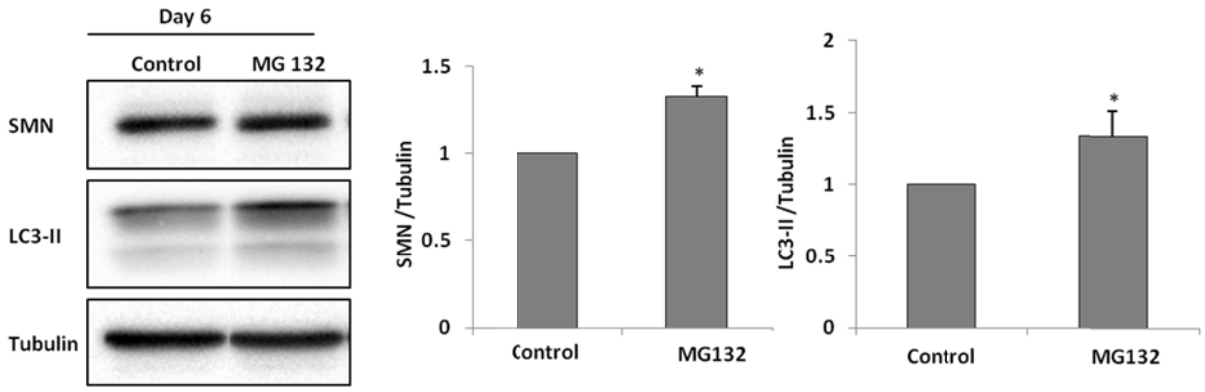

*Figure 47. Effect of proteasome inhibitor MG 132 on Smn and LC3-II protein level. MNs were purified and 6 days after plating, cells were treated with MG 132. Protein extracts were obtained and submitted to Western blot. Membranes were probed with anti-Smn or anti-LC3 antibody. Same membranes were re-probed with anti-tubulin as a loading control. Graph represents the level of Smn or LC3-II protein expression in comparison with tubulin. Bar indicates a significant difference in MG 132 treated condition corresponds to quantification of three independent experiments. Student's t test (*p<0.05).*

2. 1. Effect of MG-132 on MN cell survival

To measure the effect of proteasome inhibitor on MN survival, MNs were plated approximately 15,000 cells per dish in the presence of NTFs. On day 6, cells were treated or left un-treated with MG-132 (2.5 µM). Images were taken using the inverted microscope at several time periods (t0, t12, t24, t36) and the image took at t0 were considered as the number of cells present at the initial period. MG-132 treated cells showed axonal degeneration and cells died after 36 hrs treatment. MNs morphological observation indicates that proteasome inhibition causes neurite degeneration and MN cell death.

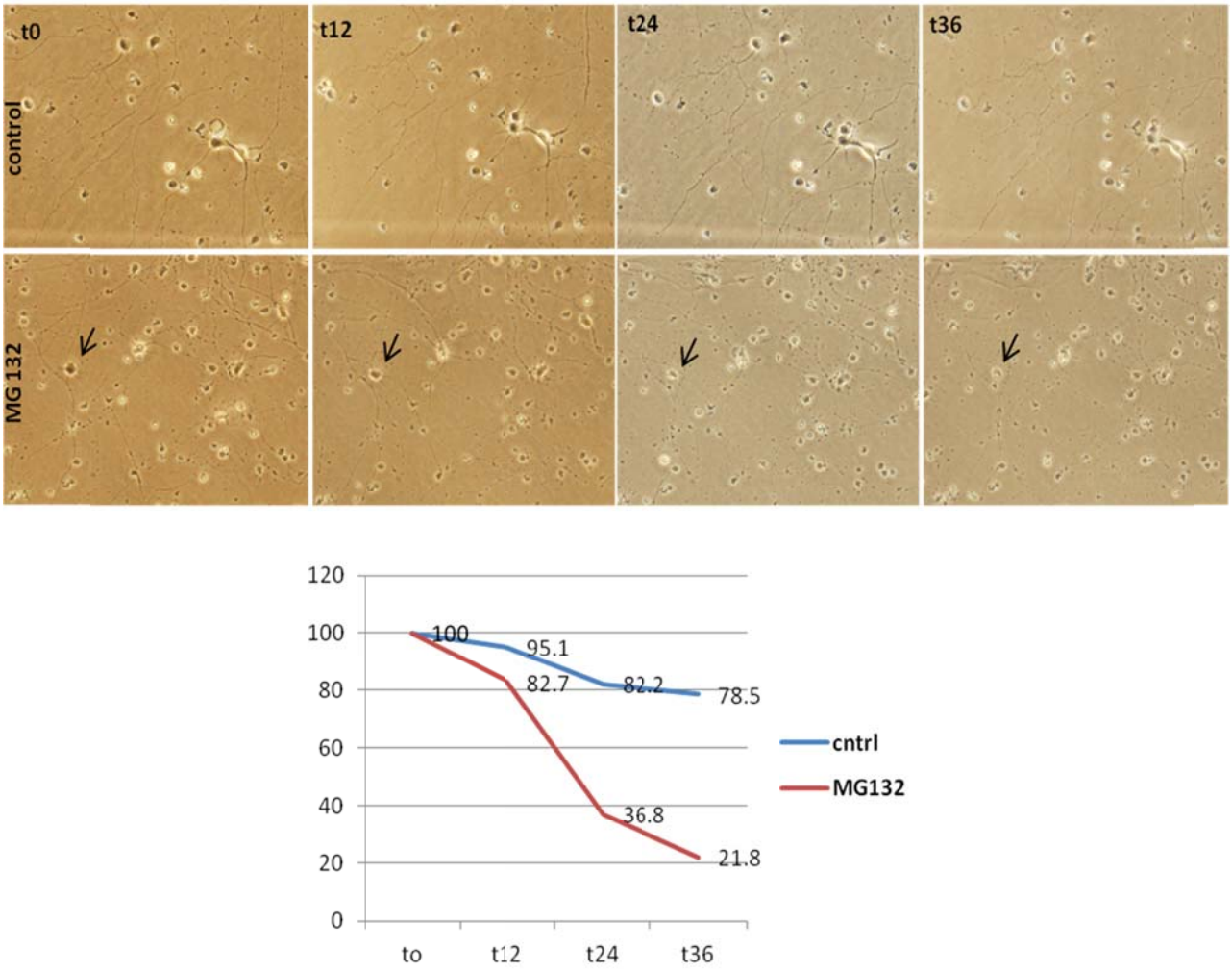

Figure 48. Effect of MG-132 on MN cell survival. Images taken from cells treated or non-treated with MG-132 at different interval of time (t0, t12, t24, t36) on day 6. Graph represents the percentage of cell survival in control (indicated by the blue line) or MG-132 (indicated by red line).

2.2. Effect of proteasome inhibitor MG 132 on SMA mutant MN

We also analysed the effect of MG 132 on Smn protein level in mtSMA and WT MNs. Tails from SMA E12.5 embryos were submitted to genotype. WT and mtSMA spinal cords were dissected and MNs were obtained. Six days after plating cells were treated with MG 132 (2.5 µM) for 12 hrs. Cells were lysated and extracts were submitted to Western blot analysis. An increased Smn protein expression were observed in MG 132 treated MNs compared to the non-treated condition. We also observed an increase of LC3-II in MG-132 treated cells. This result suggests that MG-132 protects Smn from proteasome degradation in SMA MNs.

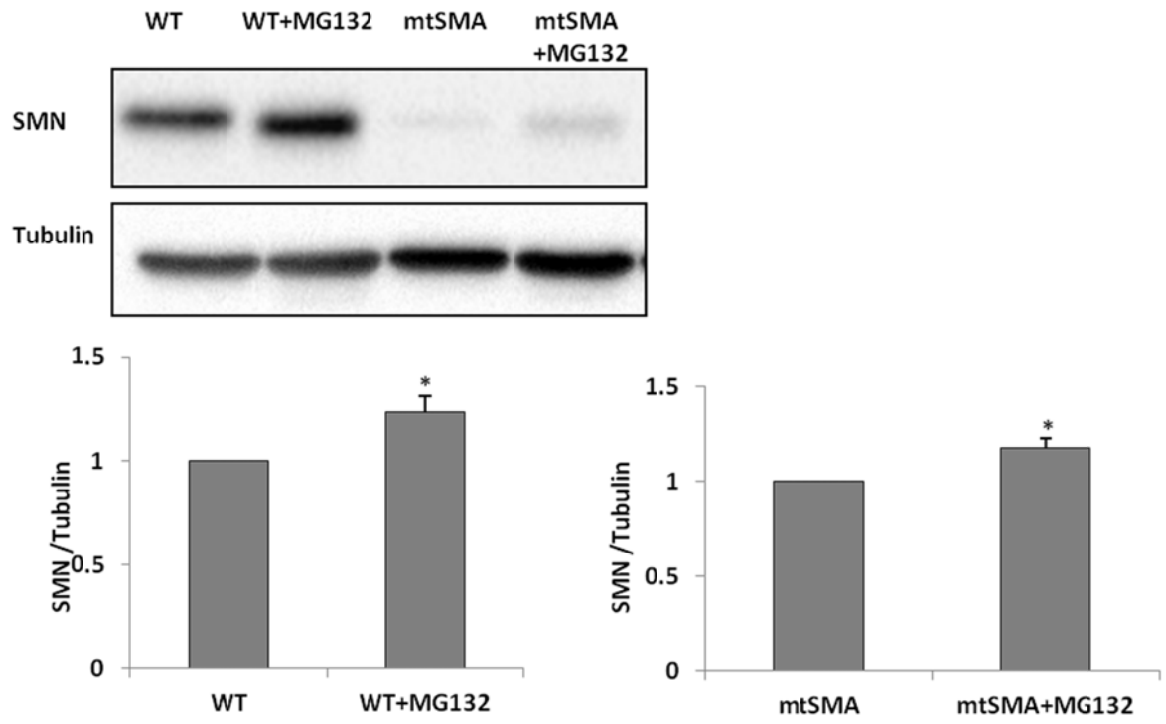

Figure 49. Effect of MG 132 on SMA mutant MN cultures. WT and mtSMA cells were treated with MG-132 6 days after plating. Protein extracts were obtained and submitted to Western blot analysis. Membranes probed with antibody against the Smn. The same membranes were re-probed with anti-tubulin as a loading control. Graph represents the expression of Smn protein in the different conditions and corresponds to quantification of three independent experiments. Student's t test p<0.005 WT; p<0.05 mtSMA.

3. Effect of autophagy modulators on Smn protein expression level

In Smn reduced MNs we have observed changes of the autophagy markers. Because the level of Smn is a central feature in SMA pathogenesis, we decided to induce or to inhibit the autophagy process in order to analyze the effect of its regulation in Smn protein level.

3.1. Effect of autophagy blockers on Smn protein level

Autophagy is a multi-step process, it initiates with the phagophore formation, double membrane autophagosome and fuse with the lysosome for digestion. To analyse the effect of autophagy inhibition on Smn protein level in MNs, cells were treated with different drugs that block autophagy process at a different level.

3.1.1. Effect of the late stage autophagy blocker Bafilomycin A1 and Ammonium Chloride on Smn protein level

BafA1 prevents the fusion of the autophagosome with the lysosome. BafA1 directly interfere with the maturation of autophagosome by inhibiting fusion with the lysosome

(Akitsugu Yamamoto et al. 1998). Ammonium chloride (NH_4Cl) is also an autophagy blocker that neutralise the lysosomal pH and blocks autolysosomal degradation (Sun et al. 2014).

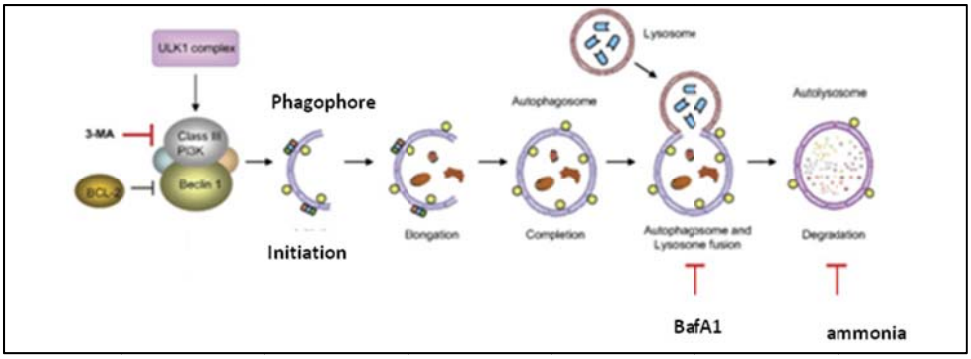

Figure 50. Autophagy process blocked by BafA1 or NH_4Cl at a late stage of autophagosome fusion with the lysosome. Blocking of autophagy increases the accumulation of autophagosomes inside the MN.

MNs were treated with BafA1 (50 nM) for 4 hrs or NH_4Cl (10 mM) for 4 hrs on day 6 after plating. Protein extracts were obtained and submitted to Western blot analysis. MN treated with Baf A1, or NH_4Cl shows decreased Smn protein expression level compared to non-treated cells. The same membranes were probed with the ant-LC3 antibody showing an increase of LC3-II band. These results together indicate that the late inhibitors of the autophagy process cause an increase of autophagosome number and the reduction of Smn protein level.

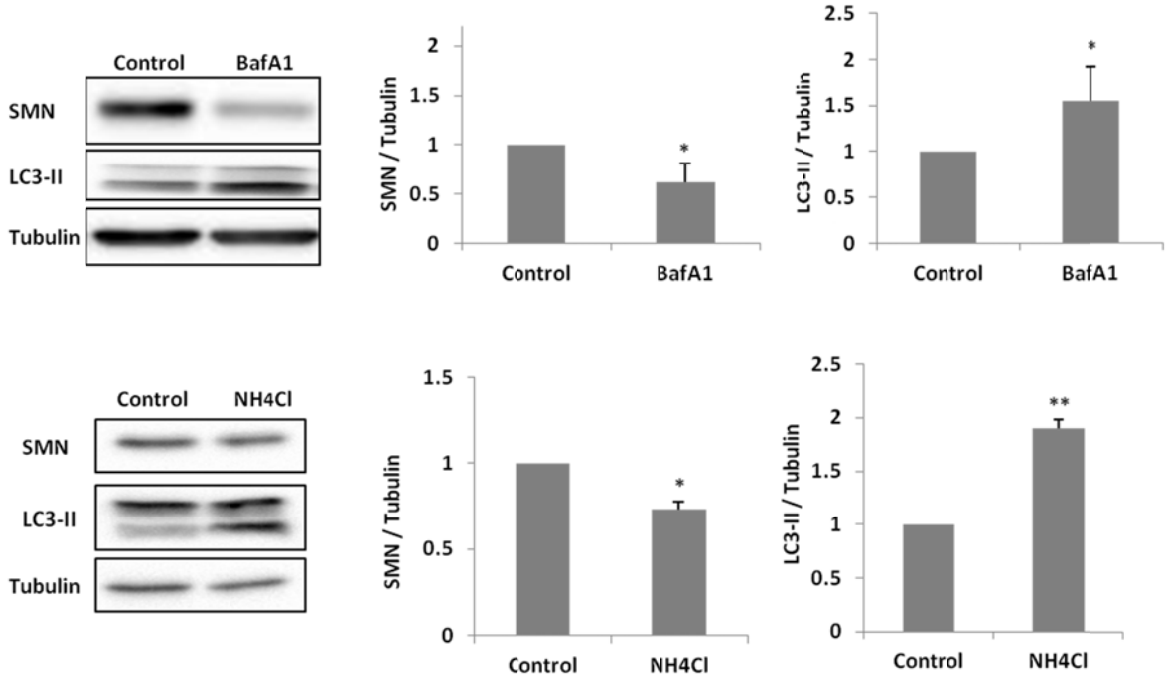

*Figure 51. MNs treated with BafA1 (50 nM) or NH_4Cl (10 mM). Protein extracts were obtained and submitted to Western blot analysis and membranes probed with anti-Smn or anti-LC3 II antibody. Membranes reprobed with anti-tubulin considered as a loading control. Graph shows percentage of Smn or LC3-II protein expression level in comparison with tubulin and corresponds to quantification of three independent experiments. Bar represents the mean of three independent experiments. *p<0.05; **p<0.005 Student's t test.*

3.2.2. Effect of early stage of autophagy blockers: 3-methyladenine on Smn protein level

3-methyladenine treatment blocks the phagophore formation step in the early stage of autophagy process. It blocks class III PI3K binding to Beclin 1 that initiate phagophore formation and prevent autophagy (Wu et al. 2010).

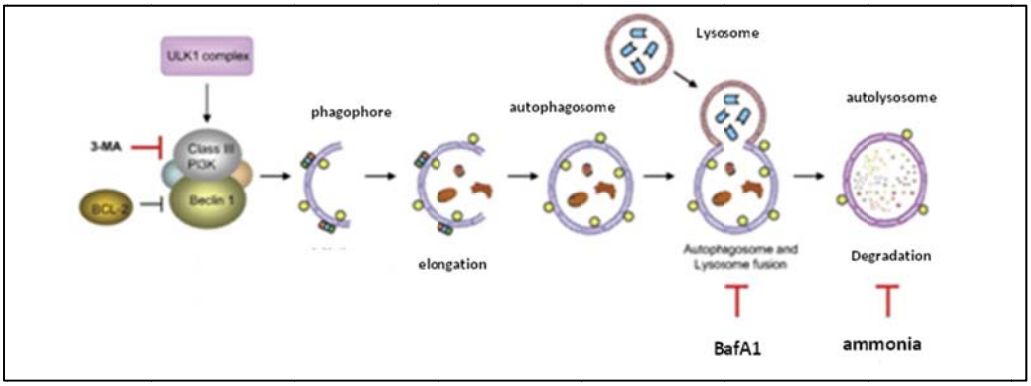

Figure 52. Autophagy process blocked by 3-MA, at an early stage by blocking class III PI3K binding with Beclin 1 and inhibit activation of phagophore and autophagosome formation.

To measure the effect of an early stage autophagy blocker, cells were treated with 3-MA (5 mM) for 12 hrs, 6 days after plating. After treatment, protein extracts were obtained and

submitted to Western blot analysis. The result shows increased Smn protein level in 3-MA treated cells. The same membranes were probed with the anti-LC3 antibody and no differences were observed in LC3-II protein level. These results together show that early stage autophagy blocker increases Smn protein level in MNs. Compared to the previous data, inhibition of phagophore formation increases Smn protein level in MNs whereas late-stage inhibition decreases Smn.

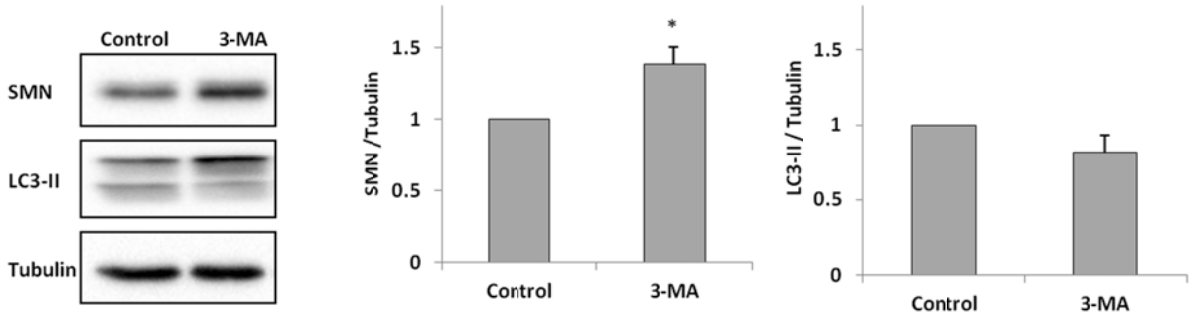

*Figure 53. Western blot analysis showing the effect of 3-MA, early stage autophagy inhibitor on MN. Cells treated with 3-MA for 12hrs on Day 6. Protein extraction obtained and submitted to Western blot analysis. Membranes probed with anti-Smn or anti-LC3-II antibody and re-probed again with anti-tubulin considered as a loading control. Graphs represent the level of Smn or LC3-II compared with tubulin and correspond to quantification of three independent experiments. *p<0.005 Student's t test (left graph).*

3.1.2.b. Effect of early stage autophagy blocker 3-MA on SMA mutant MNs

To measure the effect of 3-MA on mtSMA MNs, E13 SMA mouse embryos were submitted to genotyping. WT and mtSMA MNs were obtained and plated. Six days later cells were treated with 3-MA (5 mM) for 12 hrs. Protein extracts were obtained and submitted to Western blot analysis. The result shows an increase of Smn level in 3-MA treated cultures compared to non-treated cells in both WT and mtSMA MNs. Our result suggests that 3-MA increases Smn protein level in mtSMA MNs.

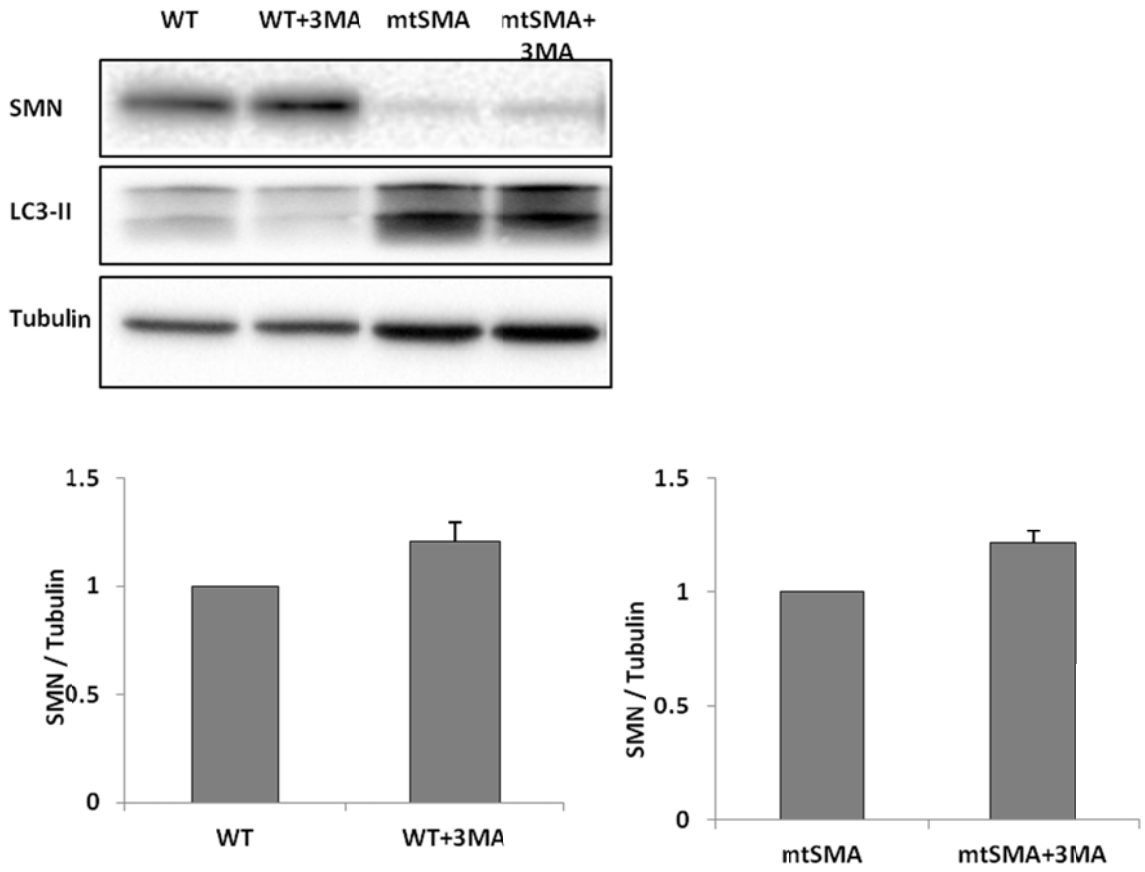

Figure 54. Effect of 3-MA, early stage autophagy inhibitor on SMA MNs. WT and mtSMA MNs were treated with 3-MA (5mM) for 12 hrs on Day 6. Membranes were probed with anti-Smn or anti-LC3-II antibodies and reprobed with antibody against α-tubulin considered as a loading control.

3.1.3. Effect of autophagy blocker and a proteasome inhibitor on Smn protein expression level

Proteasome and autophagy are the most relevant machineries responsible for protein degradation. Proteasome inhibition activates autophagy as an alternative effect to maintain cellular homeostasis. We treated the cells with the proteasome inhibitor MG-132 (2.5 µM for 12hrs) and the autophagy inhibitors BafA1 (50 nM for 4hrs) or NH_4CL (10 mM for 4hrs) or 3-MA (5 mM for 12hrs). Protein extracts were submitted to western blot using anti-Smn or anti-LC3 antibodies. Results shown in Figure 55 indicate that treatment with BafA1 or NH4Cl increased LC3-II and decreased Smn protein level, and 3-MA did not change LC3-II level and increased Smn protein. When cells were co-treated with MG-132 together with Baf1 or NH_4Cl, MG-132 reverted the effect of BafA1 or NH_4Cl and the levels of Smn increased significantly. This result suggests that autophagosome increase may induce proteasome activation. In MG132 and 3-MA co-treated cells, Smn measures indicate that no significant differences when MG132 was added to 3-MA cells suggesting that inhibition of the

autophagosome formation and proteasome activation have not an additive effect on Smn stability.

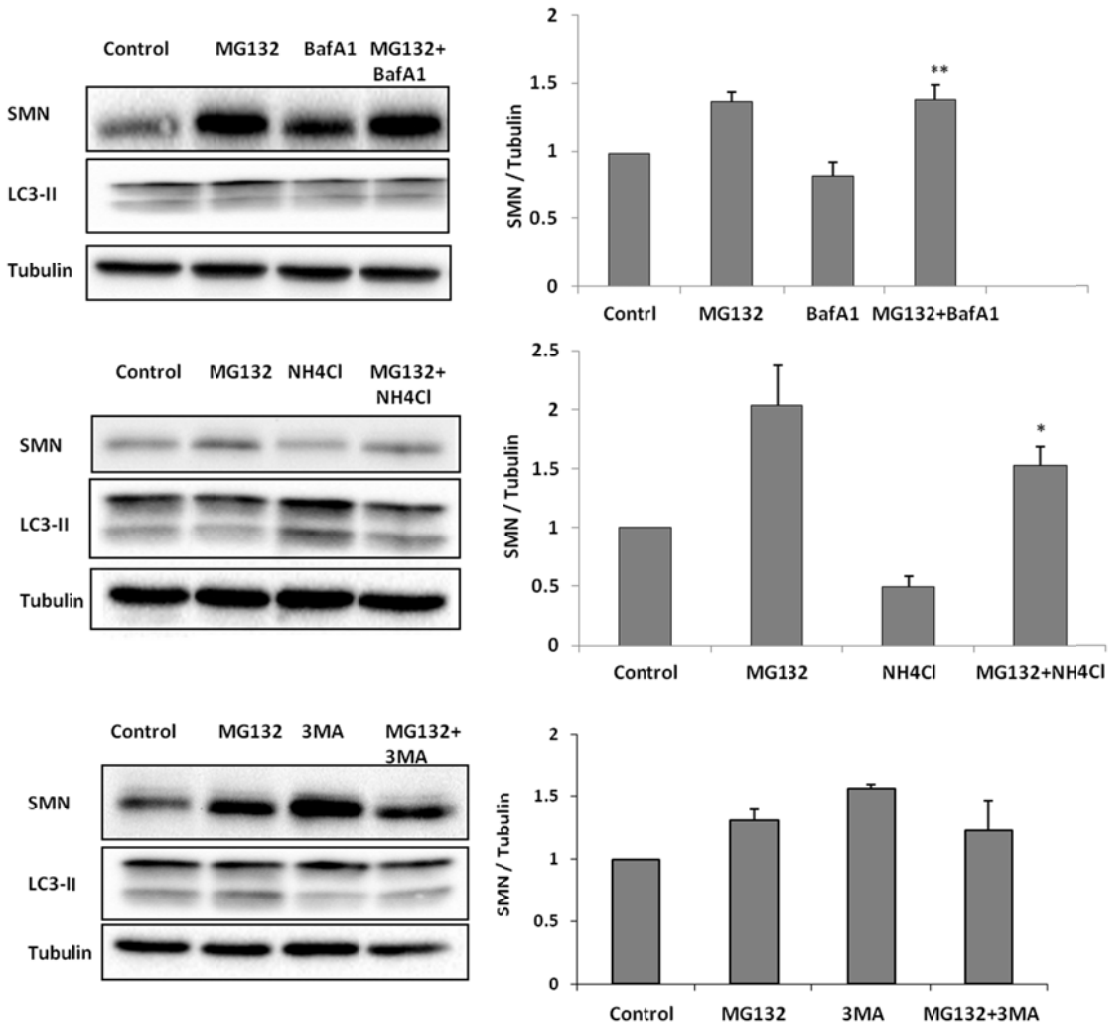

*Figure 55. Western blot analysis showing MNs treated with MG 132 and BafA1 or NH_4Cl or 3-MA. Protein extracts were obtained and submitted to Western blot. Membrane was probed with anti-Smn or anti-LC3-II antibodies and reprobed with anti α-tubulin antibody as a loading control. Graph shows Smn protein expression level compared to tubulin and corresponds to the quantification of three independent experiments. *p<0.01 and **p<0.0001 one way ANOVA Bonferroni's multiple comparison test.*

3.2. Effect of autophagy inducers on Smn protein expression level

3.2.1.a. Effect of Rapamycin (mTOR-dependent autophagy pathway) treatment on Smn protein level

Rapamycin is upstream of mTOR and activates autophagy in nutrient-deprived conditions (Hosokawa et al. 2009) (Figure 56).

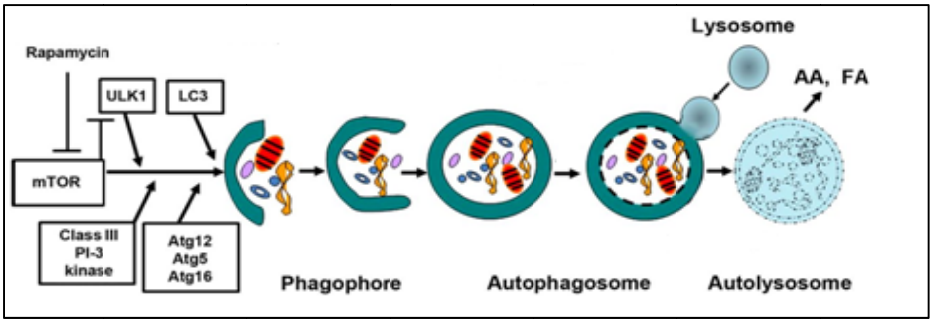

Figure 56. Rapamycin, autophagy inducer inhibits mTOR and mediate ULK1, PI3K, Beclin 1 activation of the autophagy pathway.

To measure the effect of Rapamycin treatment on Smn protein expression, 6 days after plating MNs were treated with Rapamycin (100- mM) for 6 hrs. Protein extracts were obtained and Smn and LC3 protein level were analysed by western blot. The results show that rapamycin treatment induced both Smn and LC3-II protein increase.

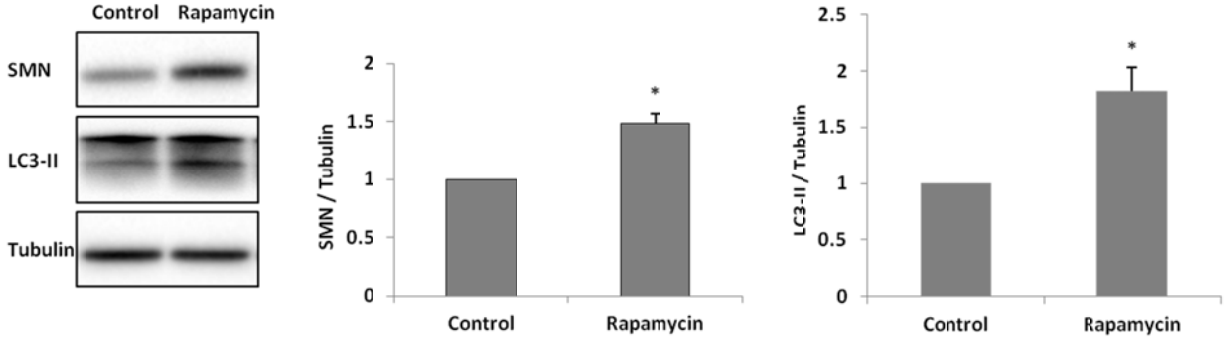

*Figure 57. Western blot analysis showing the effect of mTOR dependent autophagy inducer, rapamycin treatment on Smn protein level in MNs. Membranes were probed with anti-Smn or anti-LC3 antibody, and the same membranes were reprobed with tubulin as a loading control. Graph represents Smn or LC3-II protein expression level in comparison with tubulin. Graphs represent the mean of more than three independent experiments. *p<0.05 Student's t test.*

b. Effect of Curcumin (mTOR-dependent autophagy pathway) treatment on Smn protein level

Curcumin induces autophagy through mTOR dependent pathway by AMPK signalling activation and mTOR inhibition (Xiao et al. 2013; Aoki et al. 2007). To measure the effect of curcumin on Smn protein level, cells were treated with 500 nM of Curcumin for 12hrs on day 6 after plating. The results showed that Curcumin treatment increases Smn protein level in MNs. We also measured changes in LC3-II protein level, no significant differences of LC3-II level were observed in curcumin treated cultures compared to non-treated cells.

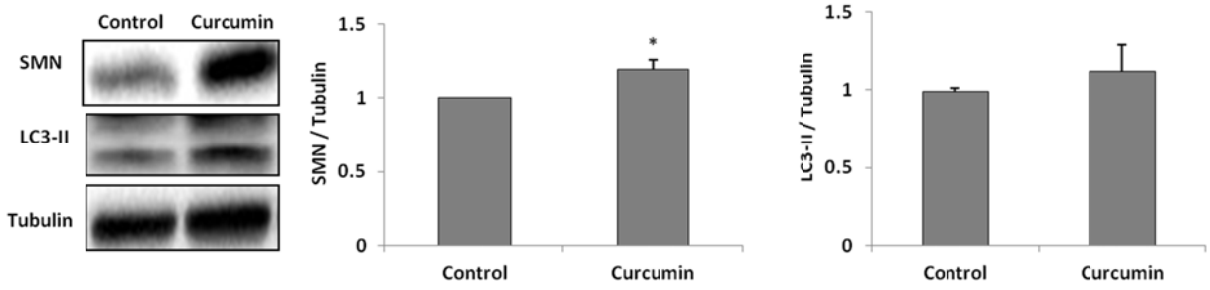

*Figure 58. Western blot analysis of Smn protein level in curcumin treated. Membranes were probed with antibodies against Smn or LC3-II, and the same membranes were reprobed with anti-tubulin antibody as a loading control. Graphs represent Smn or LC3-II protein expression level by comparison with tubulin and corresponds to the quantification of three independent experiments. *p<0.05 Student's t test in measures of Smn western blot.*

3.2.2. Effect of mTOR-independent autophagy inducers on Smn protein level

Autophagy can also be induced through an mTOR-independent pathway the phosphatidyl inositol dependent pathway which is activated by Ca^{2+} signaling. To measure the effect of mTOR independent autophagy inducer cells treated with trehalose (100 mM) for 6 hrs or resveratrol (5 µM) for 12 hrs, six days after plating. Protein extracts were obtained and submitted to Western blot analysis. The results show that both trehalose and resveratrol increased Smn protein level compared to non-treated cells as well as LC3-II protein.

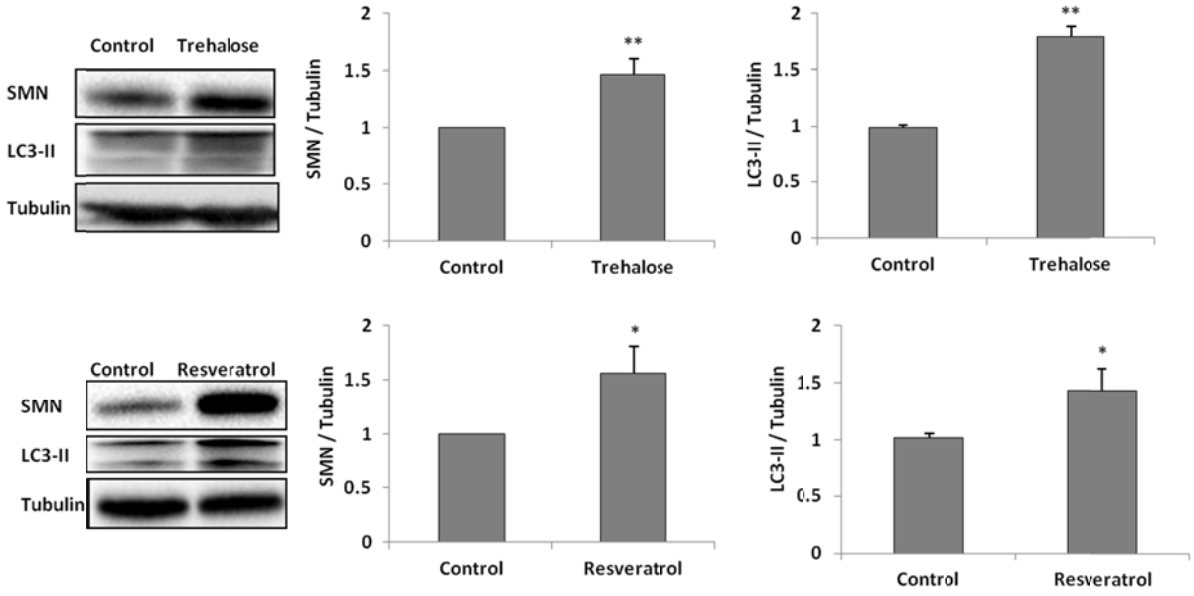

Figure 59. Western blot analysis of protein extracts from trehalose- or resveratrol-treated cultures. Membranes were probed with anti-Smn or anti-LC3 antibodies, and the same membranes were reprobed with anti-tubulin as a loading control. Graph represents the level of Smn protein expression by comparison with tubulin and quantification was done from three independent experiment. Graphs of trehalose treatment **p<0.005 Student's t test; graphs of resveratrol treatment *p<0.05 Student's t test.

4. Calpain inhibition increases Smn protein level

4.1. Effect of calpain reduction on Smn protein level in MNs

Calpains are calcium-activated cysteine proteases involved in numerous cellular processes. Calpains typically perform the cleavage of their substrates to regulate their activity (Vosler et al. 2008). To measure the effect of calpain inhibition on Smn protein level in MNs, cells were transduced with EV or shcalpain (shCpn) lentivirus and six days after plating protein extracts were obtained and submitted to Western blot analysis. Results shown in Figure 60 demonstrate that calpain knockdown increased Smn protein level in MNs, indicating the role of calpain protease in the regulation of SMN protein.

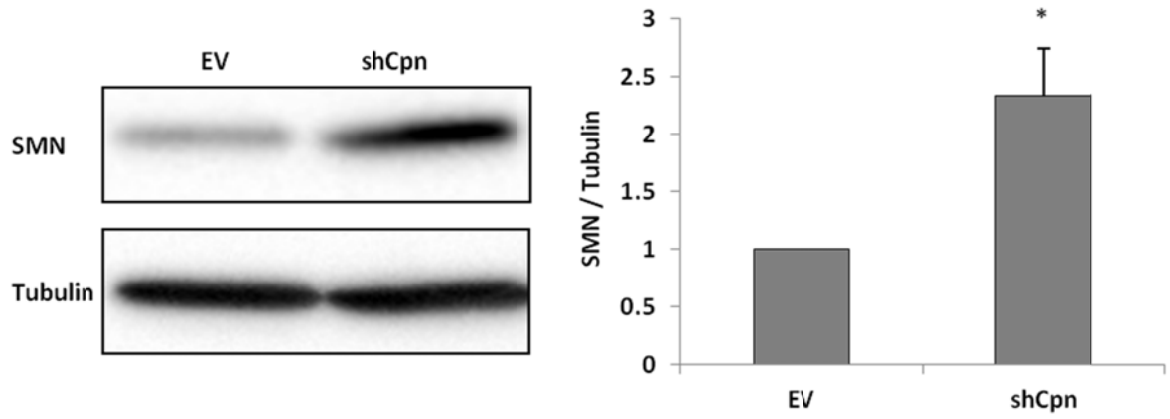

*Figure 60. Effect of calpain reduction on Smn protein level in MNs. MNs were transduced with EV or shCpn, and cell lysates were obtained after 6 days of transduction. Protein extracts were analysed by western blot. Membranes were probed with anti-Smn antibody and the same membranes were reprobed with anti-tubulin as a loading control. Graph shows the level of Smn protein by comparison with tubulin. Bars indicate the mean of three independent experiments, *p<0.05 Student's t test.*

4.2. Effect of calpain inhibition and autophagy inhibitor on Smn protein level

It has been described that calpain activity induces autophagy (Menzies et al. 2014). To further analyze the involvement of autophagy on Smn regulation by calpain, we have decided to add the autophagy inhibitor NH_4Cl (10 mM for 4 hrs) to shCpn or EV control cultures. Protein extracts were obtained and submitted to Western blot analysis. The result shows that NH_4Cl treatment decreased Smn protein level both in EV and shCpn condition compared with untreated control.

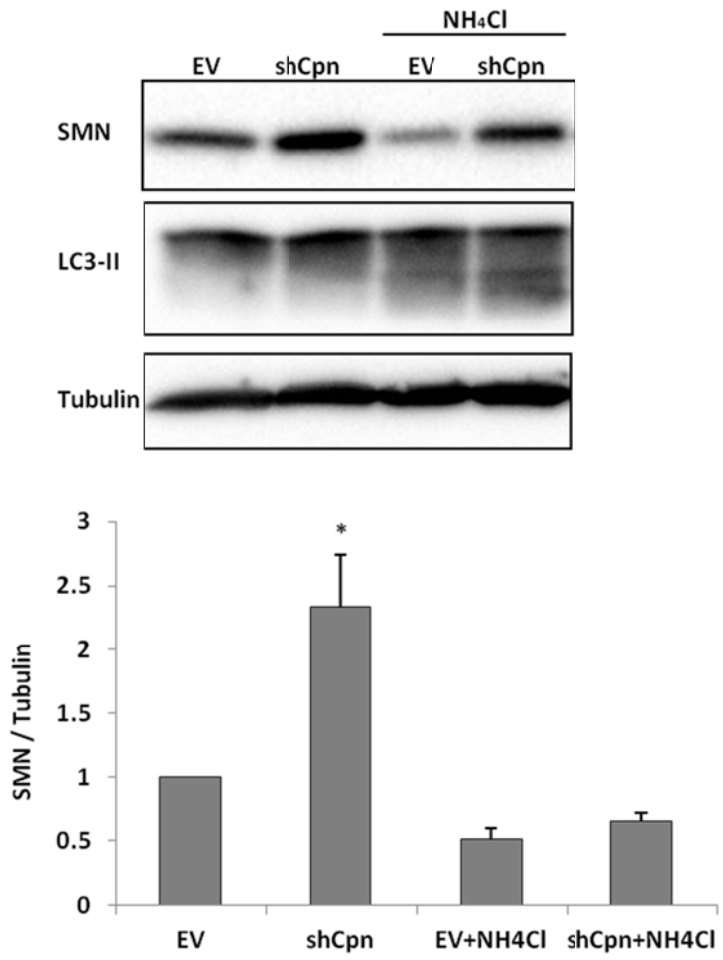

*Figure 61. Effect of calpain knockdown and autophagy blocker NH_4CL treatment on Smn protein level. EV or shCpn MNs were treated or not with NH4Cl, Protein extracts were obtained and submitted to western blot. Membranes were probed with anti-Smn or anti-LC3II antibody and the same membrane reprobed with tubulin antibody as a loading control. Graph represents the quantification of Smn protein by comparison with tubulin. Bars indicate the mean of three independent experiments. *p<0.05 student's t test.*

Discussion

DISCUSSION

The main objective of our work is to study the origin of MN degeneration originated by SMN protein reduction. To this end, we have used a primary culture of Smn-reduced spinal cord MNs from embryonic CD1 mouse as a suitable *in vitro* model of SMA. Our primary goal was to analyze the response of these MNs to Smn knockdown when they are isolated from their environment. Changes in phenotype will be indicative of changes in intracellular protein and pathways that will help us to understand the physiopathology of SMA disease. In this context, we reduced the Smn protein expression using RNA interference method.

From our previous results we have showed that the Smn reduction causes neurite degeneration and MN cell death in a later stage. The neurite degeneration observed consists of swelling and disruptions, and the cell death has not the features of apoptosis (Garcera et al, 2011). Similar results have been described in a cellular model of reduced Smn in NSC34 MN-like cell line. Smn-reduced NSC34 cells show changes in the soma size, reduction of the number of somas bearing neurites, and mitochondrial dysfunction (Acsadi et al., 2009). All these results together demonstrate that Smn reduction induce morphological changes that may be the origin of MN degeneration.

SMN is present throughout neurites and growth cones (Zhang et al., 2003). Therefore reduced level of Smn in MNs can disrupt neurites and this disruption can lead to neuronal degeneration. Axonal swelling and varicosities have been also described in SMA mice and patients (McGovern et al., 2008; Burghes and Beattie 2009). Consequently morphological cell responses to Smn reduction observed in several *in vivo* and *in vitro* models suggest that neurite degeneration and cell body changes may be parallel events. However, we need more experiments to determine whether neurite degeneration lead to cell death or vice versa. Therefore, from our results and others (Hilliard 2009; King et al., 2009) we propose that elucidating the mechanism/s involved in axon degeneration may be important in the understanding of SMA disease.

Regarding to the cell death observed in Smn-reduced MNs, our previous work demonstrated that over-expression of the anti-apoptotic protein Bcl-xL was able to prevent cell death in these cells, without affecting Smn protein level. Bcl-xL is a member of the BH3-only family of proteins that negatively controls apoptosis. It regulates mitochondrial outer membrane

permeabilization through its binding to the pro-apoptotic proteins Bax and Bak (Merry & Korsmeyer 1997). Thus, Bcl-xL is one of the essential mitochondrial mechanisms regulating cell death. Because Bcl-xL is an anti-apoptotic protein and the cell death observed in Smn-reduced MNs did not have the characteristics of apoptosis (caspase 3 activation and nuclear DNA fragmentation), we concluded that a caspase independent mechanism might be involved in MN cell death when Smn protein level is reduced. It has been previously suggested that mitochondria can also promote cell death in a caspase-independent process (Susin et al.,1999). In this context, it is known that the anti-apoptotic proteins Bcl-2 and Bcl-xL are important factors in the regulation of the autophagy process. Bcl-2 and Bcl-xL inhibit Beclin 1-mediated autophagy through their binding to Beclin 1 (Pattingre et al., 2005).

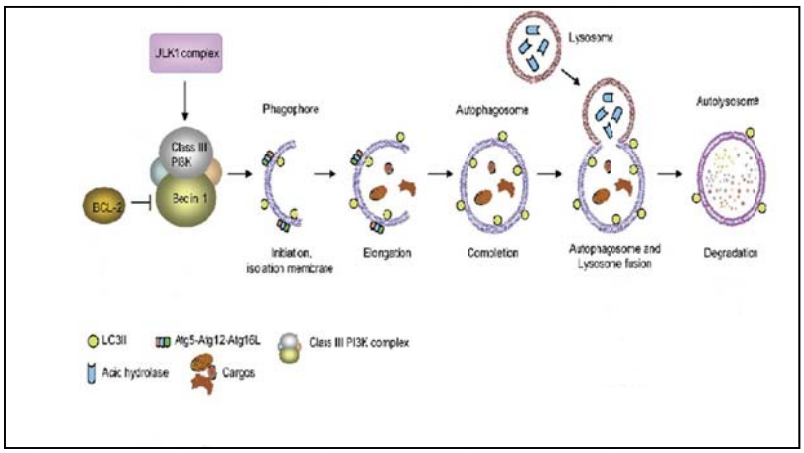

Figure 62. Bcl-2 and Bcl-xL proteins are anti-autophagy proteins by their binding to Beclin 1 and inhibiting the activation of autophagosome initiation.

Smn reduction causes alteration of the autophagy level in MNs

We analyzed the autophagy in Smn-reduced MNs in order to study the molecular process that leads Bcl-xL protein to prevent the degenerative events caused by Smn reduction (Garcera et al. 2011). After electron microscopy analysis of autophagosomes and western blot analysis of proteins, we observed an increase of autophagosome formation that can be prevented by Bcl-xL over-expression. We have also observed an increase of LC3-II and Beclin 1 protein levels. Together these results demonstrated the deregulation of the autophagy process in SMN-reduced MNs. To study the level of autophagy in SMA disease, we have used the type I SMA mice model. The levels of the autophagy marker LC3-II were also increased in cultured MNs obtained from embryonic SMA transgenic mice. Therefore using

two different *in vitro* approaches to the analysis we concluded that Smn protein reduction causes an increment of the autophagosome number as well as of the autophagy-related proteins Beclin-1 and LC3-II. LC3-II increase was counteracted by Bcl-xL overexpression, which promotes morphological recovery of Smn-reduced MNs. These results together suggest that an increase of Beclin 1-dependent autophagy could be one of the mechanisms responsible for MNs degeneration in the Smn knockdown model. In this context, over-expression of Bcl-xL may bind to Beclin1 and inhibits the autophagy process, thereby reducing the autophagy mediated neurite degeneration and cell death in MNs.

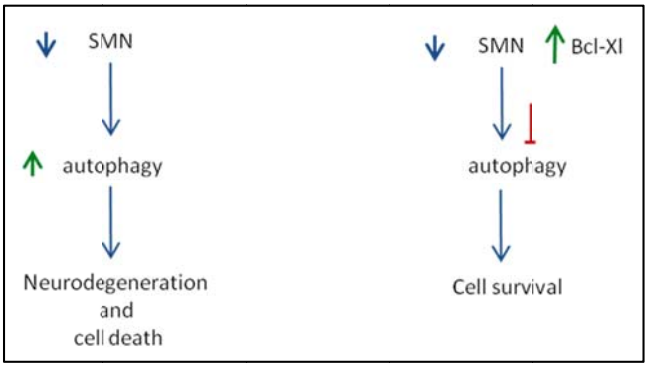

Figure 63. Bcl-xL over-expression decreases autophagy through autophagy reduction.

We considered two effective methods to monitor the autophagy process (i) autophagosome detection by electron microscopy and (ii) the presence of LC3-II in cells (Menzies et al. 2012; Barth et al. 2010). In our *in vitro* model, electron microscopy shows increased number of autophagosomes into the MNs cytoplasm and the western blot protein analysis shows increased LC3-II levels in total cell extracts. In both cases, the parameters are indicating an increase of the autophagosome number and it can be the consequence of either enhanced autophagosome synthesis or reduced autophagosome turnover. In autophagosome studies, it is important to analyze which of the above process is occurring. Enhanced autophagosome synthesis frequently correlates with increased delivery of autophagic substrates to lysosomes for degradation (increased flux). Decreased LC3-II degradation can occur at any point after autophagosome formation including: delayed trafficking to the lysosome, reduced fusion between autophagosomes and lysosomes, or impaired lysosomal proteolytic activity. LC3-II levels will rise over the experiment if the number of molecules generated exceeds the number of degraded. Conversely, decreased levels of LC3-II can be observed if there is a drop in autophagosome synthesis (associated with decreased

autophagic flux) or if autophagic flux is induced (Gurney et al. 2015; Menzies et al. 2012). LC3-II levels will decrease even if there is an increase in synthesis if the number of molecules generated in the experimental time-frame is less that the number of degraded. To distinguish between different possible interpretations of increased or decreased levels of LC3-II, it can be measured under conditions where autophagosome degradation is blocked, for example, in the presence of lysosomal protease inhibitors. One candidate can be the proton pump inhibitor BafA1 (Figure 64).

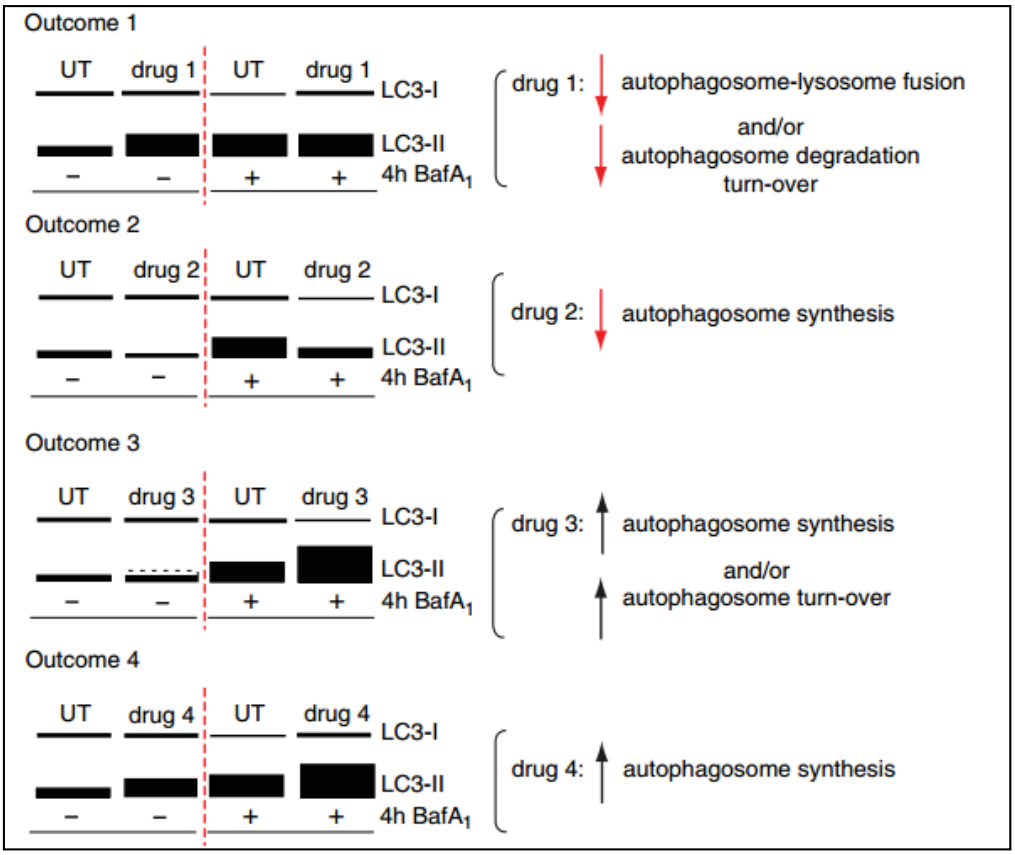

Figure 64. Theoretical scenarios for measurement of LC3 flux. The effect of a particular compound on autophagosome degradation can be inferred by comparing drug treatment in the absence or presence of inhibitors of lysosomal proteolysis (such as BafA1). The four possible outcomes of autophagy modulation by a drug are shown (drug 1-4) (Menzies et al. 2012).

Inhibition of autophagy in the presence of BafA1 the increase of LC3-II would be indicative of efficient autophagic flux whereas failure of LC3-II to increase would suggest a defect or delay earlier in the process, prior to degradation at the autolysosome (Gurney et al. 2015). Our results in the presence of BafA1 indicated that Smn reduction causes increased autophagosome synthesis without affecting autophagic flux.

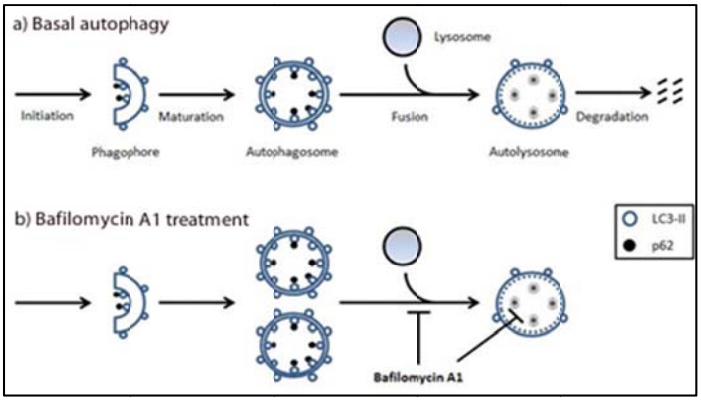

Figure 65. BafA1 treatment increases the number autophagosomes through its inhibition of lysosomal fusion.

The autophagic pathway has emerged as an essential component to maintain cellular homeostasis. In many tissues both constitutive and stress-induced autophagy are cardinal for their maintenance. Therefore, autophagy malfunctions might be the reason for cellular degeneration. When autophagosome formation is compromised or autophagosomes are accumulated, cell function and survival may be reduced. Although neuronal autophagy is substantially reduced as compared to that found in other cellular types under conditions of acute starvation (Mizushima et al. 2008), the integrity of the nervous system is more dependent on basal autophagy than that of other tissues. In neurons, autophagy is the main route for the degradation of toxic substances. In some neurodegenerative disorders, especially those caused by aggregates of mutant forms of specific proteins, there is an accumulation of autophagosome that may interfere with intracellular trafficking or may become a source of cytotoxic products (Ravikumar et al. 2004) (Wong & Cuervo 2010). It is possible that massive autophagosome accumulation perturbs axonal transport. In SMA, there are no evidences of accumulation of toxic proteins aggregates in MNs or in other cells, but microtubule-mediated axonal transport can be perturbed (Chevalier-Larsen & Holzbaur 2006). The origin of the axonal degeneration is not clear, although autophagosome accumulation may be contributing to this process. Our results demonstrated that spinal cord MNs from embryonic transgenic type I SMA mice have increased accumulation of LC3-II protein. We analyzed using immunofluorescence technique the LC3-II aggregates in E15 and E18 mtSMA MNs. Our results show an increase of autophagosome accumulation in mtSMA compared to the wild type condition, indicating that changes in the autophagy process may begin at the embryonic stage. We also measured the LC3-II accumulations in the postnatal stage P1, P3, and P5, and it was also evident the elevated level of autophagosome in these

MNs. In P3 MNs we observed an increased number of LC3-II punctate in both wild type and mtSMA. However, when mtSMA LC3-II staining is higher compared to the with wild type condition. Therefore, all these results together suggest that autophagy is impaired during embryonic development and the autophagosome accumulation can be one if the mechanisms involved in MN degeneration although we still don't know the origin. Further analysis of the cause of elevated autophagy could help to find therapeutic targets to reduce MN degeneration in SMA disease.

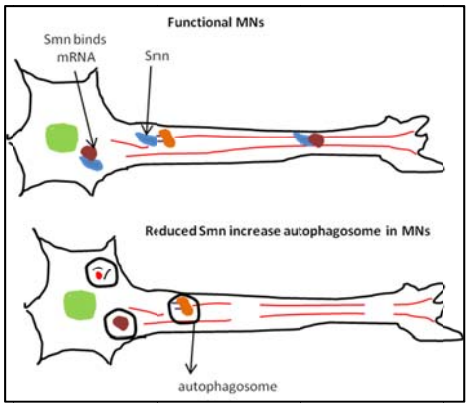

Figure 66. SMN protein is necessary for mRNA transport and β-actin delivery in axonal terminals. Reduced SMN protein can be the origin of axonal transport impairment and autophagosome accumulation which in turn can exacerbate axonal disruption.

There are some evidences that pharmacological stimulation of autophagic flux constitutes a promising clinical strategy for the treatment of neurodegenerative disorders. Nonetheless, in some cases autophagy induction may be detrimental, in particular, when the disease mechanism relies on autophagosome accumulation or defective autophagic cargo accumulation. In some of these cases, pharmacological or genetic approaches for stimulating autophagic flux by ameliorating lysosomal biogenesis or enhancing autophagosome-lysosome fusion should be considered.

Proteasome degradation of SMN protein in MNs

The correlation between *SMN2* copy number and SMA severity indicates that increasing SMN protein levels may ameliorate the disease phenotype. The mechanisms to increase SMN protein have been actively invesatigated as a therapeutic option for SMA treatment. Our results show that the pharmacological inhibition of proteasome increases SMN protein level in MNs. The proteasome process is an important mechanism of intracellular protein

degradation (Lecker et al. 2006). It has been described that SMN can be ubiquitinated by Mib1 (Kwon et al. 2013) and degreaded by the proteasome (Chang et al. 2004). In our MN culture system, MG-132 proteasome inhibitor treatment increases Smn protein level reinforcing the previosuly reported role of proteasome in SMN protein stability. Intracellular SMN protein level includes the full-length functional SMN protein (FL-SMN) and the truncated Δ7SMN protein. FL-SMN can oligomerize and form large multiprotein complexes that are highly stable. In contrast, Δ7SMN or SMN mutants are unable to oligomerize and they have a shorter half-life. But SMN and Δ7SMN can associate each other and this association stabilizes Δ7SMN protein turnover and ameliorate the SMA phenotype by increasing the amount of oligomeric SMN (Le et al. 2005). SMN protein stability can be manipulated pharmacologically by regulating its incorporation into the SMN complex, i.e., PKA activation inhibits SMN degradation due to greater incorporation of SMN into SMN-Gemin complex (Burnett et al. 2009). Identifying compounds that promote SMN complex formation could provide a novel avenue for SMA therapeutics development.

MG-132 can reduce the degradation of SMN in SMA phenotype and promote the SMN increase in MNs. Even the results support that MG-132 could be a good target for SMA treatment, one of the significant concerns about the use of proteasome inhibitors is that they might be cytotoxic. However, recent studies suggest that proteasome inhibition protects cells from cancer proliferation in cellular and animal models (Adams 2002; Hara et al. 2001; Matsunaga et al. 2009; Hideshima et al. 2001). Moreover, the use of proteasome inhibitors have been approved by FDA (Food and Drug Administration) for the treatment of multiple myelomas (Twombly 2003; Orlowski et al. 2002). Therefore, the use of proteasome inhibitors can be considered as a therapeutic tool destined to increase cellular SMN.

SMN is a multifunctional protein present in all cell types. Even SMA pathogenesis is mainly related to spinal cord MNs degeneration, it has been recently evidenced that in SMA patients and mouse models there are abnormalities in several cell types, tissues and organs aside from spinal cord MNs (Hamilton & Gillingwater 2013). The selective depletion of SMN in mouse MNs led to a dystrophic phenotype rather than full-blown SMA, thereby demonstrating that SMN loss in cells and tissues other than MNs is required to generate SMA disease pathogenesis (Park et al. 2010). One explanation for the relatively mild SMA phenotype of the mice with the selective depletion of SMN in MNs is that the presence of

normal SMN levels in non-neuronal tissue serves to modulate disease severity. Particularly important, in light to the research describing the multi-system phenotypes observed in SMA models, will be therapies that target the whole spectrum of systemic defects.

Effect of autophagy modulators on SMN protein

Late stage autophagy inhibitor decreases SMN protein level

Autophagy modulators can also regulate SMN protein stability. In the present work, we demonstrate that autophagosome formation is increased in SMA MNs. We Increasing or decreasing the autophagy process, SMN protein level can be regulated and it may lead to new therapeutic options. Our results show that inhibition of autophagy with BafA1 and NH4Cl decreases Smn protein level. BafA1 and NH4Cl inhibit autophagy at a late stage of the process when the autophagosome fuses with the lysosome, it prevents the autophagolysosome degradation and causes and increase of autophagosome accumulation. In this context, it has been recently described that autophagy inhibition can induce proteasome activity that leads to protein degradation (Wang & Wang 2015; Wang et al. 2013). Therefore, our results indicate that the decrease of SMN protein in BafA1 or NH4CL treated cells can be the result of the autophagosome number increase and the activation of the proteasome.

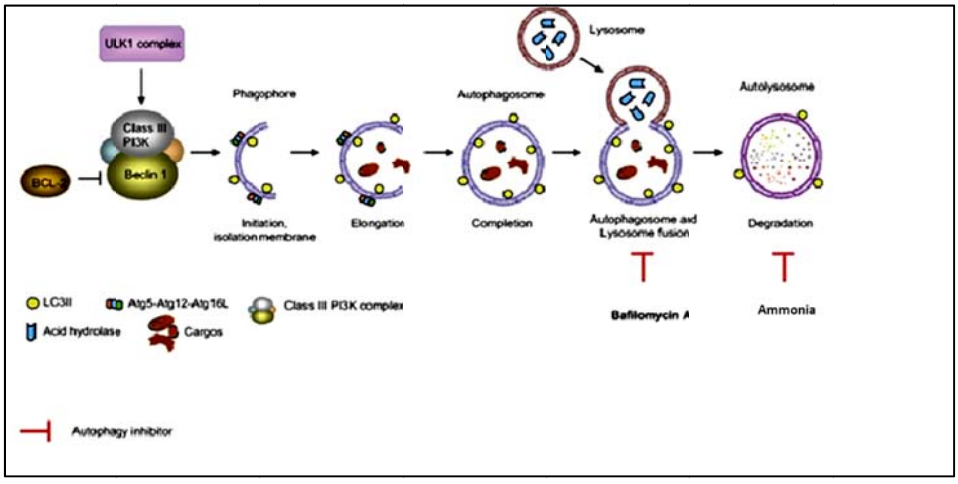

Figure 67. Autophagy inhibitors BafA1 and NH4Cl block the fusion of autophagosomes with lysosomes and increase the accumulation of autophagosomes.

Early stage autophagy inhibitor increase SMN protein level

3-MA treatment inhibits autophagy at the early stage of phagophore formation by inhibiting the class III PI3-K (Ito et al. 2007; Egami & Araki 2008). Our results demonstrate that 3-MA treatment increases SMN protein level in MNs and suggests that the early stage autophagy blocker prevent the formation of autophagosomes and proteasome activation.

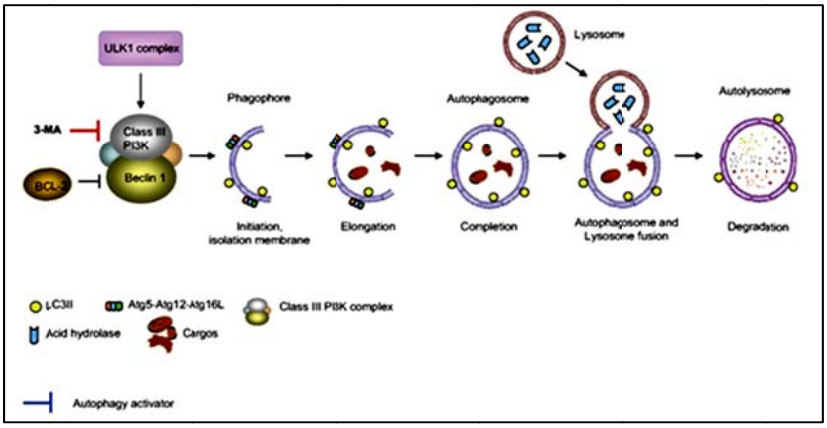

Figure 68. 3-MA inhibits class III PI3-K and phagophore formation. Initial inhibition of autophagy by 3-MA increases SMN protein level in MNs.

Inhibition of proteasome activates autophagy in MNs

MG-132 treatment increases Smn and LC3-II proteins indicating that proteasome inhibition increases autophagy activity. Proteasome is a major degradative pathway and its inhibition initiates ER stress as an alternative response and, activates the autophagy process (Ding et al. 2007; Dominguez-Bautista et al. 2015; Menzies et al. 2000). To inhibit the autophagy induced by MG-132 we treated these cells with the autophagy inhibitors BafA1 and NH4CL. Our results demonstrated even in the presence of both proteasome and autophagy inhibitors, the level of Smn protein is similar to the MG-132 condition. Therefore, in MNs the level of SMN is mainly regulated by the proteasome activity.

It has been previously described that in skeletal muscle cells the use of autophagy together with proteasome inhibitors decreases nearly 50% of protein degradation (Seiliez et al. 2014) suggesting an important contribution of both proteolytic systems to total protein degradation. These data identify the autophagy process as a potential target for strategies aimed at improving muscle protein retention and fillet yield through reductions in protein degradation. In our SMA MNs model autophagy increase and autophagosome accumulation

may activate the proteasome system which in turn contributes to reduce the compromised levels of SMN protein. Preclinical studies demonstrate that autophagy inhibition with hydroxychloroquine augments the antimyeloma efficacy of the proteasome inhibitor bortezomib and a phase I trial combining both drugs for relapsed or refractory myeloma have not shown toxic effects (Vogl et al. 2014). Thus, combined targeting of proteasomal and autophagic protein degradation is therefore feasible and a potentially useful strategy for improving outcomes in therapy.

Autophagy induction increases Smn protein level#

Rapamycin treatment regulates mTOR-dependent autophagy after nutrient deprivation (Berger et al. 2006). Rapamycin induced autophagy also reduces the neurodegeneration caused by toxic accumulation (Williams et al. 2006). In neurons, the axonal trafficking is much more important because the transport of various mRNA and protein along the axons are necessary for their function. Therefore, presence of rapamycin may help in removing unwanted products from cell through increased autophagolysosomal clearance (Zhang et al. 2011). Curcumin is also an mTOR dependent autophagy inducer (Lee et al. 2011; Han et al. 2012). Our results show that treatment of rapamycin or curcumin increases LC3-II and Smn protein level. We also used mTOR-independent autophagy inducers, trehalose and resveratrol, and increased level of Smn and LC3-II were observed. Similar results have been described in a cellular model of Smn reduced PC12 cell lines where Resveratrol increases neurite elongation independent of SMN protein level. In SMA patient fibroblasts rapamycin treatment increases SMN protein level (Bora-Tatar & Erdem-Yurter 2014; Sakla & Lorson 2008). All these results together demonstrate that treatment using autophagy inducers (both mTOR dependent and independent) increases Smn protein level in MNs.

Calcium signaling and SMN regulation

Inhibition of calpain increases Smn protein level

Calcium is playing an important role in maintaining cellular physiology in neurons. Increases of intracellular calcium concentration in some pathological conditions activate the calcium-sensitive protease calpain. Calpain is localized in the cytosol in its inactive form in the absence of calcium. Upon an increase in intracellular calcium level, calpain is translocated to

the membrane, and there, it becomes activated. Activated calpain has a number of substrates such as growth factor receptors, cytoskeletal proteins, microtubule-associated proteins and mitochondria, for different cellular mechanisms such as progression of cell cycle, differentiation, apoptosis, synaptic plasticity, CNS development in neurons (Sorimachi et al. 1997; Jánossy et al. 2004). It is well-known that calpain regulates cell survival in response to Ca2+ signal and can be over-activated under increased Ca2+ influx (Stifanese et al. 2010). Increased Ca2+ influx causes calpain activation and results in MN cell death. This cell-death effect can be counteracted using calpain inhibitors in MNs (Gou-Fabregas et al. 2009). Deregulation of Ca2+ influx results in calcium overload, and subsequently calpain over-activation forces neurons to degeneration. Calpain activation has been shown as related to neuronal damage in ischemia, stroke, and Alzheimer and Huntington diseases (Cowan et al. 2008; Shields et al. 2000). In the present work, we demonstrate that calpain knockdown increases Smn protein level into MNs. This result suggests that calpain can be involved in SMN stability in MNs. It has been previously reported that in skeletal muscle SMN is a proteolytic target of calpain (Walker et al. 2008). In fact, S192 and F193 are two adjacent calpain cleavage sites in SMN protein (Fuentes et al. 2010). Moreover, it has been recently demonstrated that calpain mediated proteasome activation is required for axonal degeneration (Park et al. 2013) suggesting the importance of Ca2+ homeostasis, calpain activity and SMN stability in MNs.

Our results also show that inhibition of calpain increases autophagy in MNs. It has been also evidenced that calpain inhibition activates autophagy to protect cells against toxicity (Williams et al. 2008a; Menzies et al. 2014) . Calpain reduced activity decreases protein cleavage and can induce toxic protein accumulation. This protein accumulation can be cleared by an increased autophagy process(Webb et al. 2003) (Berger et al. 2006). Therefore, when calpain activity is reduced protein clearance is activated by autophagy as alternative process. In our hands, NH4Cl treatment of calpain knockdown cells, reduced the levels of SMN protein suggesting that NH4 treatment induces proteasome activity and degradation of SMN even in the presence of reduced levels of calpain. Thus, posttranslational modifications may play an important role in determining rates of SMN degradation.

In the present work we demonstrate that in MNs, SMN protein level is regulated through proteasome, calcium activated calpain and autophagy process. Alteration of these processes could be effective to increase the SMN protein level in MNs and reduce neurodegeneration in SMA. Our observations indicate that understanding SMN degradation may provide novel approaches for SMA therapeutics development, but further experiments will be required to examine these possibilities.

Conclusions

Conclusions

1. Smn knockdown causes an increase of the autophagosome marker LC3-II and the autophagy inductor Beclin 1. Elevated levels of these two proteins indicate an increase of the autophagy activity in cultured spinal cord MNs with low levels of Smn
2. Cultured embryonic MNs isolated from SMA type I transgenic mice have higher levels of LC3-II protein compared to wild type MNs
3. Immunofluorescence experiments and electron microscopy studies demonstrate an increase of LC3-II level and autophagosome number in MNs
4. Experiments using the autophagy inhibitor BafA1 indicate that the autophagy flux is not altered in Smn-reduced MNs suggesting an increase of autophagosome formation in this cellular model
5. Immunofluorescence experiments demonstrated that embryonic and postnatal spinal cord MNs from SMA type I mutant mice have higher levels of LC3-II compared to their wild type littermates
6. Treatment with the proteasome inhibitor MG-132 causes an increase of Smn protein level in non-transgenic and transgenic (wild type and SMA mutants) cultured spinal cord MNs.
7. The inhibitors of the autophagosome and lysosome fusion and autolysosome degradation BafA1 and NH_4Cl, respectively, increase LC3-II and reduce Smn protein level. MG-132 addition together with the inhibitors prevents Smn reduction. These results suggest that BafA1 and NH_4Cl treatment induce proteasome activation.
8. Treatment with the inhibitor of the autophagosome formation 3-MA does not increase LC3-II level. However, the same treatment induces Smn increase in non-transgenic and SMA transgenic cultured spinal cord MNs
9. The mTOR-dependent autophagy activators rapamycin and curcumin induce both LC3-II and Smn protein level increase in cultured spinal cord MNs. The mTOR-independent autophagy activators trehalose and resveratrol also induce LC3-II and Smn increase.
10. Calpain knockdown increases Smn protein level in MNs. The addition of the autophagy inhibitor NH_4Cl prevents Smn increase.

References

Adams, J., 2002. Proteasome inhibitors as new anticancer drugs. *Current opinion in oncology*, 14(6), pp.628–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12409653 [Accessed February 13, 2015].

De Almeida, L.P. et al., 2001. Neuroprotective effect of a CNTF-expressing lentiviral vector in the quinolinic acid rat model of Huntington's disease. *Neurobiology of disease*, 8(3), pp.433–46. Available at: http://www.sciencedirect.com/science/article/pii/S0969996101903882 [Accessed December 17, 2014].

Alsafadi, S. et al., 2009. P53 family: at the crossroads in cancer therapy. *Current medicinal chemistry*, 16(32), pp.4328–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19754415 [Accessed December 19, 2014].

Andreassi, C. et al., 2004. Phenylbutyrate increases SMN expression in vitro: relevance for treatment of spinal muscular atrophy. *European journal of human genetics : EJHG*, 12(1), pp.59–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14560316 [Accessed March 18, 2015].

Aoki, H. et al., 2007. Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. *Molecular pharmacology*, 72(1), pp.29–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17395690 [Accessed November 6, 2014].

Arora, A., Ha, C. & Park, C.B., 2004. Inhibition of insulin amyloid formation by small stress molecules. *FEBS letters*, 564(1-2), pp.121–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15094052 [Accessed November 6, 2014].

Arthur, J.S.C. et al., 2000. Disruption of the Murine Calpain Small Subunit Gene, Capn4: Calpain Is Essential for Embryonic Development but Not for Cell Growth and Division. *Molecular and Cellular Biology*, 20(12), pp.4474–4481. Available at: http://mcb.asm.org/content/20/12/4474 [Accessed March 17, 2015].

Ashkenazi, A. & Dixit, V.M., 1998. Death receptors: signaling and modulation. *Science (New York, N.Y.)*, 281(5381), pp.1305–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9721089 [Accessed December 24, 2014].

Baccon, J. et al., 2002. Identification and characterization of Gemin7, a novel component of the survival of motor neuron complex. *The Journal of biological chemistry*, 277(35), pp.31957–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12065586 [Accessed November 5, 2014].

Barth, S., Glick, D. & Macleod, K.F., 2010. Autophagy: assays and artifacts. *The Journal of pathology*, 221(2), pp.117–24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2989884&tool=pmcentrez&rendertype=abstract [Accessed November 27, 2014].

Bebee, T.W., Gladman, J.T. & Chandler, D.S., 2010. Splicing regulation of the survival motor neuron genes and implications for treatment of spinal muscular atrophy. *Frontiers in bioscience (Landmark edition)*, 15, pp.1191–204. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2921696&tool=pmcentrez&rendertype=abstract [Accessed January 6, 2015].

Berger, Z. et al., 2006. Rapamycin alleviates toxicity of different aggregate-prone proteins. *Human molecular genetics*, 15(3), pp.433–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16368705 [Accessed January 7, 2015].

Berridge, M.J., 1993. Inositol trisphosphate and calcium signalling. *Nature*, 361(6410), pp.315–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8381210 [Accessed October 19, 2014].

Bertrandy, S. et al., 1999. The RNA-binding properties of SMN: deletion analysis of the zebrafish orthologue defines domains conserved in evolution. *Human molecular genetics*, 8(5), pp.775–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10196366 [Accessed January 8, 2015].

Bladt, F. et al., 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature*, 376(6543), pp.768–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7651534 [Accessed February 18, 2015].

Blommaart, E.F. et al., 1997. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *European journal of biochemistry / FEBS*, 243(1-2), pp.240–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9030745 [Accessed October 29, 2014].

Bora-Tatar, G. & Erdem-Yurter, H., 2014. Investigations of curcumin and resveratrol on neurite outgrowth: perspectives on spinal muscular atrophy. *BioMed research international*, 2014, p.709108. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4101952&tool=pmcentrez&rendertype=abstract [Accessed February 13, 2015].

Borg, R. & Cauchi, R.J., 2014. GEMINs: potential therapeutic targets for spinal muscular atrophy? *Frontiers in neuroscience*, 8, p.325. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4197776&tool=pmcentrez&rendertype=abstract [Accessed November 10, 2014].

Borg, R. & Cauchi, R.J., 2013. The Gemin associates of survival motor neuron are required for motor function in Drosophila. *PloS one*, 8(12), p.e83878. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3877121&tool=pmcentrez&rendertype=abstract [Accessed November 10, 2014].

Bowerman, M. et al., 2014. Defects in pancreatic development and glucose metabolism in SMN-depleted mice independent of canonical spinal muscular atrophy neuromuscular pathology. *Human molecular genetics*, 23(13), pp.3432–44. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4049303&tool=pmcentrez&rendertype=abstract [Accessed November 12, 2014].

Bowman, E.J., Siebers, A. & Altendorf, K., 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proceedings of the National Academy of Sciences of the United States of America*, 85(21), pp.7972–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=282335&tool=pmcentrez&rendertype=abstract [Accessed December 28, 2014].

Boyce, V.S. & Mendell, L.M., 2014. Neurotrophic factors in spinal cord injury. *Handbook of experimental pharmacology*, 220, pp.443–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24668482 [Accessed November 6, 2014].

Breckenridge, D.G. et al., 2003. Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene*, 22(53), pp.8608–18. Available at: http://dx.doi.org/10.1038/sj.onc.1207108 [Accessed December 26, 2014].

Briese, M. et al., 2009. Deletion of smn-1, the Caenorhabditis elegans ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan. *Human molecular genetics*, 18(1), pp.97–104. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2644645&tool=pmcentrez&rendertype=abstract [Accessed January 8, 2015].

Brown, C.R. et al., 1996. Chemical chaperones correct the mutant phenotype of the delta F508 cystic fibrosis transmembrane conductance regulator protein. *Cell stress & chaperones*, 1(2), pp.117–25. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=248464&tool=pmcentrez&rendertype=abstract [Accessed November 6, 2014].

Burnett, B.G. et al., 2009. Regulation of SMN protein stability. *Molecular and cellular biology*, 29(5), pp.1107–15. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2643817&tool=pmcentrez&rendertype=abstract [Accessed January 19, 2015].

Carissimi, C. et al., 2006. Gemin8 is a novel component of the survival motor neuron complex and functions in small nuclear ribonucleoprotein assembly. *The Journal of biological chemistry*, 281(12), pp.8126–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16434402 [Accessed November 5, 2014].

Casella, G.T.B. et al., 2010. Neurotrophic factors improve muscle reinnervation from embryonic neurons. *Muscle & nerve*, 42(5), pp.788–97. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20976782 [Accessed December 4, 2014].

Castro, D. & Iannaccone, S.T., 2014. Spinal muscular atrophy: therapeutic strategies. *Current treatment options in neurology*, 16(11), p.316. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25245431 [Accessed November 5, 2014].

Cauchi, R.J., Davies, K.E. & Liu, J.-L., 2008. A motor function for the DEAD-box RNA helicase, Gemin3, in Drosophila. *PLoS genetics*, 4(11), p.e1000265. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2577925&tool=pmcentrez&rendertype=abstract [Accessed January 8, 2015].

Chan, E.Y., 2009. mTORC1 phosphorylates the ULK1-mAtg13-FIP200 autophagy regulatory complex. *Science signaling*, 2(84), p.pe51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19690328 [Accessed November 6, 2014].

Chan, Y.B. et al., 2003. Neuromuscular defects in a Drosophila survival motor neuron gene mutant. *Human molecular genetics*, 12(12), pp.1367–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12783845 [Accessed November 5, 2014].

Chang, H.-C. et al., 2004. Degradation of survival motor neuron (SMN) protein is mediated via the ubiquitin/proteasome pathway. *Neurochemistry international*, 45(7), pp.1107–12. Available at: http://www.sciencedirect.com/science/article/pii/S0197018604000907 [Accessed November 13, 2014].

Chang, J.G. et al., 2001. Treatment of spinal muscular atrophy by sodium butyrate. *Proceedings of the National Academy of Sciences of the United States of America*, 98(17), pp.9808–13. Available at: http://www.pnas.org/content/98/17/9808.full [Accessed February 28, 2015].

Chang, T. et al., 2011. Brief report: phenotypic rescue of induced pluripotent stem cell-derived motoneurons of a spinal muscular atrophy patient. *Stem cells (Dayton, Ohio)*, 29(12), pp.2090–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21956898 [Accessed November 11, 2014].

Chen, E.W. & Chiu, A.Y., 1992. Early stages in the development of spinal motor neurons. *The Journal of comparative neurology*, 320(3), pp.291–303. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1535349 [Accessed January 26, 2015].

Chen, M. et al., 2000. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nature medicine*, 6(7), pp.797–801. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10888929 [Accessed November 20, 2014].

Chen, M.-L. et al., 2013. Resveratrol attenuates vascular endothelial inflammation by inducing autophagy through the cAMP signaling pathway. *Autophagy*, 9(12), pp.2033–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24145604 [Accessed November 6, 2014].

Chen, Q. & Haddad, G.G., 2004. Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals. *The Journal of experimental biology*, 207(Pt 18), pp.3125–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15299033 [Accessed October 26, 2014].

Chevalier-Larsen, E. & Holzbaur, E.L.F., 2006. Axonal transport and neurodegenerative disease. *Biochimica et biophysica acta*, 1762(11-12), pp.1094–108. Available at: http://www.sciencedirect.com/science/article/pii/S0925443906000731 [Accessed April 1, 2015].

Chinnaiyan, A.M., 1999. The Apoptosome: Heart and Soul of the Cell Death Machine. *Neoplasia*, 1(1), pp.5–15. Available at: http://www.sciencedirect.com/science/article/pii/S147655869980030X [Accessed December 24, 2014].

Cho, S. & Dreyfuss, G., 2010. A degron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity. *Genes & development*, 24(5), pp.438–42. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2827839&tool=pmcentrez&rendertype=abstract [Accessed November 5, 2014].

Ciechanover, A., Orian, A. & Schwartz, A.L., 2000. The ubiquitin-mediated proteolytic pathway: Mode of action and clinical implications. *Journal of Cellular Biochemistry*, 77(S34), pp.40–51. Available at: http://doi.wiley.com/10.1002/(SICI)1097-4644(2000)77:34+<40::AID-JCB9>3.0.CO;2-6 [Accessed January 8, 2015].

Cifuentes-Diaz, C. et al., 2001. Deletion of murine SMN exon 7 directed to skeletal muscle leads to severe muscular dystrophy. *The Journal of cell biology*, 152(5), pp.1107–14. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2198815&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

Cifuentes-Diaz, C. et al., 2002. Neurofilament accumulation at the motor endplate and lack of axonal sprouting in a spinal muscular atrophy mouse model. *Human molecular genetics*, 11(12),

pp.1439–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12023986 [Accessed November 5, 2014].

Cortes, C.J. & La Spada, A.R., 2014. The many faces of autophagy dysfunction in Huntington's disease: from mechanism to therapy. *Drug discovery today*, 19(7), pp.963–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24632005 [Accessed October 28, 2014].

Corti, S. et al., 2010. Embryonic stem cell-derived neural stem cells improve spinal muscular atrophy phenotype in mice. *Brain : a journal of neurology*, 133(Pt 2), pp.465–81. Available at: http://brain.oxfordjournals.org/content/133/2/465.long [Accessed November 8, 2014].

Corti, S. et al., 2012. Genetic correction of human induced pluripotent stem cells from patients with spinal muscular atrophy. *Science translational medicine*, 4(165), p.165ra162. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23253609 [Accessed January 29, 2015].

Corti, S. et al., 2009. Motoneuron transplantation rescues the phenotype of SMARD1 (spinal muscular atrophy with respiratory distress type 1). *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(38), pp.11761–71. Available at: http://www.jneurosci.org/content/29/38/11761.long [Accessed November 11, 2014].

Cory, S. & Adams, J.M., 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nature reviews. Cancer*, 2(9), pp.647–56. Available at: http://dx.doi.org/10.1038/nrc883 [Accessed July 19, 2014].

Cowan, C.M. et al., 2008. Polyglutamine-modulated striatal calpain activity in YAC transgenic huntington disease mouse model: impact on NMDA receptor function and toxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(48), pp.12725–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19036965 [Accessed April 1, 2015].

Cutler, N.S., Heitman, J. & Cardenas, M.E., 1999. TOR kinase homologs function in a signal transduction pathway that is conserved from yeast to mammals. *Molecular and cellular endocrinology*, 155(1-2), pp.135–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10580846 [Accessed November 6, 2014].

D'Amico, A. et al., 2011. Spinal muscular atrophy. *Orphanet journal of rare diseases*, 6, p.71. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3231874&tool=pmcentrez&rendertype=abstract [Accessed October 31, 2014].

Dale, J.M. et al., 2011. The spinal muscular atrophy mouse model, SMAΔ7, displays altered axonal transport without global neurofilament alterations. *Acta neuropathologica*, 122(3), pp.331–41. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3286646&tool=pmcentrez&rendertype=abstract [Accessed December 4, 2014].

Das, A. et al., 2005. Calpain activation in apoptosis of ventral spinal cord 4.1 (VSC4.1) motoneurons exposed to glutamate: Calpain inhibition provides functional neuroprotection. *Journal of Neuroscience Research*, 81(4), pp.551–562. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15968645 [Accessed March 17, 2015].

Ding, W.-X. et al., 2007. Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. *The American journal of pathology*, 171(2), pp.513–24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1934546&tool=pmcentrez&rendertype=abstract [Accessed November 5, 2014].

Dominguez-Bautista, J.A. et al., 2015. Loss of lysosome-associated membrane protein 3 (LAMP3) enhances cellular vulnerability against proteasomal inhibition. *European Journal of Cell Biology*. Available at: http://www.sciencedirect.com/science/article/pii/S0171933515000126 [Accessed February 4, 2015].

Dubrez-Daloz, L., Dupoux, A. & Cartier, J., 2008. IAPs: more than just inhibitors of apoptosis proteins. *Cell cycle (Georgetown, Tex.)*, 7(8), pp.1036–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18414036 [Accessed December 19, 2014].

Durbec, P. et al., 1996. GDNF signalling through the Ret receptor tyrosine kinase. *Nature*, 381(6585), pp.789–93. Available at: http://dx.doi.org/10.1038/381789a0 [Accessed March 14, 2015].

Dutta, R. et al., 2007. Activation of the ciliary neurotrophic factor (CNTF) signalling pathway in cortical neurons of multiple sclerosis patients. *Brain : a journal of neurology*, 130(Pt 10), pp.2566–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17898009 [Accessed November 10, 2014].

Dykxhoorn, D.M., Novina, C.D. & Sharp, P.A., 2003. Killing the messenger: short RNAs that silence gene expression. *Nature reviews. Molecular cell biology*, 4(6), pp.457–67. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12778125 [Accessed July 13, 2014].

Ebens, A. et al., 1996. Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. *Neuron*, 17(6), pp.1157–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8982163 [Accessed March 14, 2015].

Eberwine, J. et al., 2001. Local translation of classes of mRNAs that are targeted to neuronal dendrites. *Proceedings of the National Academy of Sciences of the United States of America*, 98(13), pp.7080–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=34626&tool=pmcentrez&rendertype=abstract [Accessed December 12, 2014].

Efeyan, A., Zoncu, R. & Sabatini, D.M., 2012. Amino acids and mTORC1: from lysosomes to disease. *Trends in molecular medicine*, 18(9), pp.524–33. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3432651&tool=pmcentrez&rendertype=abstract [Accessed July 16, 2014].

Egami, Y. & Araki, N., 2008. Characterization of Rab21-positive tubular endosomes induced by PI3K inhibitors. *Experimental cell research*, 314(4), pp.729–37. Available at: http://www.sciencedirect.com/science/article/pii/S0014482707005538 [Accessed February 13, 2015].

El-Husseini, A. el-D. et al., 2001. Polarized targeting of peripheral membrane proteins in neurons. *The Journal of biological chemistry*, 276(48), pp.44984–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11546762 [Accessed March 14, 2015].

Elmore, S., 2007. Apoptosis: a review of programmed cell death. *Toxicologic pathology*, 35(4), pp.495–516. Available at: http://tpx.sagepub.com/content/35/4/495.full [Accessed July 10, 2014].

Epple, U.D. et al., 2001. Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. *Journal of bacteriology*, 183(20), pp.5942–55. Available at: http://jb.asm.org/content/183/20/5942.abstract?ijkey=168a7180d11ce9339d179621dc356dccc1bb2d30&keytype2=tf_ipsecsha [Accessed December 26, 2014].

Eskelinen, E.-L. et al., 2014. Seeing is believing: The impact of electron microscopy on autophagy research. *Autophagy*, 7(9), pp.935–956. Available at: http://dx.doi.org/10.4161/auto.7.9.15760 [Accessed December 9, 2014].

Ethell, D.W. & Buhler, L.A., 2003. Fas Ligand-Mediated Apoptosis in Degenerative Disorders of the Brain. *Journal of Clinical Immunology*, 23(6), pp.439–446. Available at: http://link.springer.com/10.1023/B:JOCI.0000010420.96419.a8 [Accessed December 24, 2014].

Fischer, U., Liu, Q. & Dreyfuss, G., 1997. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell*, 90(6), pp.1023–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9323130 [Accessed February 6, 2015].

Fournier Le Ray, C. & Fontaine-Perus, J., 1991. Influence of spinal cord stimulation on the innervation pattern of muscle fibers in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 11(12), pp.3840–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1744693 [Accessed December 7, 2014].

Frank, D.A. & Greenberg, M.E., 1996. Signal transduction pathways activated by ciliary neurotrophic factor and related cytokines. *Perspectives on developmental neurobiology*, 4(1), pp.3–18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9169915 [Accessed December 17, 2014].

Friese, A. et al., 2009. Gamma and alpha motor neurons distinguished by expression of transcription factor Err3. *Proceedings of the National Academy of Sciences of the United States of America*, 106(32), pp.13588–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2716387&tool=pmcentrez&rendertype=abstract [Accessed November 24, 2014].

Fuentes, J.L., Strayer, M.S. & Matera, A.G., 2010. Molecular determinants of survival motor neuron (SMN) protein cleavage by the calcium-activated protease, calpain. *PloS one*, 5(12), p.e15769. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3012718&tool=pmcentrez&rendertype=abstract [Accessed January 12, 2015].

Furuya, N. et al., 2005. The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. *Autophagy*, 1(1), pp.46–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16874027 [Accessed November 5, 2014].

Garcera, A. et al., 2011. A new model to study spinal muscular atrophy: neurite degeneration and cell death is counteracted by BCL-X(L) Overexpression in motoneurons. *Neurobiology of disease*, 42(3), pp.415–26. Available at: http://www.sciencedirect.com/science/article/pii/S096999611100057X [Accessed November 27, 2014].

Garcera, A. et al., 2013. Survival motor neuron protein reduction deregulates autophagy in spinal cord motoneurons in vitro. *Cell death & disease*, 4, p.e686. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3702296&tool=pmcentrez&rendertype=abstract [Accessed September 30, 2014].

Gershoni, M., Templeton, A.R. & Mishmar, D., 2009. Mitochondrial bioenergetics as a major motive force of speciation. *BioEssays : news and reviews in molecular, cellular and developmental biology*, 31(6), pp.642–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19408245 [Accessed December 16, 2014].

Glading, A., Lauffenburger, D.A. & Wells, A., 2002. Cutting to the chase: calpain proteases in cell motility. *Trends in cell biology*, 12(1), pp.46–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11854009 [Accessed March 17, 2015].

Goldberg, A.L., 2003. Protein degradation and protection against misfolded or damaged proteins. *Nature*, 426(6968), pp.895–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14685250 [Accessed December 9, 2014].

Goll, D.E. et al., 2003. The calpain system. *Physiological reviews*, 83(3), pp.731–801. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12843408 [Accessed November 6, 2014].

Gordon, P.B. et al., 1993. Dependence of hepatocytic autophagy on intracellularly sequestered calcium. *The Journal of biological chemistry*, 268(35), pp.26107–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8253727 [Accessed December 27, 2014].

Gou-Fabregas, M. et al., 2009. Specific vulnerability of mouse spinal cord motoneurons to membrane depolarization. *Journal of neurochemistry*, 110(6), pp.1842–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19627436 [Accessed October 29, 2014].

Grumbles, R.M. et al., 2009. Neurotrophic factors improve motoneuron survival and function of muscle reinnervated by embryonic neurons. *Journal of neuropathology and experimental neurology*, 68(7), pp.736–46. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2727878&tool=pmcentrez&rendertype=abstract [Accessed December 4, 2014].

Grunwald, D.J. & Eisen, J.S., 2002. Headwaters of the zebrafish -- emergence of a new model vertebrate. *Nature reviews. Genetics*, 3(9), pp.717–24. Available at: http://dx.doi.org/10.1038/nrg892 [Accessed November 11, 2014].

Grzeschik, S.M. et al., 2005. Hydroxyurea enhances SMN2 gene expression in spinal muscular atrophy cells. *Annals of neurology*, 58(2), pp.194–202. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16049920 [Accessed March 18, 2015].

Gupta, S.C. et al., 2012. Discovery of curcumin, a component of golden spice, and its miraculous biological activities. *Clinical and experimental pharmacology & physiology*, 39(3), pp.283–99. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3288651&tool=pmcentrez&rendertype=abstract [Accessed October 23, 2014].

Gurney, M.A. et al., 2015. Measuring cardiac autophagic flux in vitro and in vivo. *Methods in molecular biology (Clifton, N.J.)*, 1219, pp.187–97. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25308270 [Accessed November 16, 2014].

Hamakubo, T. et al., 1986. Distribution of calpains I and II in rat brain. *J. Neurosci.*, 6(11), pp.3103–3111. Available at: http://www.jneurosci.org/content/6/11/3103.short [Accessed March 17, 2015].

Hamilton, G. & Gillingwater, T.H., 2013. Spinal muscular atrophy: going beyond the motor neuron. *Trends in molecular medicine*, 19(1), pp.40–50. Available at: http://www.sciencedirect.com/science/article/pii/S1471491412002109 [Accessed March 23, 2015].

Han, J. et al., 2012. Curcumin induces autophagy to protect vascular endothelial cell survival from oxidative stress damage. *Autophagy*, 8(5), pp.812–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22622204 [Accessed March 1, 2015].

Hannon, G.J. & Rossi, J.J., 2004. Unlocking the potential of the human genome with RNA interference. *Nature*, 431(7006), pp.371–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15372045 [Accessed February 4, 2015].

Hao, L.T., Burghes, A.H. & Beattie, C.E., 2011. Generation and Characterization of a genetic zebrafish model of SMA carrying the human SMN2 gene. *Molecular neurodegeneration*, 6(1), p.24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3080329&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

Hara, T. et al., 2001. Degradation of p27(Kip1) at the G(0)-G(1) transition mediated by a Skp2-independent ubiquitination pathway. *The Journal of biological chemistry*, 276(52), pp.48937–43. Available at: http://www.jbc.org/content/276/52/48937.long [Accessed February 13, 2015].

Hayashi-Nishino, M. et al., 2009. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. *Nature cell biology*, 11(12), pp.1433–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19898463 [Accessed August 18, 2014].

He, C. & Klionsky, D.J., 2009. Regulation mechanisms and signaling pathways of autophagy. *Annual review of genetics*, 43, pp.67–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2831538&tool=pmcentrez&rendertype=abstract [Accessed July 12, 2014].

Heckman, C.J. & Enoka, R.M., 2012. Motor unit. *Comprehensive Physiology*, 2(4), pp.2629–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23720261 [Accessed December 3, 2014].

Heier, C.R. et al., 2010. Arrhythmia and cardiac defects are a feature of spinal muscular atrophy model mice. *Human molecular genetics*, 19(20), pp.3906–18. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2947406&tool=pmcentrez&rendertype=abstract [Accessed November 13, 2014].

Heinrich, P.C. et al., 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *The Biochemical journal*, 334 (Pt 2, pp.297–314. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1219691&tool=pmcentrez&rendertype=abstract [Accessed December 17, 2014].

Hemelaar, J. et al., 2003. A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L. *The Journal of biological chemistry*, 278(51), pp.51841–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14530254 [Accessed November 5, 2014].

Henderson, C. et al., 1994. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science*, 266(5187), pp.1062–1064. Available at: http://www.sciencemag.org/content/266/5187/1062.abstract [Accessed December 5, 2014].

Henderson, C.E., 1996. Role of neurotrophic factors in neuronal development. *Current opinion in neurobiology*, 6(1), pp.64–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8794045 [Accessed December 15, 2014].

Heras-Sandoval, D. et al., 2014. The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. *Cellular signalling*, 26(12), pp.2694–2701. Available at: http://www.sciencedirect.com/science/article/pii/S0898656814002848 [Accessed November 3, 2014].

Hideshima, T. et al., 2001. Novel therapies targeting the myeloma cell and its bone marrow microenvironment. *Seminars in oncology*, 28(6), pp.607–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11740818 [Accessed February 13, 2015].

Hirokawa, N. & Takemura, R., 2005. Molecular motors and mechanisms of directional transport in neurons. *Nature reviews. Neuroscience*, 6(3), pp.201–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15711600 [Accessed October 12, 2014].

Hoetelmans, R.W.M. et al., 2003. The role of various Bcl-2 domains in the anti-proliferative effect and modulation of cellular glutathione levels: a prominent role for the BH4 domain. *Cell proliferation*, 36(1), pp.35–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12558659 [Accessed December 19, 2014].

Hollis, E.R. & Tuszynski, M.H., 2011. Neurotrophins: potential therapeutic tools for the treatment of spinal cord injury. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*, 8(4), pp.694–703. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3250295&tool=pmcentrez&rendertype=abstract [Accessed December 15, 2014].

Hosokawa, N. et al., 2009. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Molecular biology of the cell*, 20(7), pp.1981–91. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2663915&tool=pmcentrez&rendertype=abstract [Accessed November 2, 2014].

Hsieh-Li, H.M. et al., 2000. A mouse model for spinal muscular atrophy. *Nature genetics*, 24(1), pp.66–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10615130 [Accessed February 2, 2015].

Ito, S. et al., 2007. 3-Methyladenine suppresses cell migration and invasion of HT1080 fibrosarcoma cells through inhibiting phosphoinositide 3-kinases independently of autophagy inhibition. *International journal of oncology*, 31(2), pp.261–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17611681 [Accessed February 13, 2015].

J, B., 2008. The regulation and function of Class III PI3Ks: novel roles for Vps34. Available at: http://www.biochemj.org/bj/410/0001/bj4100001.htm [Accessed December 29, 2014].

Jánossy, J. et al., 2004. Calpain as a multi-site regulator of cell cycle. *Biochemical pharmacology*, 67(8), pp.1513–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15041468 [Accessed April 7, 2015].

Jeandet, P. et al., 2013. Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. *International journal of molecular sciences*, 14(7), pp.14136–70. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3742236&tool=pmcentrez&rendertype=abstract [Accessed October 31, 2014].

Jezierski, A. et al., 2014. Neuroprotective effects of GDNF-expressing human amniotic fluid cells. *Stem cell reviews*, 10(2), pp.251–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24415130 [Accessed November 10, 2014].

Kabeya, Y. et al., 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO journal*, 19(21), pp.5720–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=305793&tool=pmcentrez&rendertype=abstract [Accessed August 5, 2014].

Kamada, Y., Sekito, T. & Ohsumi, Y., 2004. Autophagy in yeast: a TOR-mediated response to nutrient starvation. *Current topics in microbiology and immunology*, 279, pp.73–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14560952 [Accessed November 6, 2014].

Kariya, S. et al., 2008. Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Human molecular genetics*, 17(16), pp.2552–69. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2722888&tool=pmcentrez&rendertype=abstract [Accessed November 11, 2014].

Kernochan, L.E. et al., 2005. The role of histone acetylation in SMN gene expression. *Human molecular genetics*, 14(9), pp.1171–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15772088 [Accessed March 18, 2015].

Kerr, J.F., Wyllie, A.H. & Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer*, 26(4), pp.239–57. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2008650&tool=pmcentrez&rendertype=abstract [Accessed January 14, 2015].

Kim, D.-H. et al., 2002. mTOR Interacts with Raptor to Form a Nutrient-Sensitive Complex that Signals to the Cell Growth Machinery. *Cell*, 110(2), pp.163–175. Available at: http://www.sciencedirect.com/science/article/pii/S0092867402008085 [Accessed December 19, 2014].

Kim, J.H., 2014. Brain-derived neurotrophic factor exerts neuroprotective actions against amyloid β-induced apoptosis in neuroblastoma cells. *Experimental and therapeutic medicine*, 8(6), pp.1891–1895. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4218707&tool=pmcentrez&rendertype=abstract [Accessed November 10, 2014].

Kitamura, K. et al., 2007. Hepatocyte growth factor promotes endogenous repair and functional recovery after spinal cord injury. *Journal of neuroscience research*, 85(11), pp.2332–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17549731 [Accessed December 17, 2014].

Klionsky, D.J., 2000. Autophagy as a Regulated Pathway of Cellular Degradation. *Science*, 290(5497), pp.1717–1721. Available at: http://www.sciencemag.org/content/290/5497/1717 [Accessed December 27, 2014].

Klionsky, D.J. et al., 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy*, 8(4), pp.445–544. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3404883&tool=pmcentrez&rendertype=abstract [Accessed July 10, 2014].

Kobayashi, T., Askanas, V. & Engel, W.K., 1987. Human muscle cultured in monolayer and cocultured with fetal rat spinal cord: importance of dorsal root ganglia for achieving successful functional innervation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 7(10), pp.3131–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3668620 [Accessed December 7, 2014].

Kong, L. et al., 2009. Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(3), pp.842–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2746673&tool=pmcentrez&rendertype=abstract [Accessed October 29, 2014].

Krakora, D. et al., 2013. Synergistic effects of GDNF and VEGF on lifespan and disease progression in a familial ALS rat model. *Molecular therapy : the journal of the American Society of Gene Therapy*, 21(8), pp.1602–10. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3734670&tool=pmcentrez&rendertype=abstract [Accessed October 23, 2014].

Kwon, D.Y. et al., 2013. The E3 ubiquitin ligase mind bomb 1 ubiquitinates and promotes the degradation of survival of motor neuron protein. *Molecular biology of the cell*, 24(12), pp.1863–71. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3681692&tool=pmcentrez&rendertype=abstract [Accessed February 14, 2015].

Laplante, M. & Sabatini, D.M., 2012. mTOR signaling in growth control and disease. *Cell*, 149(2), pp.274–93. Available at: http://www.sciencedirect.com/science/article/pii/S0092867412003510 [Accessed July 9, 2014].

Le, T.T. et al., 2005. SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length

SMN. *Human molecular genetics*, 14(6), pp.845–57. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15703193 [Accessed November 5, 2014].

Lecker, S.H., Goldberg, A.L. & Mitch, W.E., 2006. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *Journal of the American Society of Nephrology : JASN*, 17(7), pp.1807–19. Available at: http://jasn.asnjournals.org/content/17/7/1807.full [Accessed July 20, 2014].

Lee, J.-A. & Gao, F.-B., 2009. Inhibition of autophagy induction delays neuronal cell loss caused by dysfunctional ESCRT-III in frontotemporal dementia. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(26), pp.8506–11. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2726650&tool=pmcentrez&rendertype=abstract [Accessed December 29, 2014].

Lee, N., Spearry, R.P., et al., 2013. Muscle ciliary neurotrophic factor receptor α promotes axonal regeneration and functional recovery following peripheral nerve lesion. *The Journal of comparative neurology*, 521(13), pp.2947–65. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3992866&tool=pmcentrez&rendertype=abstract [Accessed November 10, 2014].

Lee, N., Rydyznski, C.E., et al., 2013. The contribution of ciliary neurotrophic factor receptors to adult motor neuron survival in vivo is specific to insult type and distinct from that for embryonic motor neurons. *The Journal of comparative neurology*, 521(14), pp.3217–25. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3986355&tool=pmcentrez&rendertype=abstract [Accessed November 10, 2014].

Lee, Y.J. et al., 2011. Involvement of ROS in Curcumin-induced Autophagic Cell Death. *The Korean journal of physiology & pharmacology : official journal of the Korean Physiological Society and the Korean Society of Pharmacology*, 15(1), pp.1–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3062078&tool=pmcentrez&rendertype=abstract [Accessed November 6, 2014].

Lefebvre, S. et al., 1995. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*, 80(1), pp.155–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7813012 [Accessed November 5, 2014].

Levine, B. & Klionsky, D.J., 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Developmental cell*, 6(4), pp.463–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15068787 [Accessed July 19, 2014].

Li, M. et al., 2000. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science (New York, N.Y.)*, 288(5464), pp.335–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10764647 [Accessed November 20, 2014].

Liang, X.H. et al., 1998. Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *Journal of virology*, 72(11), pp.8586–96. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=110269&tool=pmcentrez&rendertype=abstract [Accessed December 1, 2014].

Lin, L.F. et al., 1993. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science (New York, N.Y.)*, 260(5111), pp.1130–2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8493557 [Accessed November 6, 2014].

Liu, Q. et al., 1997. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell*, 90(6), pp.1013–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9323129 [Accessed March 19, 2015].

Liu, Q. & Dreyfuss, G., 1996. A novel nuclear structure containing the survival of motor neurons protein. *The EMBO journal*, 15(14), pp.3555–65. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=451956&tool=pmcentrez&rendertype=abstract [Accessed November 5, 2014].

Liu, R. et al., 2005. Trehalose differentially inhibits aggregation and neurotoxicity of beta-amyloid 40 and 42. *Neurobiology of disease*, 20(1), pp.74–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16137568 [Accessed November 6, 2014].

Liu, X.-S. et al., 2014. Human umbilical cord mesenchymal stem cells infected with adenovirus expressing HGF promote regeneration of damaged neuron cells in a Parkinson's disease model. *BioMed research international*, 2014, p.909657. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4167956&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Locksley, R.M., Killeen, N. & Lenardo, M.J., 2001. The TNF and TNF Receptor Superfamilies. *Cell*, 104(4), pp.487–501. Available at: http://www.sciencedirect.com/science/article/pii/S0092867401002379 [Accessed December 24, 2014].

Lorson, C.L. & Androphy, E.J., 2000. An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. *Human molecular genetics*, 9(2), pp.259–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10607836 [Accessed November 5, 2014].

Lu, Y. et al., 2013. Two pore channel 2 (TPC2) inhibits autophagosomal-lysosomal fusion by alkalinizing lysosomal pH. *The Journal of biological chemistry*, 288(33), pp.24247–63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3745369&tool=pmcentrez&rendertype=abstract [Accessed December 28, 2014].

Maeda, M. et al., 2014. Transcriptome profiling of spinal muscular atrophy motor neurons derived from mouse embryonic stem cells. *PloS one*, 9(9), p.e106818. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4156416&tool=pmcentrez&rendertype=abstract [Accessed October 31, 2014].

Maina, F. et al., 2001. Coupling Met to specific pathways results in distinct developmental outcomes. *Molecular cell*, 7(6), pp.1293–306. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11430831 [Accessed December 18, 2014].

Malkus, K.A., Tsika, E. & Ischiropoulos, H., 2009. Oxidative modifications, mitochondrial dysfunction, and impaired protein degradation in Parkinson's disease: how neurons are lost in the Bermuda triangle. *Molecular neurodegeneration*, 4, p.24. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2701947&tool=pmcentrez&rendertype=abstract [Accessed November 4, 2014].

Marinkovic, P. et al., 2012. Axonal transport deficits and degeneration can evolve independently in mouse models of amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences of the United States of America*, 109(11), pp.4296–301. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3306689&tool=pmcentrez&rendertype=abstract [Accessed December 4, 2014].

Mariño, G., Madeo, F. & Kroemer, G., 2011. Autophagy for tissue homeostasis and neuroprotection. *Current opinion in cell biology*, 23(2), pp.198–206. Available at: http://www.sciencedirect.com/science/article/pii/S0955067410001729 [Accessed November 14, 2014].

Markowitz, J.A., Singh, P. & Darras, B.T., 2012. Spinal muscular atrophy: a clinical and research update. *Pediatric neurology*, 46(1), pp.1–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22196485 [Accessed January 12, 2015].

Masu, Y. et al., 1993. Disruption of the CNTF gene results in motor neuron degeneration. *Nature*, 365(6441), pp.27–32. Available at: http://dx.doi.org/10.1038/365027a0 [Accessed December 17, 2014].

Matsunaga, K. et al., 2009. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nature cell biology*, 11(4), pp.385–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19270696 [Accessed October 25, 2014].

Mattson, M.P., 2007. Calcium and neurodegeneration. *Aging cell*, 6(3), pp.337–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17328689 [Accessed December 5, 2014].

Mattson, M.P. & Chan, S.L., 2003. Calcium orchestrates apoptosis. *Nature cell biology*, 5(12), pp.1041–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14647298 [Accessed December 15, 2014].

McLean, D.L. & Fetcho, J.R., 2011. Movement, technology and discovery in the zebrafish. *Current opinion in neurobiology*, 21(1), pp.110–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3057273&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

McWhorter, M.L. et al., 2003. Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. *The Journal of cell biology*, 162(5), pp.919–31. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1761110&tool=pmcentrez&rendertype=abstract [Accessed October 29, 2014].

Mehrpour, M. et al., 2010. Overview of macroautophagy regulation in mammalian cells. *Cell research*, 20(7), pp.748–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20548331 [Accessed October 25, 2014].

Meister, G., Eggert, C. & Fischer, U., 2002. SMN-mediated assembly of RNPs: a complex story. *Trends in cell biology*, 12(10), pp.472–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12441251 [Accessed February 6, 2015].

Melki, J. et al., 1990. Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. *Nature*, 344(6268), pp.767–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1970420 [Accessed December 29, 2014].

Menzies, F.M. et al., 2014. Calpain inhibition mediates autophagy-dependent protection against polyglutamine toxicity. *Cell death and differentiation*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25257175 [Accessed November 3, 2014].

Menzies, F.M. et al., 2012. Measurement of autophagic activity in mammalian cells. *Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.]*, Chapter 15, p.Unit 15.16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22422474 [Accessed January 18, 2015].

Menzies, F.M., Ravikumar, B. & Rubinsztein, D.C., Protective roles for induction of autophagy in multiple proteinopathies. *Autophagy*, 2(3), pp.224–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16874096 [Accessed January 7, 2015].

Merry, D.E. & Korsmeyer, S.J., 1997. Bcl-2 gene family in the nervous system. *Annual review of neuroscience*, 20, pp.245–67. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9056714 [Accessed January 18, 2015].

Middleton, G., 2000. Cytokine-induced Nuclear Factor Kappa B Activation Promotes the Survival of Developing Neurons. *The Journal of Cell Biology*, 148(2), pp.325–332. Available at: http://jcb.rupress.org/content/148/2/325?ijkey=e670748192f7ca3f3c264e12e5bb4b56a1106818&keytype2=tf_ipsecsha [Accessed December 17, 2014].

Miguel-Aliaga, I. et al., 1999. The Caenorhabditis elegans orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability. *Human molecular genetics*, 8(12), pp.2133–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10545592 [Accessed January 7, 2015].

Mindell, J.A., 2012. Lysosomal acidification mechanisms. *Annual review of physiology*, 74, pp.69–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22335796 [Accessed November 22, 2014].

Mizushima, N. et al., 1998. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. *The Journal of biological chemistry*, 273(51), pp.33889–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9852036 [Accessed November 5, 2014].

Mizushima, N. et al., 2008. Autophagy fights disease through cellular self-digestion. *Nature*, 451(7182), pp.1069–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2670399&tool=pmcentrez&rendertype=abstract [Accessed July 10, 2014].

Mizushima, N., 2007. Autophagy: process and function. *Genes & development*, 21(22), pp.2861–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18006683 [Accessed July 14, 2014].

Monani, U.R. et al., 2003. A transgene carrying an A2G missense mutation in the SMN gene modulates phenotypic severity in mice with severe (type I) spinal muscular atrophy. *The Journal of cell biology*, 160(1), pp.41–52. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2172739&tool=pmcentrez&rendertype=abstract [Accessed October 28, 2014].

Monani, U.R. et al., 2000. The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. *Human molecular genetics*, 9(3), pp.333–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10655541 [Accessed November 5, 2014].

Mondello, C. & Scovassi, A.I., 2010. Apoptosis: a way to maintain healthy individuals. *Sub-cellular biochemistry*, 50, pp.307–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20012589 [Accessed December 19, 2014].

Murone, M., Rosenthal, A. & de Sauvage, F.J., 1999. Sonic hedgehog signaling by the Patched–Smoothened receptor complex. *Current Biology*, 9(2), pp.76–84. Available at: http://www.sciencedirect.com/science/article/pii/S0960982299800189 [Accessed March 14, 2015].

Nagy, P. et al., 2014. How and why to study autophagy in Drosophila: it's more than just a garbage chute. *Methods (San Diego, Calif.)*. Available at: http://www.sciencedirect.com/science/article/pii/S104620231400379X [Accessed December 8, 2014].

Nakamura, T. et al., 1989. Molecular cloning and expression of human hepatocyte growth factor. *Nature*, 342(6248), pp.440–3. Available at: http://www.nature.com/nature/journal/v342/n6248/pdf/342440a0.pdf [Accessed December 18, 2014].

Naryshkin, N.A. et al., 2014. SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy. *Science*, 345(6197), pp.688–693. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25104390 [Accessed August 7, 2014].

Nixon, R.A., 2003. The calpains in aging and aging-related diseases. *Ageing Research Reviews*, 2(4), pp.407–418. Available at: http://www.sciencedirect.com/science/article/pii/S1568163703000291 [Accessed March 17, 2015].

Oppenheim, R.W. et al., 2001. Cardiotrophin-1, a Muscle-Derived Cytokine, Is Required for the Survival of Subpopulations of Developing Motoneurons. *J. Neurosci.*, 21(4), pp.1283–1291. Available at: http://www.jneurosci.org/content/21/4/1283.long [Accessed December 17, 2014].

Oppenheim, R.W., 1996. Neurotrophic survival molecules for motoneurons: an embarrassment of riches. *Neuron*, 17(2), pp.195–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8780643 [Accessed December 15, 2014].

Orlowski, R.Z. et al., 2002. Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 20(22), pp.4420–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12431963 [Accessed February 13, 2015].

Paratcha, G., Ledda, F. & Ibáñez, C.F., 2003. The Neural Cell Adhesion Molecule NCAM Is an Alternative Signaling Receptor for GDNF Family Ligands. *Cell*, 113(7), pp.867–879. Available at: http://www.sciencedirect.com/science/article/pii/S0092867403004355 [Accessed February 16, 2015].

Park, G.-H. et al., 2010. Reduced survival of motor neuron (SMN) protein in motor neuronal progenitors functions cell autonomously to cause spinal muscular atrophy in model mice expressing the human centromeric (SMN2) gene. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 30(36), pp.12005–19. Available at: http://www.jneurosci.org/content/30/36/12005.short [Accessed April 1, 2015].

Park, J.Y. et al., 2013. Calcium-dependent proteasome activation is required for axonal neurofilament degradation. *Neural regeneration research*, 8(36), pp.3401–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4146008&tool=pmcentrez&rendertype=abstract [Accessed April 7, 2015].

Patten, I. & Placzek, M., 2000. The role of Sonic hedgehog in neural tube patterning. *Cellular and molecular life sciences : CMLS*, 57(12), pp.1695–708. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11130176 [Accessed November 24, 2014].

Pattingre, S. et al., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell*, 122(6), pp.927–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16179260 [Accessed July 9, 2014].

Paushkin, S. et al., 2002. The SMN complex, an assemblyosome of ribonucleoproteins. *Current opinion in cell biology*, 14(3), pp.305–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12067652 [Accessed February 6, 2015].

Pearn, J., 1978. Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. *Journal of medical genetics*, 15(6), pp.409–13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1013753&tool=pmcentrez&rendertype=abstract [Accessed November 5, 2014].

Pelvig, D.P. et al., 2008. Neocortical glial cell numbers in human brains. *Neurobiology of aging*, 29(11), pp.1754–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17544173 [Accessed November 6, 2014].

Peterson, W.M. et al., 2000. Ciliary Neurotrophic Factor and Stress Stimuli Activate the Jak-STAT Pathway in Retinal Neurons and Glia. *J. Neurosci.*, 20(11), pp.4081–4090. Available at: http://www.jneurosci.org/content/20/11/4081?ijkey=2dd36b12142068e8efaca4af50f6b08a084967e0&keytype2=tf_ipsecsha [Accessed December 17, 2014].

Petiot, A. et al., 2000. Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *The Journal of biological chemistry*, 275(2), pp.992–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10625637 [Accessed November 5, 2014].

Poças, G.M. et al., 2015. α-Synuclein modifies mutant huntingtin aggregation and neurotoxicity in Drosophila. *Human molecular genetics*, 24(7), pp.1898–907. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25452431 [Accessed March 13, 2015].

Pun, S. et al., 2006. Selective vulnerability and pruning of phasic motoneuron axons in motoneuron disease alleviated by CNTF. *Nature neuroscience*, 9(3), pp.408–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16474388 [Accessed December 4, 2014].

Rajendra, T.K. et al., 2007. A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle. *The Journal of cell biology*, 176(6), pp.831–41. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2064057&tool=pmcentrez&rendertype=abstract [Accessed January 8, 2015].

Ramesh, T. et al., A genetic model of amyotrophic lateral sclerosis in zebrafish displays phenotypic hallmarks of motoneuron disease. *Disease models & mechanisms*, 3(9-10), pp.652–62. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2931540&tool=pmcentrez&rendertype=abstract [Accessed January 9, 2015].

Ravikumar, B. et al., 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nature genetics*, 36(6), pp.585–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15146184 [Accessed December 18, 2014].

Ravikumar, B. et al., 2010. Plasma membrane contributes to the formation of pre-autophagosomal structures. *Nature cell biology*, 12(8), pp.747–57. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2923063&tool=pmcentrez&rendertype=abstract [Accessed September 1, 2014].

Reiling, J.H. & Sabatini, D.M., 2006. Stress and mTORture signaling. *Oncogene*, 25(48), pp.6373–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17041623 [Accessed December 17, 2014].

Riessland, M. et al., 2010. SAHA ameliorates the SMA phenotype in two mouse models for spinal muscular atrophy. *Human molecular genetics*, 19(8), pp.1492–506. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20097677 [Accessed January 5, 2015].

Robles, E., Huttenlocher, A. & Gomez, T.M., 2003. Filopodial calcium transients regulate growth cone motility and guidance through local activation of calpain. *Neuron*, 38(4), pp.597–609. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12765611 [Accessed March 17, 2015].

Ross, C.A. & Poirier, M.A., 2004. Protein aggregation and neurodegenerative disease.

Rossoll, W. et al., 2003. Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *The Journal of cell biology*, 163(4), pp.801–12. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2173668&tool=pmcentrez&rendertype=abstract [Accessed October 20, 2014].

Roth, K.A. & D'Sa, C., 2001. Apoptosis and brain development. *Mental retardation and developmental disabilities research reviews*, 7(4), pp.261–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11754520 [Accessed November 19, 2014].

Roy, N. et al., 1995. The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell*, 80(1), pp.167–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7813013 [Accessed December 29, 2014].

Roy, S. et al., 2005. Axonal transport defects: a common theme in neurodegenerative diseases. *Acta neuropathologica*, 109(1), pp.5–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15645263 [Accessed March 13, 2015].

Rubinsztein, D.C., Codogno, P. & Levine, B., 2012. Autophagy modulation as a potential therapeutic target for diverse diseases. *Nature reviews. Drug discovery*, 11(9), pp.709–30. Available at: http://dx.doi.org/10.1038/nrd3802 [Accessed July 9, 2014].

Ruchaud, S. et al., 2002. Caspase-6 gene disruption reveals a requirement for lamin A cleavage in apoptotic chromatin condensation. *The EMBO journal*, 21(8), pp.1967–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=125972&tool=pmcentrez&rendertype=abstract [Accessed December 19, 2014].

Russman, B.S., 2007. Spinal muscular atrophy: clinical classification and disease heterogeneity. *Journal of child neurology*, 22(8), pp.946–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17761648 [Accessed January 5, 2015].

Saelens, X. et al., 2004. Toxic proteins released from mitochondria in cell death. *Oncogene*, 23(16), pp.2861–74. Available at: http://dx.doi.org/10.1038/sj.onc.1207523 [Accessed November 26, 2014].

Sakla, M.S. & Lorson, C.L., 2008. Induction of full-length survival motor neuron by polyphenol botanical compounds. *Human genetics*, 122(6), pp.635–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17962980 [Accessed February 20, 2015].

Sapp, E. et al., 1997. Huntingtin localization in brains of normal and Huntington's disease patients. *Annals of neurology*, 42(4), pp.604–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9382472 [Accessed November 6, 2014].

Sarkar, S. et al., 2005. Lithium induces autophagy by inhibiting inositol monophosphatase. *The Journal of cell biology*, 170(7), pp.1101–11. Available at: http://jcb.rupress.org/content/170/7/1101 [Accessed December 7, 2014].

Sarkar, S., 2013. Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. *Biochemical Society transactions*, 41(5), pp.1103–30. Available at: http://www.biochemsoctrans.org/bst/041/1103/bst0411103.htm [Accessed December 19, 2014].

Schmelzle, T. et al., 2004. Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. *Molecular and cellular biology*, 24(1), pp.338–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=303340&tool=pmcentrez&rendertype=abstract [Accessed October 21, 2014].

Schrank, B. et al., 1997. Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proceedings of the National Academy of Sciences of the United States of America*, 94(18), pp.9920–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=23295&tool=pmcentrez&rendertype=abstract [Accessed November 5, 2014].

Segal, R.A., 2003. Selectivity in neurotrophin signaling: theme and variations. *Annual review of neuroscience*, 26, pp.299–330. Available at: http://www.annualreviews.org/doi/full/10.1146/annurev.neuro.26.041002.131421?url_ver=Z3

9.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed& [Accessed January 26, 2015].

Seglen, P.O. & Gordon, P.B., 1982. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 79(6), pp.1889–92. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=346086&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Seiliez, I., Dias, K. & Cleveland, B.M., 2014. Contribution of the autophagy-lysosomal and ubiquitin-proteasomal proteolytic systems to total proteolysis in rainbow trout (Oncorhynchus mykiss) myotubes. *American journal of physiology. Regulatory, integrative and comparative physiology*, 307(11), pp.R1330–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25274907 [Accessed February 20, 2015].

Shababi, M. et al., 2010. Cardiac defects contribute to the pathology of spinal muscular atrophy models. *Human molecular genetics*, 19(20), pp.4059–71. Available at: http://hmg.oxfordjournals.org/content/19/20/4059.long [Accessed November 13, 2014].

Shaid, S. et al., 2013. Ubiquitination and selective autophagy. *Cell death and differentiation*, 20(1), pp.21–30. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3524631&tool=pmcentrez&rendertype=abstract [Accessed September 15, 2014].

Shang, L. et al., 2011. Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK. *Proceedings of the National Academy of Sciences of the United States of America*, 108(12), pp.4788–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3064373&tool=pmcentrez&rendertype=abstract [Accessed October 24, 2014].

Shields, D.C. et al., 2000. Calpain activity and expression increased in activated glial and inflammatory cells in penumbra of spinal cord injury lesion. *Journal of neuroscience research*, 61(2), pp.146–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10878587 [Accessed April 1, 2015].

Shimizu, K. & Stopfer, M., 2013. Gap junctions. *Current biology : CB*, 23(23), pp.R1026–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24309273 [Accessed December 7, 2014].

Shin, S., Dalton, S. & Stice, S.L., 2005. Human motor neuron differentiation from human embryonic stem cells. *Stem cells and development*, 14(3), pp.266–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15969621 [Accessed January 29, 2015].

Sigrist, S.J. et al., 2014. Spermidine-triggered autophagy ameliorates memory during aging. *Autophagy*, 10(1), pp.178–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24262970 [Accessed November 20, 2014].

Smith, D.A., 2014. A new mechanokinetic model for muscle contraction, where force and movement are triggered by phosphate release. *Journal of muscle research and cell motility*, 35(5-6), pp.295–306. Available at: http://link.springer.com/article/10.1007/s10974-014-9391-z/fulltext.html [Accessed December 7, 2014].

Song, Y. et al., 2014. Rapamycin Plays a Neuroprotective Effect after Spinal Cord Injury via Anti-Inflammatory Effects. *Journal of biochemical and molecular toxicology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25171343 [Accessed November 16, 2014].

Sorimachi, H., Ishiura, S. & Suzuki, K., 1997. Structure and physiological function of calpains. *The Biochemical journal*, 328 (Pt 3, pp.721–32. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1218978&tool=pmcentrez&rendertype=abstract [Accessed April 7, 2015].

Stahl, N. et al., 1995. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science (New York, N.Y.)*, 267(5202), pp.1349–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7871433 [Accessed December 17, 2014].

Stifanese, R. et al., 2010. Adaptive Modifications in the Calpain/Calpastatin System in Brain Cells after Persistent Alteration in Ca 2+ Homeostasis. *Journal of Biological Chemistry*, 285(1), pp.631–643. Available at: http://www.jbc.org/content/285/1/631 [Accessed April 7, 2015].

Stifani, N., 2014. Motor neurons and the generation of spinal motor neuron diversity. *Frontiers in cellular neuroscience*, 8, p.293. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4191298&tool=pmcentrez&rendertype=abstract [Accessed November 4, 2014].

Stockli, K.A., 1991. Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. *The Journal of Cell Biology*, 115(2), pp.447–459. Available at: http://jcb.rupress.org/content/115/2/447?ijkey=223cdfe0202eaa9fcc8e7ea7f4a6617fd938489a&keytype2=tf_ipsecsha [Accessed December 17, 2014].

Suliman, A. et al., 2001. Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and -independent pathways. *Oncogene*, 20(17), pp.2122–33. Available at: http://www.nature.com/onc/journal/v20/n17/full/1204282a.html [Accessed December 24, 2014].

Sumner, C.J. et al., 2003. Valproic acid increases SMN levels in spinal muscular atrophy patient cells. *Annals of neurology*, 54(5), pp.647–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14595654 [Accessed March 18, 2015].

Sun, R. et al., 2014. Ammonium chloride inhibits autophagy of hepatocellular carcinoma cells through SMAD2 signaling. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25342595 [Accessed December 28, 2014].

Tanaka, M. et al., 2004. Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. *Nature medicine*, 10(2), pp.148–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14730359 [Accessed November 6, 2014].

Taylor, R.C., Cullen, S.P. & Martin, S.J., 2008. Apoptosis: controlled demolition at the cellular level. *Nature reviews. Molecular cell biology*, 9(3), pp.231–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18073771 [Accessed July 11, 2014].

Thomas, N.H. & Dubowitz, V., The natural history of type I (severe) spinal muscular atrophy. *Neuromuscular disorders : NMD*, 4(5-6), pp.497–502. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7881295 [Accessed January 7, 2015].

Thompson, C.B., 1995. Apoptosis in the pathogenesis and treatment of disease. *Science (New York, N.Y.)*, 267(5203), pp.1456–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7878464 [Accessed December 19, 2014].

Tolkovsky, A.M., 2009. Mitophagy. *Biochimica et biophysica acta*, 1793(9), pp.1508–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19289147 [Accessed November 6, 2014].

Tooze, S.A. & Yoshimori, T., 2010. The origin of the autophagosomal membrane. *Nature cell biology*, 12(9), pp.831–5. Available at: http://dx.doi.org/10.1038/ncb0910-831 [Accessed November 25, 2014].

Torres, J. et al., 2012. Efficient differentiation of embryonic stem cells into mesodermal precursors by BMP, retinoic acid and Notch signalling. *PloS one*, 7(4), p.e36405. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3340340&tool=pmcentrez&rendertype=abstract [Accessed December 7, 2014].

Tovar-Y-Romo, L.B. et al., 2014. Trophic factors as modulators of motor neuron physiology and survival: implications for ALS therapy. *Frontiers in cellular neuroscience*, 8, p.61. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3937589&tool=pmcentrez&rendertype=abstract [Accessed September 8, 2014].

Treanor, J.J. et al., 1996. Characterization of a multicomponent receptor for GDNF. *Nature*, 382(6586), pp.80–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8657309 [Accessed December 17, 2014].

Turner, D.A. et al., 2014. Wnt/ -catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic stem cells. *Development*, 141(22), pp.4243–4253. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25371361 [Accessed November 4, 2014].

Twombly, R., 2003. First Proteasome Inhibitor Approved for Multiple Myeloma. *JNCI Journal of the National Cancer Institute*, 95(12), pp.845–845. Available at: http://jnci.oxfordjournals.org/content/95/12/845.full [Accessed January 16, 2015].

Ulukaya, E., Acilan, C. & Yilmaz, Y., 2011. Apoptosis: why and how does it occur in biology? *Cell biochemistry and function*, 29(6), pp.468–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21773978 [Accessed November 20, 2014].

Valori, C.F. et al., 2010. Systemic delivery of scAAV9 expressing SMN prolongs survival in a model of spinal muscular atrophy. *Science translational medicine*, 2(35), p.35ra42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20538619 [Accessed January 29, 2015].

Vega, Q.C., 1996. Glial Cell Line-derived Neurotrophic Factor Signals through the RET Receptor and Activates Mitogen-activated Protein Kinase. *Journal of Biological Chemistry*, 271(39), pp.23619–23622. Available at: http://www.jbc.org/content/271/39/23619 [Accessed March 14, 2015].

Vicencio, J.M., Lavandero, S. & Szabadkai, G., 2010. Ca2+, autophagy and protein degradation: thrown off balance in neurodegenerative disease. *Cell calcium*, 47(2), pp.112–21. Available at: http://www.sciencedirect.com/science/article/pii/S0143416009002073 [Accessed December 5, 2014].

Vitriol, E.A. & Zheng, J.Q., 2012. Growth cone travel in space and time: the cellular ensemble of cytoskeleton, adhesion, and membrane. *Neuron*, 73(6), pp.1068–81. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3319373&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Vogl, D.T. et al., 2014. Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. *Autophagy*, 10(8), pp.1380–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24991834 [Accessed February 20, 2015].

Vosler, P.S., Brennan, C.S. & Chen, J., 2008. Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. *Molecular neurobiology*, 38(1), pp.78–100. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2726710&tool=pmcentrez&rendertype=abstract [Accessed December 5, 2014].

Wadman, R.I. et al., 2012. Drug treatment for spinal muscular atrophy type I. *The Cochrane database of systematic reviews*, 4, p.CD006281. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22513939 [Accessed March 31, 2015].

Walker, M.P. et al., 2008. SMN complex localizes to the sarcomeric Z-disc and is a proteolytic target of calpain. *Human molecular genetics*, 17(21), pp.3399–410. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2566527&tool=pmcentrez&rendertype=abstract [Accessed February 17, 2015].

Wang, C. & Wang, X., 2015. The interplay between autophagy and the ubiquitin-proteasome system in cardiac proteotoxicity. *Biochimica et biophysica acta*, 1852(2), pp.188–194. Available at: http://www.sciencedirect.com/science/article/pii/S092544391400249X [Accessed January 20, 2015].

Wang, R. et al., 2014. Resveratrol protects neurons and the myocardium by reducing oxidative stress and ameliorating mitochondria damage in a cerebral ischemia rat model. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, 34(3), pp.854–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25199673 [Accessed November 6, 2014].

Wang, X.J. et al., 2013. A novel crosstalk between two major protein degradation systems: regulation of proteasomal activity by autophagy. *Autophagy*, 9(10), pp.1500–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23934082 [Accessed November 15, 2014].

Webb, J.L. et al., 2003. Alpha-Synuclein is degraded by both autophagy and the proteasome. *The Journal of biological chemistry*, 278(27), pp.25009–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12719433 [Accessed January 10, 2015].

Wee, C.D. et al., 2014. Targeting SR Proteins Improves SMN Expression in Spinal Muscular Atrophy Cells. *PloS one*, 9(12), p.e115205. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4266657&tool=pmcentrez&rendertype=abstract [Accessed December 17, 2014].

Van Weering, D.H. & Bos, J.L., 1998. Signal transduction by the receptor tyrosine kinase Ret. *Recent results in cancer research. Fortschritte der Krebsforschung. Progrès dans les recherches sur le cancer*, 154, pp.271–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10027007 [Accessed March 14, 2015].

Weisenhorn, D.M. et al., 1999. Cellular aspects of trophic actions in the nervous system. *International review of cytology*, 189, pp.177–265. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10333580 [Accessed December 17, 2014].

Wichterle, H. et al., 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell*, 110(3), pp.385–97. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12176325 [Accessed January 13, 2015].

Will, C.L. & Lührmann, R., 2001. Spliceosomal UsnRNP biogenesis, structure and function. *Current Opinion in Cell Biology*, 13(3), pp.290–301. Available at: http://www.sciencedirect.com/science/article/pii/S0955067400002118 [Accessed March 18, 2015].

Williams, A. et al., 2006. Aggregate-prone proteins are cleared from the cytosol by autophagy: therapeutic implications. *Current topics in developmental biology*, 76, pp.89–101. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17118264 [Accessed December 17, 2014].

Williams, A. et al., 2008a. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nature chemical biology*, 4(5), pp.295–305. Available at: http://dx.doi.org/10.1038/nchembio.79 [Accessed August 18, 2014].

Williams, A. et al., 2008b. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nature chemical biology*, 4(5), pp.295–305. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2635566&tool=pmcentrez&rendertype=abstract [Accessed August 18, 2014].

Winkler, C. et al., 2005. Reduced U snRNP assembly causes motor axon degeneration in an animal model for spinal muscular atrophy. *Genes & development*, 19(19), pp.2320–30. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1240041&tool=pmcentrez&rendertype=abstract [Accessed March 18, 2015].

Winter, C.G. et al., 1995. A role for ciliary neurotrophic factor as an inducer of reactive gliosis, the glial response to central nervous system injury. *Proceedings of the National Academy of Sciences*, 92(13), pp.5865–5869. Available at: http://www.pnas.org/content/92/13/5865?ijkey=4d0660b1d15bec6eb55fc2e541c9c5330e414993&keytype2=tf_ipsecsha [Accessed December 17, 2014].

Wirth, B., 2000. An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA). *Human mutation*, 15(3), pp.228–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10679938 [Accessed February 14, 2015].

Wong, E. & Cuervo, A.M., 2010. Autophagy gone awry in neurodegenerative diseases. *Nature neuroscience*, 13(7), pp.805–11. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4038747&tool=pmcentrez&rendertype=abstract [Accessed September 17, 2014].

Wong, W.-K. et al., 2014. Hepatocyte Growth Factor Promotes Long-Term Survival and Axonal Regeneration of Retinal Ganglion Cells after Optic Nerve Injury: Comparison with CNTF and BDNF. *CNS neuroscience & therapeutics*, 20(10), pp.916–29. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24992648 [Accessed November 10, 2014].

Wu, Y.-T. et al., 2010. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *The Journal of biological chemistry*, 285(14), pp.10850–61. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2856291&tool=pmcentrez&rendertype=abstract [Accessed September 21, 2014].

Xiao, K. et al., 2013. Curcumin induces autophagy via activating the AMPK signaling pathway in lung adenocarcinoma cells. *Journal of pharmacological sciences*, 123(2), pp.102–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24048094 [Accessed November 6, 2014].

Yamamoto, A. et al., 1998. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell structure and function*, 23(1), pp.33–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9639028 [Accessed November 6, 2014].

Yamamoto, A. et al., 1998. Bafilomycin A1 Prevents Maturation of Autophagic Vacuoles by Inhibiting Fusion between Autophagosomes and Lysosomes in Rat Hepatoma Cell Line, H-4-II-E Cells. *Cell Structure and Function*, 23(1), pp.33–42. Available at: https://www.jstage.jst.go.jp/article/csf1975/23/1/23_1_33/_article [Accessed December 1, 2014].

Yamamoto, Y. et al., 1997. Hepatocyte growth factor (HGF/SF) is a muscle-derived survival factor for a subpopulation of embryonic motoneurons. *Development (Cambridge, England)*, 124(15), pp.2903–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9247333 [Accessed December 15, 2014].

Ylä-Anttila, P. et al., 2009. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. *Autophagy*, 5(8), pp.1180–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19855179 [Accessed November 5, 2014].

Yong, J., Wan, L. & Dreyfuss, G., 2004. Why do cells need an assembly machine for RNA-protein complexes? *Trends in cell biology*, 14(5), pp.226–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15130578 [Accessed March 19, 2015].

Young, A.R.J. et al., 2006. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. *Journal of cell science*, 119(Pt 18), pp.3888–900. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16940348 [Accessed November 5, 2014].

Zanetta, C., Nizzardo, M., et al., 2014. Molecular therapeutic strategies for spinal muscular atrophies: current and future clinical trials. *Clinical therapeutics*, 36(1), pp.128–40. Available at: http://www.sciencedirect.com/science/article/pii/S0149291813011004 [Accessed January 21, 2015].

Zanetta, C., Riboldi, G., et al., 2014. Molecular, genetic and stem cell-mediated therapeutic strategies for spinal muscular atrophy (SMA). *Journal of cellular and molecular medicine*, 18(2), pp.187–96. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3930406&tool=pmcentrez&rendertype=abstract [Accessed December 24, 2014].

Zhang, H.L. et al., 2003. Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(16), pp.6627–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12878704 [Accessed January 16, 2015].

Zhang, X. et al., 2014. MTOR-independent, autophagic enhancer trehalose prolongs motor neuron survival and ameliorates the autophagic flux defect in a mouse model of amyotrophic lateral sclerosis. *Autophagy*, 10(4), pp.588–602. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24441414 [Accessed January 9, 2015].

Zhang, X. et al., 2011. Rapamycin treatment augments motor neuron degeneration in SOD1(G93A) mouse model of amyotrophic lateral sclerosis. *Autophagy*, 7(4), pp.412–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21193837 [Accessed November 16, 2014].

Zhu, T. et al., 2014. GDNF and NT-3 Induce Progenitor Bone Mesenchymal Stem Cell Differentiation into Neurons in Fetal Gut Culture Medium. *Cellular and molecular neurobiology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25301495 [Accessed November 10, 2014].

Zoghbi, H.Y. & Orr, H.T., 2000. Glutamine repeats and neurodegeneration. *Annual review of neuroscience*, 23, pp.217–47. Available at: http://www.annualreviews.org/doi/abs/10.1146/annurev.neuro.23.1.217 [Accessed December 28, 2014].

Zoncu, R., Efeyan, A. & Sabatini, D.M., 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews. Molecular cell biology*, 12(1), pp.21–35. Available at: http://dx.doi.org/10.1038/nrm3025 [Accessed July 13, 2014].