Universitat de Lleida

A Study on the Role of NF-kB Signaling Pathway Members in Regulating Survival Motor Neuron Protein level and in the Pathogenesis of Spinal Muscular Atrophy

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TESI DOCTORAL

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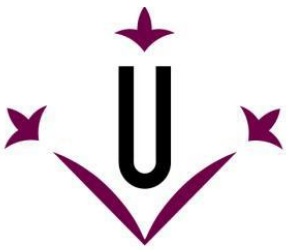

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DEPARTAMENT DE MEDICINA EXPERIMENTAL

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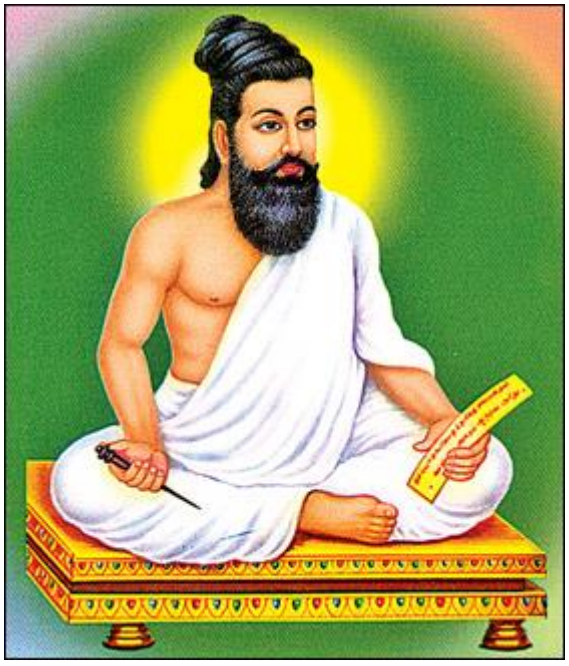

Thiruvalluvar

தெய்வத்தான் ஆகா தெனினும் முயற்சிதன்
மெய்வருத்தக் கூலி தரும்.

.....திருக்குறள்

Transliteration: Dheivathaan agadheninum, muyarchi than
mei varutha kooli tharum
……..Thirukkural

Meaning: "Something may not be achievable even by God or through God's help. But the effort exerted to attain that non-achievable will yield its deserving result.

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I would like to thank my supervisor Dr. Rosa Soler for her patience and guidance throughout the project. How true is the saying “Choose your boss, not your work.” Her positive outlook and confidence in my research inspired me and gave me confidence. She taught me even how to use knife and forceps while eating. Thank you, Rosa, for all the things you did especially the support you gave me during the worst days.

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I dedicate this work to my late friend

Mohammed Iqbal

Abstract

Abstract

SPinal Muscular Atrophy (SMA) is a neuromuscular disorder caused by mutation or loss in *SMN1* gene, encoding the ubiquitously expressed Survival Motor Neuron (SMN) protein. SMA is characterized by muscle atrophy and spinal cord motoneurons (MNs) degeneration. The molecular events behind the selective vulnerability of these MNs to the low level of SMN protein are still unknown. The nuclear factor-κB (NF-κB) pathway has recently been emerged having a vital role related to MN survival, and in neurodegenerative disorders. The NF-κB transcription factors regulate genes related to several cellular processes. In the present work, we have analyzed the ability of NF-κB pathway members to regulate Smn and their possible role in SMA pathogenesis. The NF-κB pathway activation is associated with IKKα/IKKβ phosphorylation, and RelA/p50 nuclear translocation (canonical) or IKKα homodimer phosphorylation, and RelB/p52 nuclear translocation (non-canonical). The inhibition of different protein members of both canonical, and non-canonical pathways using shRNA lentiviral transduction methodology in a primary culture of isolated embryonic spinal cord MNs reveals that the selective reduction of RelA induced the reduction of Smn whereas RelB protein reduction had no effect on Smn. In our culture system, reduction of IKKα or IKKβ proteins showed opposite effects on Smn. RT-PCR studies indicate that the shIKKβ-transduced MNs showed increased *Smn* mRNA levels, whereas it was not observed changes in *Smn* mRNA in the case of shIKKα- or shRelA-transduced MNs. The double knock-down of IKKα and IKKβ in MNs showed Smn reduction. The knockdown of IKKα and/or IKKβ showed a decrease in RelA phosphorylation, where the phosphorylation of RelA enable RelA/p50 release from its inhibitor in the cytoplasm and facilitates their nuclear translocation. Also, the CREB, one of the transcription factors for Smn was decreased in shIKKα, or in shIKKα- plus IKKβ-transduced MNs, and as well as in shRelA-transduced MNs. But, the shIKKβ MNs exhibited reduced p-RelA but increased CREB level. The shCREB-transduced MNs decreased Smn level, authenticating the regulatory role of CREB on Smn. We observed a reduction in IKKα, IKKβ and p-RelA levels in shSmn-tranduced MNs, and in MNs from a severe type SMA mouse model. Our results show the ability of NF-κB canonical pathway to regulate Smn level and, conversely, this pathway is also altered in Smn-deficient MNs. Together, these observations suggest that the NF-κB pathway has a role in SMA pathogenesis, and could be a therapeutic target for SMA.

Resum

L'atròfia muscular espinal (AME) és una malaltia neuromuscular causada per mutació o deleció en el gen *SMN1*, que codifica per la proteïna ubiqua SMN (de l'anglès s*urvival motor neuron*). L'AME es caracteritza per atròfia muscular i degeneració de les motoneurones (MN) de la medul·la espinal. Els esdeveniments moleculars que causen la vulnerabilitat específica de les MN amb nivells baixos de proteïna SMN encara no es coneixen. La via de l'NF-κB (*nuclear factor*-κB) ha destacat recentment ja que sembla jugar un paper cabdal en la supervivència de les MN i en les malalties neurodegeneratives. Els factors de transcripció NF-κB regulen gens relacionats amb molts processos cel·lulars. En aquest treball hem analitzat la capacitat dels membres de la via de l'NF-κB de regular la proteïna Smn i el seu possible rol en la patogènesi de l'AME. L'activació de la via de l'NF-κB està associada a la fosforilació de l'IKKα/IKKβ i la translocació nuclear del factor RelA/p50 (via canònica) o la fosforilació de l'homodímer d'IKKα i la translocació nuclear del factor RelB/p52 (via no canònica). La inhibició de diferents membres d'aquestes vies (tant la canònica com la no canònica) usant la metodologia de transducció amb lentivirus amb shRNA en cultius primaris de MN embrionàries aïllades de ratolí hem demostrat que una reducció selectiva del factor RelA provoca una reducció de la proteïna Smn, mentre que una reducció del factor RelB no té cap efecte en els nivells de l'Smn. En el nostre model cel·lular, la reducció dels nivells de l'IKKα o l'IKKβ provoca un efecte oposat en l'Smn. Mitjançant la tècnica de la RT-PCR hem observat que la transducció de les MN amb l'shIKKβ provoca un augment dels nivells de l'mRNA de *Smn*, mentre que la transducció amb l'shIKKα o l'shRelA no modifica l'expressió de Smn. El doble *knockdown* de l'IKKα i l'IKKβ a les MN mostra una reducció de l'Smn. El *knockdown* selectiu de l'IKKα o l'IKKβ presenta una reducció de la fosforilació del RelA, es coneix que aquesta fosforilació en permet l'alliberament del seu inhibidor al citosol i en facilita la translocació nuclear. També la proteïna CREB, un dels factors de transcripció coneguts de l'Smn, disminueix amb la transducció de les MN amb els shIKKα o amb l'IKKα i l'IKKβ alhora, així com amb l'shRelA. Ara bé, les motoneurones amb l'shIKKβ mostren una reducció de la fosforilació de RelA però un augment dels nivells de CREB. La transducció de les MN amb l'shCREB disminueix els nivells de l'Smn recolzant el paper regulador de CREB en l'Smn. Hem observat una reducció de l'IKKα, l'IKKβ i de la fosforilació de RelA amb la transducció de les MN amb l'shSmn i en les MN del model murí sever de l'AME. Els nostres resultats mostren l'habilitat de la via canònica de l'NF-κB de regular els nivells de l'Smn i que aquesta via també es troba alterada en les MN deficients en la proteïna Smn. En conjunt, aquestes observacions suggereixen que la via de l'NF-κB pot tenir un rol en la patogènesi i ser, a la vegada, una possible diana terapèutica per l'AME.

Resumen

La Atrofia Muscular Espinal (SMA) es un trastorno neuromuscular causado por la mutación o deleción del gen SMN1, el cual codifica para la proteína que se expresa ubicuamente SMN (del inglés Survival Motor Neuron). La AME se caracteriza por atrofia muscular y degeneración de las motoneuronas de la médula espinal (MN). Los eventos moleculares detrás de la vulnerabilidad selectiva de las MN con niveles bajos de la proteína SMN se desconocen. La vía del factor nuclear-kB (NF-kB) ha sido implicada recientemente en la supervivencia de las MNs, así como en trastornos neurodegenerativos. Los factores de transcripción NF-kB regulan genes relacionados con varios procesos celulares. En este trabajo hemos analizado la capacidad de los miembros de la vía del NF-κB de regular la proteína SMN y su posible rol en la patogénesis del AME. La activación de la vía del NF-κB está asociada a la fosforilación de IKKα / IKKβ y la translocación nuclear del factor RelA/ p50 (vía canónica) o la fosforilación del homodímero de IKKα y la translocación nuclear del factor RelB / p52 (vía no canónica). Hemos realizado la inhibición de diferentes miembros de estas vías (tanto la canónica como la no canónica) usando la metodología de shRNA, y la transducción mediante el uso de lentivirus, en cultivos primarios de MN embrionarias aisladas de ratón. Hemos demostrado que una reducción selectiva del factor RelA provoca una reducción de la proteína SMN, mientras que una reducción del factor RelB no tiene ningún efecto en los niveles de la SMN. En nuestro modelo celular, la reducción de las proteínas IKKα o IKKβ mostró efectos opuestos sobre la proteína Smn. Mediante la técnica de PCR, hemos observado que la transducción de las MN con el shIKKβ provoca un aumento de los niveles de mRNA de SMN, mientras que la transducción con el shIKKα o el shRelA no cambian los niveles de RNA de SMN. El doble knockdown de IKKα e IKKβ en las MN muestra una reducción de SMN. El knockdown selectivo de IKKα o IKKβ presenta una reducción de la fosforilación del RelA, esta fosforilación permite la liberación de su inhibidor en el citosol y facilita la translocación nuclear. La proteína CREB, uno de los factores de transcripción conocidos para SMN, disminuye con la transducción de las MN con shIKKα o con IKKα e IKKβ a la vez, así como con shRelA. Ahora bien, las motoneuronas transducidas con shIKKβ muestran una reducción de la fosforilación de RelA pero un aumento de los niveles de la proteína CREB. La transducción de las MN con el shCREB disminuyó los niveles de la proteína SMN apoyando el papel regulador de CREB sobre SMN.

En las MN transducidas con el shSMN hemos observado una disminución de IKKα e IKKβ, al igual que en las MN aisladas del modelo de ratón de AME (Smn-/-; *SMN2*+/+). Nuestros resultados muestran la capacidad de la vía canónica de regular SMN, y a la inversa, la alteración de la vía en las MN con niveles deficientes en Smn. En conjunto estas observaciones sugieren que la vía de NF-kappa B tiene un papel en la patogénesis SMA, y podría ser una diana terapéutica para el AME. Smn y, a la inversa, esta vía también se altera en los MNs Smn deficientes. En conjunto, estas observaciones sugieren que la vía de NF-κB tiene un papel en la patogénesis SMA, y podría ser una diana terapéutica para SMA.

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Abbreviations

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
Acetyl Choline Receptor	AChR
Acrylamide with N, N'-methylene bisacrylamide	Acri: Bis
Adeno-associated vectors	AAV
Adenosine 5′-triphosphate magnesium salt	Mg-ATP
Adenosine triphosphate	ATP
Ammonium per sulphate	APS
Amyotrophic Lateral Sclerosis	ALS
Analysis of variance	ANOVA
Antisense morpholino	MO
Antisense oligonucleotides	ASOs
Antisense oligonucleotides	ASOs
Aphidicolin	APH
Argonaute 2	Ago 2
Artemin	ARTN
AU-rich element	ARE
Blood Brain Barrier	BBB
Bone Morphogenetic Proteins	BMPs
Bovine Serum Albumin	BSA
Brain- Derived Neurotrophic Factor	BDNF
Calcium/calmodulin-dependent protein kinase type IV	CAMKIV
calmodulin-dependent kinase II	CaMKII
cAMP response element-binding protein	CREB
cAMP response elements	CRE
Cardiotrophin-1	CT-1
Cardiotrophin-Like Cytokine-1	CLC
Casein kinase II	CKII
Cell wall assembly regulator SMI1	SMI-1
Cellular inhibitor of apoptosis 1	cIAP
Ciliary Neurotrophic Factor	CNTF
Ciliary Neurotrophic Factor Receptor α	CNTFRα
c-Jun N-terminal kinases	JNKs
COUP transcription factor 2	COUP-TFII
CREB binding protein	CBP
Cytochrome c oxidase subunit 2	Cox-2
Cytomegalovirus	CMV
Decapping Scavenger enzyme	DcpS
Deoxynucleotide triphosphates	dNTPs
Deoxyribonucleic acid	DNA
Dimethyl sulfoxide	DMSO
Dulbecco's Modified Eagle's medium	DMEM
E3 ubiquitin ligase, mind bomb 1	Mib1
Elongation factor 1-alpha	EF-1α
Endothelial transcription factor GATA-2	GATA-2

Epidermal growth factor	EGF
Estrogen-related receptor gamma	ERR3
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid	EGTA
ETS domain-containing protein Elk-1	Elk-1
Exonic splicing enhancer	ESE
Extensor digitorum longus	EDL
Fibroblast Growth Factor Receptor Substrate 2	FrS2
Fibroblast growth factors	FGFs
fragile X mental retardation protein	FMRP
gamma-MNs	γ-MNs
GAR1 Ribonucleoprotein	GAR1
GDNF Family of Ligands	GFLS
GDNF family receptor alpha-1	GFRα1
Glial cell line-Deliverd neurotrophic factor	GDNF
Glucose-HEPES Buffer Saline	GHEBS
glyceraldehyde 3-phosphate dehydrogenase	*gapdh*
Glycogen synthase kinase 3 beta	GSK-3B
glycoprotein 130	gp130
Glycosylphosphatidylinositol	GPI
Green fluorescent protein	GFP
Growth arrest and DNA damage-inducible protein GADD45 beta	GADD45b
Growth Differentiation Factor-15	GDF-15
Guanosine 5′-triphosphate sodium salt	Na-GTP
Hepatocyte growth factor	HGF
Heat shock protein 90	Hsp90
Hepatocyte nuclear factor 3	HNF-3
Heterogeneous nuclear ribonuclear protein	hnRNPs
Histone acetyltransferases domain	HAT domain
Histone deacetylase inhibitor	HDACi
Histone deacetylases	HDAC
Horseradish peroxidase	HRP
Hu antigen D	HuD
Human Embryonic Kidney Cell line	HEK-293T
Human Hungtingtin	Htt
Hypaxial Motor Column	HMC
IM/homeobox protein Lhx3	Lim-3
Induced pluripotent stem cells	iPSCs
Inducible nitric oxide synthase	iNOS
Inhibitor of kappa B	IκB
Inhibitor of kappa B kinases	IKKs
Insulin Growth Factor	IGF-1
Interferon beta	IFN-β
Interferon gamma	IFN–γ
Interferon regulatory factor 1	IRF-1
Iroquois-class homeodomain protein IRX-3	Irx3
IκB Kinases	IKKs

KH-type splicing regulatory protein	KSRP
Lateral Motor Column	LMC
Leukemia Inhibitory Factor	LIF
LIM homeodomain transcription factor	LIM
long terminal repeats	LTRs
Luria-Bertani medium	LB-medium
manganese-dependent superoxide dismutase	Mn-SOD
MAP kinase kinase 1	MEK
Median Motor Column	MMC
Mitogen- and stress-activated protein kinase 1	MSK1
Mitogen- and stress-activated protein kinase 2	MSK2
Mitogen-activated protein kinase	MAPK
Morpholino	MO
Mouse embryonic fibroblast	MEF
Mullerian Inhibiting Substance	MIS
N, N, N, N'-tetramethylethylenediamine	TEMED
Nerve Growth Factor	NGF
Neuroblastoma X Spinal Cord Hybrid Cell Line 34	NSC-34
Neuromuscular junction	NMJ
Neurotrophic Factors	NTFs
Neurturin	NRTN
NF-κB-inducing kinase	NIK
NMDA-receptors	NMDAR
N-Methyl-D-Aspartate	NMDA
Nuclear Export Sequence	NES
Nuclear factor-kappa B	NF-κB
Nuclear localization sequence	NLS
Osmolarity (milliosmoles/liter)	mOsm/l
Oxygen-glucose deprivation	OGD
p75 Neurotrophin receptor	p75 NTR
Paraxial Mesoderm Caudalizing	PMC
Penicillin and Streptomycin	P/S
Phosphate-buffered saline	PBS
phosphatidylinositol-3 kinase	PI3-K
Phosphoinositide phospholipase C gamma	PLCγ
phospho-RelA	pRelA
Polyethyleneimine	PEI
Polymerase chain reaction	PCR
Polyvinylidene difluoride	PVDF
Preganglionic Motor Column	PGC
Programmed cell death	PCD
Prolactin	PRL
Proline, glutamic acid/aspartic acid, serine and threonine residues rich domain	PEST domain
Protein kinase A	PKA
Protein kinase ξ	PKCξ

Protein transport protein YIP1	YIP1p
Rel homology domain	RHD
Rho-associated protein kinase	ROCK
Ribonucleic acid interference	RNAi
Ribonucleoprotein	RNP
Ribonucleoprotein-Q	RNP-Q
RISC loading complex	RLC
RNA interfering silencing complex	RISC
Self-complementary adeno-associated virus 9	scAAV9
Self-inactivating	SIN
Signal transducer and activator of transcription 5	Stat5
Simian vacuolating virus 40	SV40
Slow-twitch fatigue-resistant	SFR
Smad interacting protein 1	SIP1
Small hairpin RNAs	shRNAs
Small interfering RNAs	siRNAs
Small nuclear ribonucleoproteins	snRNPs
Small nuclear RNA	snRNA
SMNDelta7	SMND7
Sonic Hedgehog	Shh
Spinal Muscular Atrophy	SMA
Spleen tyrosine kinase	Syk
Standard error of the mean	SEM
Suberoylanilide hydroxamic acid	SAHA
Survival motor neuron, mouse	Smn
Survival motor neuron protein, Homo sapiens	SMN1
Survival motor neuron-like protein 1	Yab8
The nuclear transcription factor kappa B	NF-κB
Tibialis anterior	TA
TNF receptor-associated factor 1	TRAF1
TNF receptor-associated factor 2	TRAF2
Transcription factor AP-2	AP-2
Transcriptional activation domain	TAD
Transcriptional activator GLI3	Gli3
Transcriptional repressor protein YY1	YY1
Transversus abdominis	TVA
Trichostatin A	TSA
Tris-acetate-EDTA buffer	TAE
Tropomyosin-related kinase	Trk
Tumor necrosis factor	TNF
Tyrosine kinase LIF receptor beta	LIFRβ
Ubiquitin/proteasome pathway	UPP
Vascular Endothelinal Growth Factor	VEGF
Zinc finger protein GLI1	Gli1
Zinc finger protein GLI2	Gli2

Chapter-I Introduction

Chapter-I

Introduction

In vertebrates, the nervous system, is anatomically divided into i) the central nervous system (CNS) consisting of the brain and spinal cord which coordinates the integration of all stimuli, all motor processes, hormone system regulation and organ control, and ii) the peripheral nervous system (PNS) including the nerves that carry sensory messages to the CNS and nerves that send information from the CNS to the muscles and glands. The two motor systems of PNS, a) the somatic motor division connects the brain and the spinal cord to the skeletal muscles, and b) the autonomic or visceral motor division controls the cells and axons that innervate with smooth muscles, cardiac muscles, and glands. The nervous system is composed of neural circuits, that process specific information through neurons and supporting glial cells. Functionally the nervous circuit is classified into afferent neurons, efferent neurons, and interneurons **(Figure 1).**

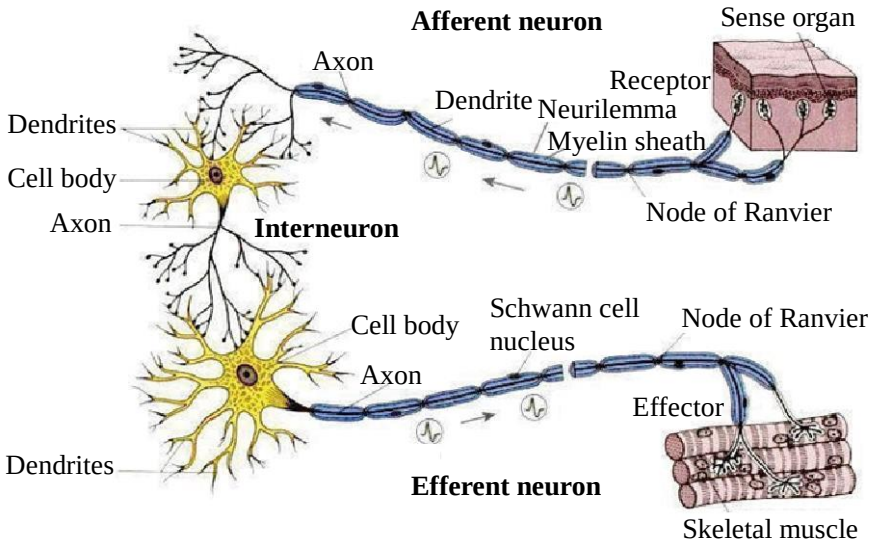

Figure 1. Functional classifications of the neural circuits: The nerve cells are classified into three types based on their functions, **i) afferent neurons** carry information towards the brain or spinal cord from receptors or sense organs**, ii) efferent neurons** carry information away from the brain or spinal cord in order to initiate an action, and **iii) Interneurons** connect neurons to each other within the same region of the brain or the spinal cord (figure adapted from https://universe-review.ca/R10-16-ANS.htm).

1.1. Motoneurons (MNs)

Motoneurons or Motor neurons are efferent neurons, which generate and transmit nerve impulses responsible for muscle contraction. The structure of an MN typically consists of i) a cell body which contains a nucleus and organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus, etc., ii) dendrites that extend outward from the cell body specialized to receive chemical signals from the axon termini of other neurons, and, iii) an axon which is a specialized for the conduction of a type of electrical impulse called an action

potential. MNs are divided into i) upper MNs, located in the cerebral cortex, and ii) lower MNs, located in the brainstem and spinal cord **(Figure 2A)**.

1.1.1. Spinal MNs

In the present study, we focus on the spinal MNs that located in the ventral horn of the spinal cord and are responsible for peripheral muscular contraction. These neurons project their axons out of the CNS to control muscle movement directly (by performing synapses with skeletal muscle) or indirectly (through the ganglia) **(Figure 2B)**.

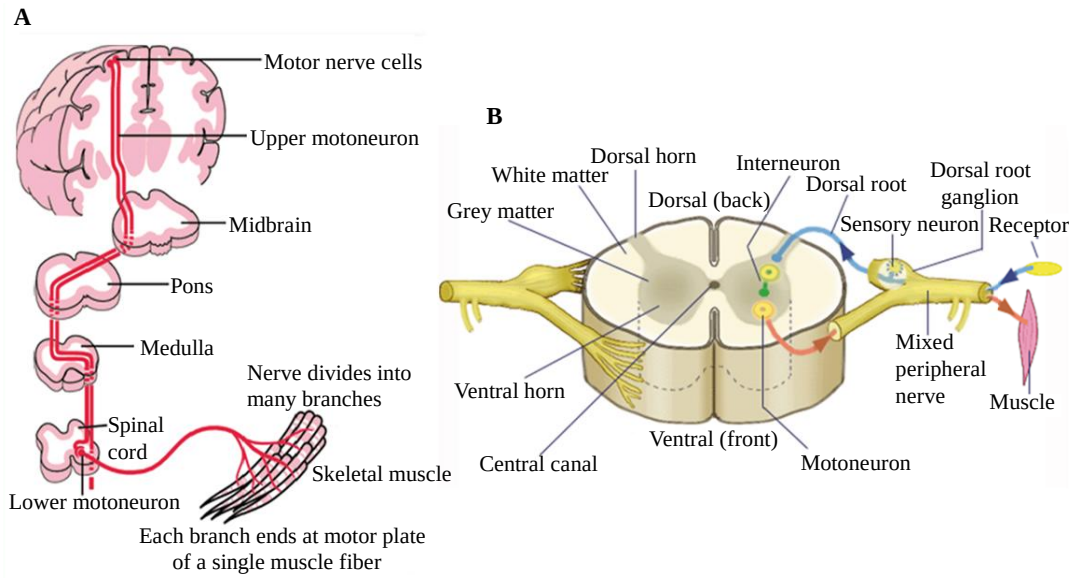

Figure 2. Classification of MNs and organization of MNs in the spinal cord: A) Upper MNs are located in the cerebral cortex and lower MNs are located in the brainstem and spinal cord (Damjanov 2000). **B)** The spinal cord MNs are located in the ventral horn establishing a connection with neuromuscular junction.

1.1.2. Classification of MNs

MNs exclusively innervate skeletal muscles that are the most abundant muscle class (Stone & Stone 2000). Depending on the muscle fiber innervations (Manuel & Zytnicki 2011), MNs can be classified into alpha- (α), beta- (β), or gamma- (γ) MNs **(Figure 3).**

α-MNs innervate extrafusal muscle fibers and direct muscle contraction. They are characterized by expressing the neuronal DNA binding protein, NeuN in the postnatal stage (Friese et al. 2009). Depending on the extrafusal fiber type they innervate α-MNs can be further divided into three different subtypes (Burke, R.E. et al., 1973), i) Slow-twitch fatigue-resistant (SFR) MNs that have a smaller cell body diameter responding to a lower stimulation threshold and are recruited first during muscle contraction. Even after the stimulation is ceased they have the capacity of maintaining a persistent activity (Lee & Heckman 1998). ii) Fast-twitch fatigable (FF) MNs that have a larger cell body and they fire once the initial

recruitment of SFR MNs and therefore giving extra strength to the activated muscle. They are essential for the movements which require short, but sharp; contraction impulses like running and jumping, and iii) Fast-twitch fatigue-resistant (FFR) MNs have intermediate characteristics between FF and SFR MNs.

β-MNs are smaller and less abundant than other MN subtypes and are poorly characterized. They innervate both intrafusal and extrafusal muscle fibers (Bessou, P. et al., 1965). They control both muscle contraction and responsiveness of the sensory feedback from muscle spindles.

γ-MNs innervate intrafusal muscle fibers and direct complex functions in motor control and are characterized by slow axonal conduction due to smaller axonal diameter. During the postnatal stage, they differ from other kinds of MNs by the expression of the transcription factor Err3 (Friese et al. 2009) and also expresses the greater amount of GFRα1, an essential receptor component for GDNF (Glial Cell-Derived Neurotrophic Factor) (Shneider et al. 2009).

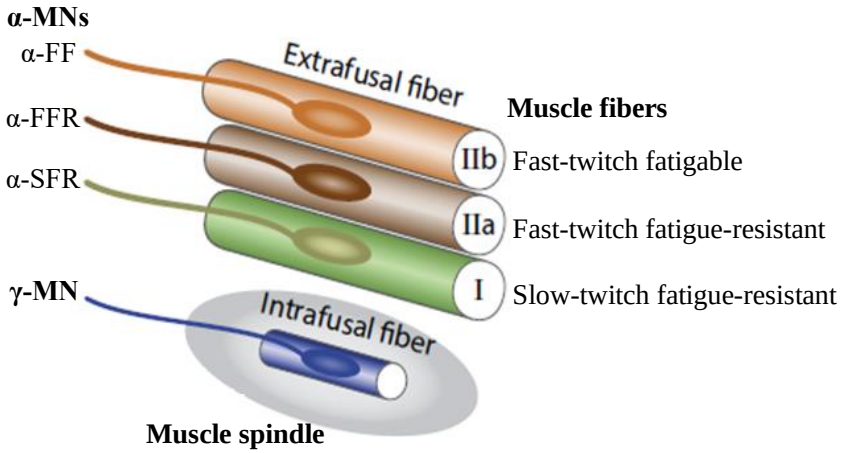

Figure 3. Classification of MNs based on muscle fiber innervation: Three types of extrafusal fibers; Fast-twitch Fatigable muscle fibers are innervated by α-FF MNs, Fast-twitch Fatigue-Resistant muscle fibers by α-FFR MNs, and Slow-twitch Fatigue-Resistant muscle fibers are innervated by α-SFR MNs. Intrafusal muscle fibers are exclusively innervated by γ-MNs (Stifani 2014)**.**

1.1.3. Development of Spinal MNs

During developmental stage, the ectodermal thickening known as neural plate merges at the terminal ends to form a single cell layered neuroepithelium neural tube (Cayuso & Martí 2005) which gives rise to the central nervous system. The caudal part of the neural tube gives rise to the spinal cord **(Figure 4A).** Classes of secreted factors like fibroblast growth factors (FGFs), Retinoids, Bone Morphogenetic Proteins (BMPs), Wnts and a Paraxial Mesoderm Caudalizing (PMC) activity have been associated with the acquisition of caudal neural character of the spinal cord (Doniach 1995; Storey et al. 1998; Bang et al. 1999; Muhr

et al. 1999). The action of a retinoid-mediated signal delivered by the potential caudal paraxial mesoderm is required for the differentiation of cells of spinal cord character (Muhr et al. 1999).

The appearance of distinct cell types at defined positions along the dorsoventral axis of the neural tube becomes marked evidence for the specification of neuronal subtypes in the spinal cord. On the ventral side, develops the floor plate cells (a specialized class of glial cell) which are differentiated by the signals triggered by an axial mesodermal cell group, the notochord (Placzek 1995). MNs and interneurons are generated at the dorsal positions of the neural tube (Jessell 2000), and the differentiation of MNs is by the inductive action of a glycoprotein called Sonic Hedgehog (Shh), secreted by cells of the notochord and floor plate cells (Tanabe et al. 1995) **(Figure 4B)**.

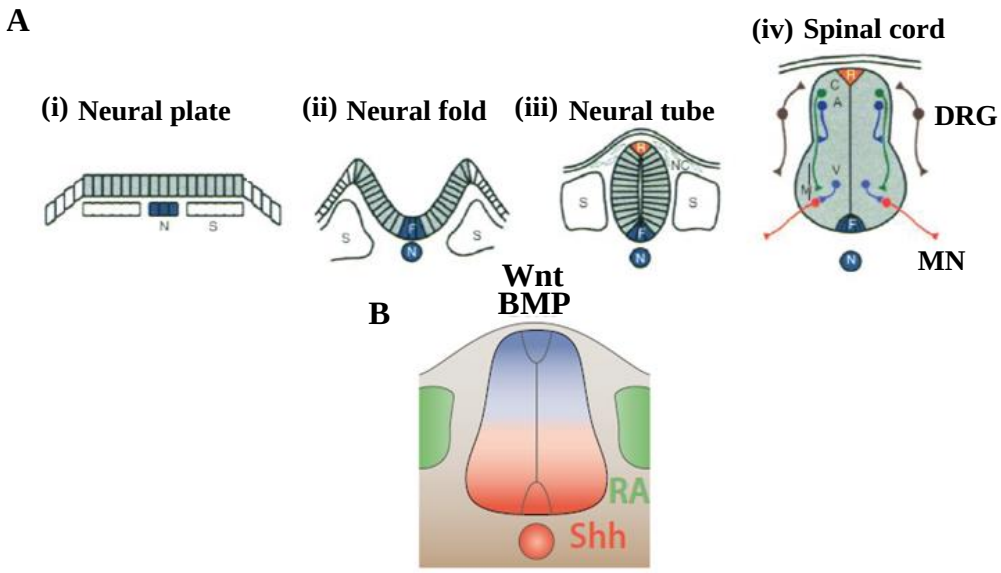

Figure 4. A) Schematic representation of spinal cord and spinal MN development: (i) Position of the neural plate about the non- neural ectoderm, the mesoderm, and the endoderm. **(ii)** Folding of the neural plate to form the neural groove. **(iii)** Dorsal closure of the neural folds to form the neural tube and neural crest. (iv) Maturation of the neural tube into the spinal cord (Tanabe & Jessell 1996). **B) Schematic representation of signals involved in the dorsoventral patterning of the neural tube:** Wnt, BMP, and Retinoic acid collaborate with Shh expressed by the floor plate and the notochord to pattern the neural tube (Stifani 2014).

1.1.3.1. Patterning in Spinal MN Development

A concentration gradient of the Shh protein along the dorsal-ventral axis is generated and the changes in Shh concentration makes five molecularly distinct classes of ventral neurons from neural progenitor whose positions are also dictated by the Shh concentration (Ericson et al. 1997) **(Figure 5A)**. A set of homeodomain proteins expressed by ventral progenitor cells acts as intermediaries in the interpretation of graded Shh signaling. These

homeodomain proteins are divided into two classes: Class I proteins (Pax7, Irx3, Dbx1, Dbx2, and Pax6) are repressed by Shh and Class II proteins (Nkx6.1 and Nkx2.2) requires Shh signaling for their expressions and the combinatorial expression profile of these two classes of homeodomain proteins defines five cardinal progenitor domains inside the ventral neural tube (Ericson et al. 1997; Briscoe et al. 2000; Briscoe et al. 1999; Jessell 2000) **(Figure 5B).**

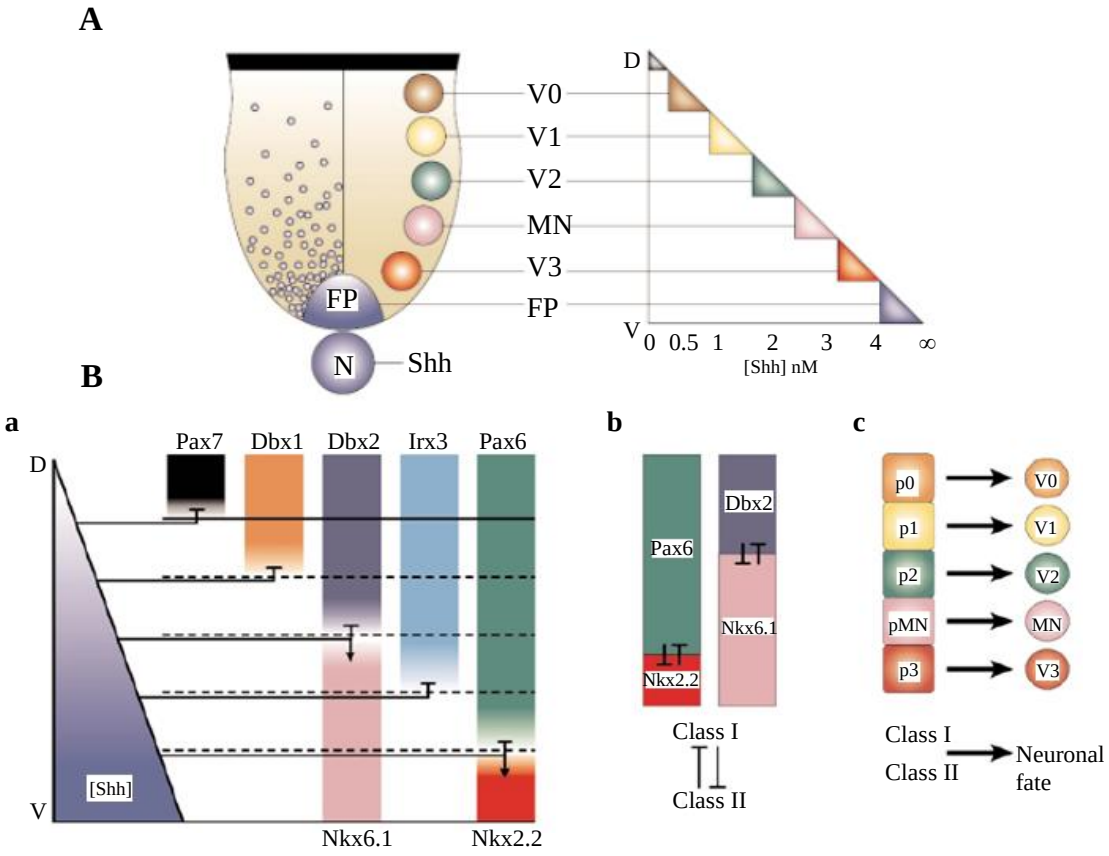

Figure 5. A) Ventral patterning is controlled through Shh expression by notochord and floor plate controls ventral pattern. The gradient of Shh activity in the ventral neural tube distributed in a ventral-high, dorsal-low profile within the ventral neural epithelium, and the position of five classes of neurons that are generated in response to graded Shh signaling. V0–V3 indicates four different classes of ventral interneurons. MN shows motor neurons. **B) a)** Shh mediates the repression of class I homeodomain proteins at different threshold concentrations and the induction of expression of class II proteins at different threshold concentrations. Shh signaling defines five progenitor domains in the ventral neural tube. **b)** The pairs of homeodomain proteins that abut a common progenitor domain boundary (Pax6 and Nkx2.2; Dbx2 and Nkx6.1) repress each other's expression. **c)** The relationship between neural progenitor (p) domains and the positions at which post-mitotic neurons are generated along the dorsoventral axis of the ventral spinal cord (Jessell 2000)**.**

MN precursor domain expresses Nkx6.1/Pax6/Olig2 (Liu et al. 2003). The activity of Nkx6.1, when unrestricted by the inhibitory effects of Irx3 and Nkx2.2 induces the expression of the homeodomain protein MNR2 which can positively auto activates its expression, induces the expression of downstream transcription factors of the LIM protein

family (LIM homeodomain transcription factor), including Islet-1, Islet-2, Lim-3 and HB9 which are considered distinct markers of MNs (Tanabe et al. 1998).

The Gli class of zinc finger transcription factors Gli1, Gli2 and Gli3 (Matise et al. 1998, Ding et al. 1998, Ruiz i Altaba 1999, Wang et al. 2000) and other Shh-regulated transcription factors like COUP-TFII are the key intermediaries in hedgehog signaling (Krishnan et al. 1997). The response of ventral neural progenitors to specific levels of Shh signaling activity dependents on BMP signaling (Liem et al. 2000).

1.1.3.2. Columnar Organization of Spinal MNs

The pattern of innervation of skeletal muscle by spinal MNs requires a high degree of spatial organization. Spinal MNs are organized into distinct anatomical columns extending along the rostrocaudal axis, the motor columns. There are four motor columns (Dasen & Jessell 2009) **(Figure 6),** and the genes encoded by the Hox transcription factors determines the specificity and organization of MNs in four columns (Jessell 2000).

- Preganglionic Motor Column (PGC) MNs projects their axons to the neurons of the sympathetic ganglia.
- Lateral Motor Column (LMC) MNs projects their axons to the ventral and dorsal muscles of the extremities and they exclusively innervate the limb muscles.
- Hypaxial Motor Column (HMC) MNs innervate and are responsible for the contraction of the intercostal muscles and the body wall in the ventral region of the body (hypaxial muscles). HMC MNs mainly innervate the respiratory muscles.
- Median Motor Column (MMC) MNs projects their axons to axial muscles (intercostal muscles and the body wall) of the dorsal region of the body (epaxial muscles).

MNs in different motor columns express specific transcription factors. All the MN precursors express Islet-1 and/or Islet-2, but, during development, the MNs show a pattern of differential expression of transcription factors, defining the functional subclasses of MNs.

Within a motor column, all the MNs that project their axons to the same skeletal muscle are termed as a pool. A typical motor pool represents all types of MNs -Fast and Slow, α and γ. Usually γ-MNs represents one-third of the MNs of a pool (Friese et al. 2009). But in the α-MNs, the ratio of FF, FR, and S varies significantly depending on the motor unit. Thus, each pool has a characteristic ratio of different subtypes of MNs from various subpopulation depending on their function, and the MNs of the pool shared morphological properties and molecular characteristics (Transcription factor profiles that confer specific expression of surface molecules such as receptors for axonal guidance, neurotrophic factors, and adhesion molecules) (Dasen & Jessell 2009; Dalla Torre di Sanguinetto et al. 2008).

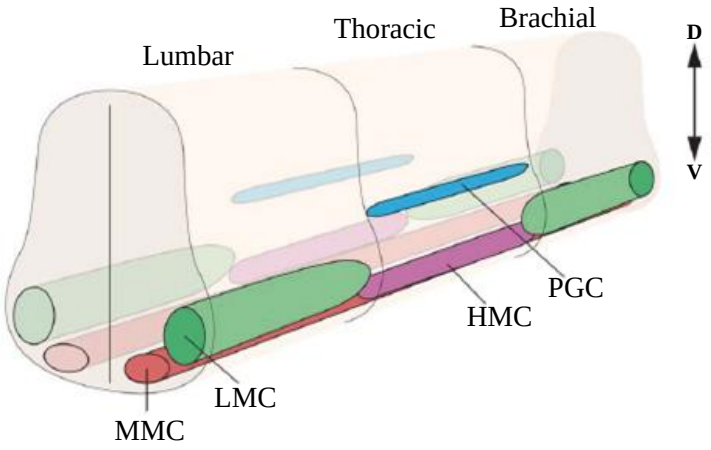

Figure 6. Schematic summarizing the segmental distribution of spinal motor columns: LMC MNs, innervating limb muscles are present in brachial and lumbar segments. PGC that contains visceral MNs are present in thoracic level, and HMC innervating the abdominal walls are also present at thoracic levels. MMC MNs, innervating proximal muscles are present all along the spinal cord (D-dorsal, V-ventral) (Allodi & Hedlund 2014).

Extracellular and intracellular signaling molecules such as neurotrophic factors or calcium are essential for the survival and functions of the neurons and non-neuronal cells of the nervous system.

1.2. Neurotrophic Factors (NTFs)

The neurotrophic factors (NTFs) are a small protein that bind to their specific receptors on the neuronal membrane and induce the activation of intracellular signaling pathways which, in turn, activate the expression of several genes associated with survival, differentiation or programmed cell death (PCD) control among others. NTFs exert survival-promoting and trophic actions first identified in embryonic neuronal cells. Throughout development, neurons require the presence of NTFs for survival and the precise establishment of neuronal populations (Purves et al. 1988; Schober & Unsicker 2001).

NTF support is essential for neurons in the spinal cord, and it is provided by different cellular sources, including astrocytes, microglia, neurons and endothelial cells. They are capable of regrowing damaged neurons during injury or degeneration (Hollis & Tuszynski 2011). Studies have shown that NTFs may protect neurons from death stimuli. In several neurodegenerative disease animal models, it has been observed that the benefits promoted by these neuroprotective factors; Parkinson´s disease (PD) (Alexi et al. 2000; Kordower et al. 2000), Huntington's disease (Alexi et al. 2000), Alzheimer's disease (AD) (Hefti 1994), Spinal Muscular Atrophy (SMA) (Simon et al. 2010), and Amyotrophic Lateral Sclerosis (ALS) (Manabe et al. 2002). A variety of NTFs families is involved in MNs survival, both *in vivo* and *in vitro* (Oppenheim 1991).

1.2.1. Neurotrophins

The neurotrophin family includes five members: Nerve Growth Factor (NGF), Brain- Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4/5 (NT-4/5), and Neurotrophin-7 (NT-7). The first known member of this family was NGF, which was discovered for their effect on survival and neurite outgrowth in the sensory neurons (Levi-montalcini & Angeletti 1968). Years later, BDNF was found (Barde et al. 1982), and then, NT-3, NT-4/5, and NT-7.

Neurotrophins, including BDNF, are initially synthesized as precursors or pro-neurotrophins, transformed to mature neurotrophins by protease cleavage (Mowla et al. 2001). These factors may perform its function by binding to two types of receptors, i) Tropomyosin-related kinase (Trk) which have three isoforms TrkA, TrkB, TrkC, and ii) p75 Neurotrophin receptor (p75 NTR). p75 NTR receptor have high affinity to all NTs in pro-form and low affinity with mature neurotrophins, whereas, Trk receptors have high specificity to address specific neurotrophins. For example, NGF preferentially binds to TrkA, BDNF and NT4/5 bind to TrkB, and NT-3 binds to TrkC (and TrkA to a lesser extent) **(Figure 7)**.

The two receptors (Trk and p75 NTR) can be expressed in the same cell to enhance the responses of neurons to neurotrophins (Miller & Kaplan 2001). Binding of neurotrophins with the Trk receptor causes its dimerization. These dimers phosphorylate each other and enhance the catalytic activity of the kinase to activate different signaling pathways **(Figure 8)**. Some of them are; PLCγ (Patapoutian & Reichardt 2001), Ras/MAPK (Marshall 1995) and PI3-K/Akt (Nguyen et al. 2009). By activating these pathways, the binding of neurotrophins to Trk promotes cell survival and differentiation. p75 NTR activation involves in both survival pathways via signaling PI3-K/Akt and NF-κB, and apoptosis via activation of the JNK pathway (Holgado-Madruga et al. 1997; Kaplan & Miller 2000; Du et al. 2006).

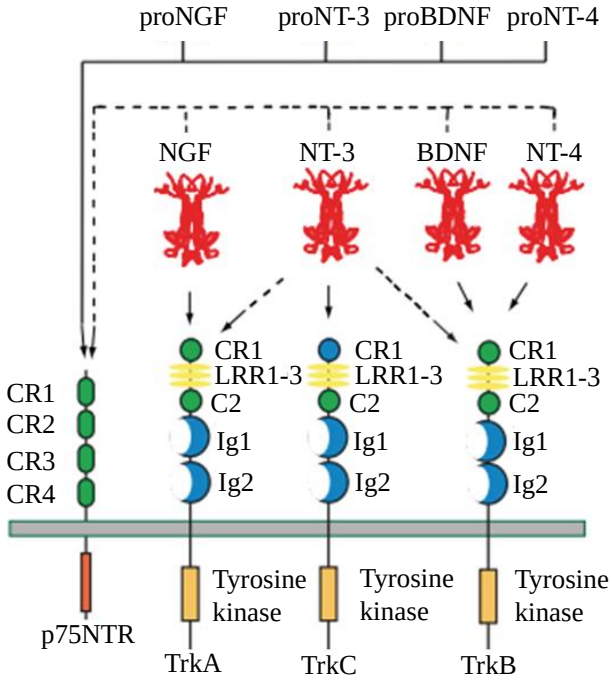

Figure 7. Neurotrophin–receptor interactions: All the proneurotrophins bind to p75NTR, but, not to the Trk receptors. p75NTR have low affinity to all the mature neurotrophins. Three isoforms of Trk receptors show specificity in interactions with the mature neurotrophin. NGF binds specifically to TrkA; BDNF and NT-4/5 recognize TrkB; NT-3 activates TrkC (Reichardt 2006).

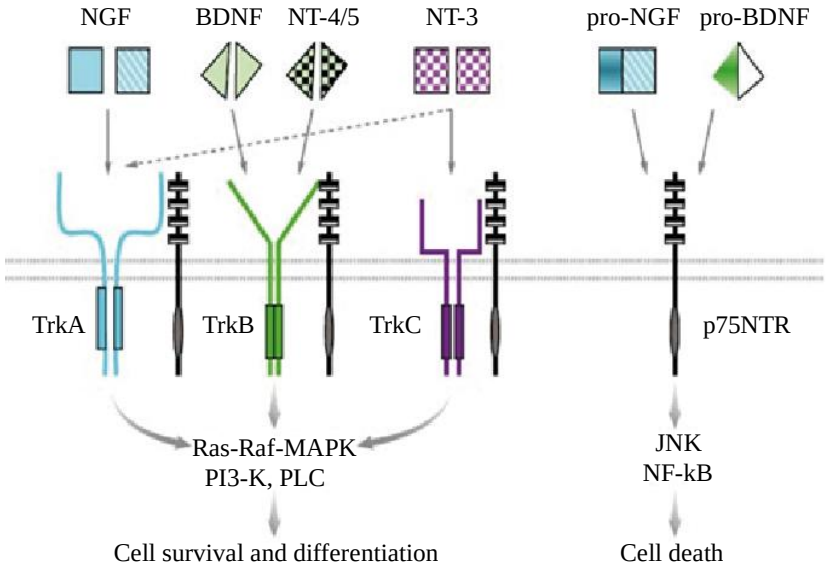

Figure 8. Pathways activated by Neurotrophins: The neurotrophins perform its functions by interacting with the two receptors Trk and p75 NTR. Through Trk receptor, neurotrophins activate PLCγ, Ras/MAPK, and PI3-K/Akt pathways. By binding with p75 NTR, neurotrophins activate JNK, PI3-K/Akt and NF-κB pathways (Pezet & McMahon 2006).

1.2.2. GDNF Family of Ligands (GFL)

The GDNF Family of Ligands (GFLs) consists of four NTFs: Glial cell line-Derived Neurotrophic Factor (GDNF), Neurturin (NRTN), Artemin (ARTN), and Persephin (PSPN).

GDNF was discovered in 1993 as a growth factor growth promoting survival of dopaminergic neurons (Lin et al. 1993). A year later, Henderson and colleagues described GDNF as a potent regulator of the survival of the MNs (Henderson et al. 1994). It has been shown that all GFLs regulate the survival and differentiation of several neuronal populations (Airaksinen & Saarma 2002).

GFLs are synthesized in the form of precursors, preproGFL and are transformed to mature forms by proteolytic cleavage. GLF share the receptor tyrosine kinase, RET. RET is activated only if the GFL is first bound to a novel class of proteins, known as GDNF family receptor-α (GFRα) receptors, which are linked to the plasma membrane by a Glycosylphosphatidylinositol (GPI) anchor. Four different GFRα receptors have been characterized (GFRα1, 2, 3 and 4), which determine the ligand specificity of GFRα-RET complex. GDNF binds to GFRα1 and then forms a complex with RET. NRTN binds to GFRα2, ARTN to GFRα3, and PSPN activates RET by binding to GFRα4. NRTN and ARTN might crosstalk weakly with GFRα1 and GDNF with GFRα2 and GFRα3. However, in mammals, at least PSPN can bind only to GFRα4 (Airaksinen et al. 1999; Baloh et al. 2000; Takahashi 2001; Lindahl et al. 2001). GDNF dimer first binds to either monomeric or dimeric GFRα1, and the GDNF–GFRα1 complex then interacts with two RET molecules, thereby inducing their homodimerization, and tyrosine autophosphorylation (Airaksinen et al. 1999). Once active, RET stimulates different signaling pathways which depend on specific adaptor proteins Src homologous and collagen-like protein (Shc) and Fibroblast Growth Factor Receptor Substrate 2 (FrS2) (Airaksinen & Saarma 2002). Activation of RET receptors directs neuronal survival by activating several intracellular signaling pathways like ERK/MAPK (Worby et al. 1996) and PI3-K/Akt (Soler et al. 1999; Gould et al. 2008). In MNs, the receptor for GDNF is expressed in the earlier period of PCD, and the depletion of GDNF during development causes the loss of 20-30% of the MNs (Gould et al. 2008) **(Figure 9).**

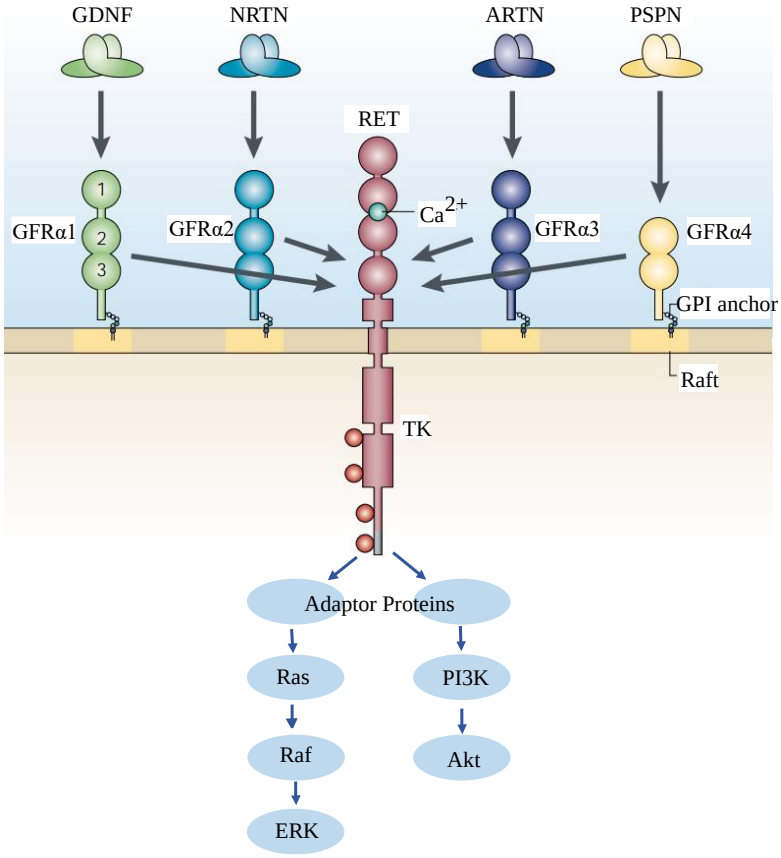

Figure 9. GDNF-family ligands and receptor interactions: GFLs activates the RET tyrosine kinase by first binding their cognate GFRα receptors. Arrows indicate the preferred ligand–receptor interactions. GFRα is attached to the plasma membrane by a GPI anchor, and they have three globular cysteine-rich domains (1, 2, 3). GFRα4 has only two domains. GFLs bind mainly to the second domain of GFRα receptors. Once activated, RET stimulates different signaling pathways like which depends on specific adaptor proteins Shc and (FrS2). RET activates pathways like ERK/MAPK, PI3-K/Akt signaling pathways (Airaksinen & Saarma 2002)**.**

1.2.3. Cytokines

The members of the Interleukin-6 (IL-6) family of cytokines involved in neuronal survival are, Ciliary Neurotrophic Factor (CNTF), Cardiotrophin-1 (CT-1), Leukemia Inhibitory Factor (LIF), and Cardiotrophin-Like Cytokine-1 (CLC). The cytokine family is described as one of the most important factors for *in vivo* and *in vitro* of MN survival (Arakawa et al. 1990; Sendtner et al. 2000). The members of this family act through their binding to a heterodimeric receptor composed by glycoprotein 130 (gp130) and tyrosine kinase receptor beta LIF (LIFRβ) (Hibi et al. 1996). Activation of these receptors (gp130/LIFRβ) requires co-activators; for CNTF and CLC the co-activator is Ciliary Neurotrophic Factor Receptor α (CNTFRα), and for CT-1 involves its binding to another co-receptor like CNTFRα, not yet identified. Once stimulated, the receptors activate different signaling pathways, including JAK/STAT, PI3-K/Akt, Ras/MAPK and NF-κB pathways by blocking the intrinsic pathway of apoptosis (Dolcet et al. 2001; Mincheva-Tasheva & Soler 2012; Gallagher et al. 2007) **(Figure 10)**.

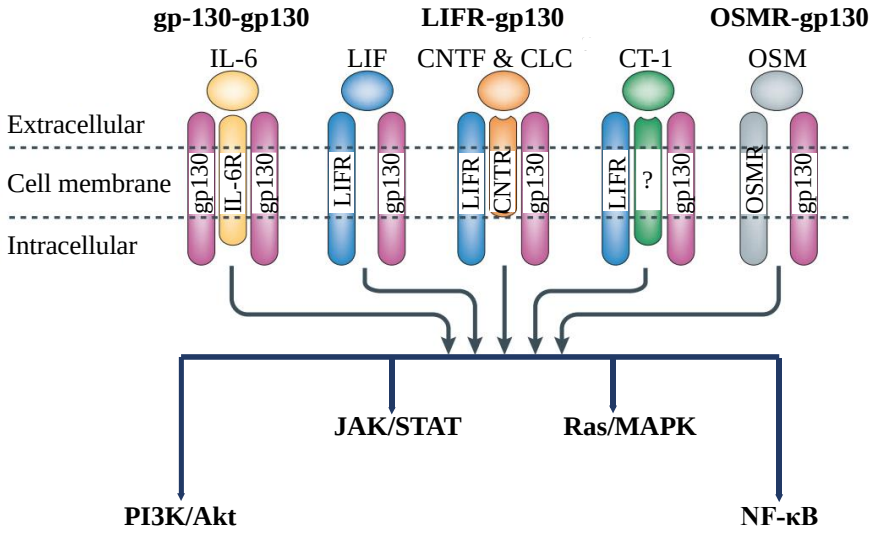

Figure 10. Cytokines and receptor interactions: The members of the cytokine family act by binding with a heterodimeric receptor composed of gp130 and LIFRβ. To get activated, these receptors (gp130/LIFRβ) requires co-activators; for CNTF and CLC, the co-activator is CNTFRα, whereas, for CT-1 another co-receptor like CNTFRα, not yet identified. Once activated, the receptors activate different signaling pathways, including JAK/STAT, PI3-K/Akt, Ras/MAPK and NF-κB pathways (Bauer et al. 2007).

CNTF was isolated as an NTF from the parasympathetic neurons of ciliary ganglion of chick embryos in 1979 (Varon et al. 1979). Later its trophic function was demonstrated in neurons and glial cells of the CNS and PNS (Vergara & Ramirez 2004). Unlike other NTFs, the CNTF is mainly expressed in the nervous tissue after PCD. CNTF synthesized by Schwann cells can be transported retrogradely to the spinal MNs (Sendtner et al. 2000). Studies in knockout mice for CNTF have shown that inhibition of CNTF does not affect the number of MNs during development, but causes atrophy and loss of a subpopulation of MNs after birth (Sendtner et al. 2000). Mice deficient for CNTFRα or gp130 receptors show a decline of 40% MNs during PCD (Burek & Oppenheim 1996). After axotomy, the neuroprotective effect of exogenous administration of CNTF has been shown in MNs (Sendtner et al. 1990). Moreover, the deletion of CNTF in a mouse model of mild SMA exacerbates the disease phenotype (Simon et al. 2010).

CT-1 was identified in 1996 as a factor which can induce hypertrophy of cardiomyocytes (Pennica et al. 1996). Later, CT-1 effects were observed on the survival of developing spinal MNs (Pennica et al., 1996b). Studies in knockout mice for CT-1 show 20-30% of the MNs during embryonic development (Holtmann et al. 2005). Unlike CNTF, CT-1 seems not essential for the survival of MNs in postnatal stages (Oppenheim et al. 2001), lack of CT-1 causes no loss of the MNs during postnatal period (Holtmann et al., 2005), however, the exogenous administration of CT-1 in a transgenic mouse model of ALS or in

murine models of SMA delays the onset of disease and modulates axonal degeneration respectively (Bordet et al. 2001).

1.2.4. Hepatocyte growth factor (HGF)

HGF is a member of the plasminogen-related growth factor family. HGF is involved in multiple actions like migration, proliferation, and differentiation of cells and tissues through its binding to the receptor c-Met (mesenchymal –epithelial transition), which is a proto-oncogene product with tyrosine kinase activity (Boros & Miller 1995). It was first identified as a potent mitogen for hepatocytes (Kinoshita et al. 1989). HGF/Met signaling enhance axonal growth and survival of peripheral sensory neurons both *in vitro* and *in vivo* (Maina et al. 1997). Binding of HGF to Met activates several intracellular pathways including the Ras/MAP kinases, FAK, PI3-K through interaction with Src tyrosine kinase and STAT transcription activation (Thompson et al. 2004) **(Figure 11)**.

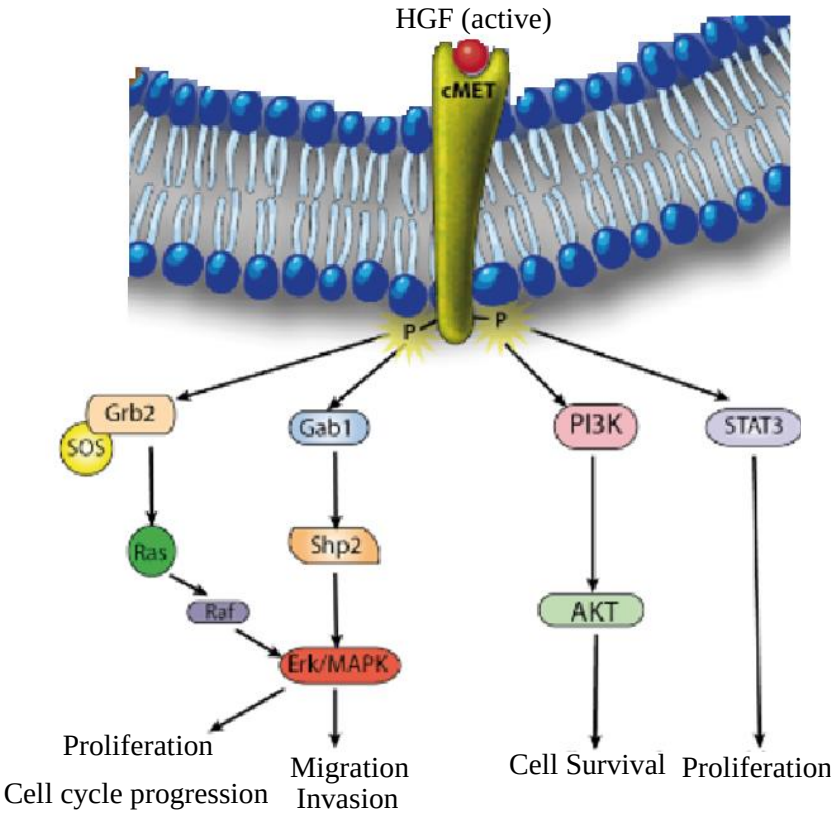

Figure 11. HGF and c-Met interactions: HGF activates several intracellular signaling pathways including the Ras/MAP kinases, FAK, PI3-K through interaction with Src tyrosine kinase and by STAT transcription activation by interacting with Met receptors (Faria et al. 2011).

Met receptor activates PI3-K and promotes outgrowth of particular MN axons *in vitro* (Maina et al. 2001). HGF is also involved in the development and axonal guiding of MNs (Wong et al. 1997) and promotes survival and axonal regeneration of MNs during spinal

cord injury (Kitamura et al. 2007; Wong et al. 2014). HGF regulates Ca^{2+} mediated neurodegeneration in PD (Liu et al. 2014). In CNS development, HGF stimulates oligodendrocyte precursor cells and neural precursors for proliferation and migration (Yan & Rivkees 2002; Garzotto et al. 2008).

A list of NTFs essential for the survival of MNs is given in **Table 1**. In our present work, MN culture is maintained using a cocktail of NTFs (BDNF, GDNF, CNTF and HGF).

NTFs essential for MN Survival
Neurotrophins
• Brain Derived Neurotrophic Factor **(BDNF)**
• Neurotrophin 3 (NT-3)
• Neurotrophin 4/5 (NT-4/5)
• Nerve Growth Factor (NGF)
GDNF Family ligands (GFL)
• Glial cell line-Derived Neurotrophic Factor **(GDNF)**
• Neurtrurin (NTN)
• Persephin (PSP)
• Artemin (ART)
Cytokines
• Ciliary Neurotrophic Factor **(CNTF)**
• Cardiotrophin-1 (CT-1)
• Leukemia inhibitory factor (LIF)
Others
• Hepatocyte Growth Factor **(HGF)**
• Insulin Growth Factor (IGF-1)
• Vascular Endothelial Growth Factor (VEGF)
• Pleiotrophin
• Neuropoietin
• Mullerian Inhibiting Substance (MIS)
• Growth Differentiation Factor-15 (GDF-15)
• Progranulin
• Angiogenin

Table 1: List of NTFs essential for MNs survival

NTFs, thus, by stimulating the intracellular signaling pathways, regulate cell survival by the activation or inhibition of gene expression that control neuronal physiology during development, adulthood and diseases (Airaksinen and Saarma 2002; Chao 2003). NF-κB signaling pathway is one among them.

1.3. Nuclear factor-kappa B (NF-κB)

The nuclear transcription factor kappa B (NF-κB) was discovered in 1986 in the laboratory of D. Baltimore as a transcription modulator of the light chain of B lymphocyte immunoglobulins (Sen & Baltimore 1986). Subsequent studies demonstrated that NF-κB is ubiquitously expressed dimeric transcription factor involved in the regulation of various cellular processes such as inflammation, adhesion, proliferation, differentiation, apoptosis and angiogenesis (Karin & Lin 2002).

1.3.1. NF-κB: Family members, Structure, and classification

NF-κB is a dimer composed of members of the Rel family of transcription factors: RelA (p65), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2) which can be classified into two subfamilies **(Figure 12),** i) Subfamily I include RelA, c-Rel, and RelB members with transactivation domain (TAD). TAD is required for the regulation, of the gene expressions (Hayden & Ghosh 2008), ii) Subfamily II includes the proteins p52, and p50 that lacks TAD and are unable to activate gene transcription after NF-κB activation. The proteins p52 and p50 are the mature form generated from their precursors p100, and p105, respectively.

Regarding its structure, all the NF-κB family members share a highly conserved N-300 amino acid terminal domain called Rel homology domain (RHD), and the nuclear localization sequence (NLS). RHD is responsible for the dimerization of NF-κB family members, the interaction between NF-κB and IκB, and the binding of NF-κB dimer with DNA (Baeuerle & Henkel 1994), whereas, the NLS is essential for the translocation of NF-κB into the nucleus (Ghosh et al. 1998).

NF-κB proteins form homo- or hetero-dimers and are retained in the cytosol in an inactive form. Even though it is possible to find different homo- or hetero-dimers of NF-κB members in mammalians, the most abundant form is the RelA/p50 heterodimer (Ghosh et al. 2002). Several stimuli like inflammation, infection, injury, oxidative stress, or NTFs can induce nuclear translocation of NF-κB. After migration into the nucleus, the NF-κB dimers bind to κB sites containing the consensus sequence GGGRNNYYCC (R=purine bases, Y=pyrimidine, and N=any base) in the promoter or enhancer regions of the target genes, therefore, regulating them by binding with co-activators or co-repressors (Mémet 2006; Hayden & Ghosh 2004; Hayden & Ghosh 2008).

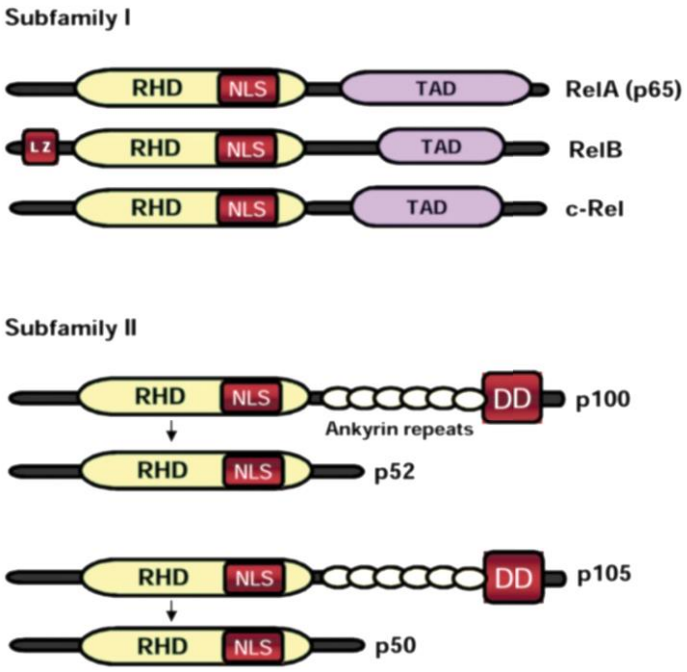

Figure 12. NF-κB Family Members: All the NF-κB family members share a highly conserved N-300 amino acid terminal domain called Rel homology domain (RHD), and the nuclear localization sequence (NLS). Subfamily I that includes the RelA, c-Rel, and RelB members consist of the transactivation domain (TAD), whereas Subfamily II that includes the proteins p52, and p50 lacks TAD domain (Mincheva-Tasheva & Soler 2012).

1.3.2. Inhibitors of NF-κB (IκB)

In the absence of stimuli, NF-κB homo- and/or heterodimers are predominantly retained in the cytoplasm, and form inactive complexes with their inhibitors, which are the members of the IκB (Inhibitor κB) protein family. However, in response to different stimuli, NF-κB dimers in the cytosol are released from its inhibitor and translocate to the nucleus where they bind to the κB sequences of the DNA and regulate the transcription of specific genes (Perkins 2006).

In mammals, six isoforms of inhibitory proteins (IκBα, IκBβ, IκBε, IκBγ, IκBδ, and Bcl-3 have been described. All of them share their three-dimensional structure and are distinguished by the number of ankyrin repeat motifs located in their amino-terminal segment, which is responsible for the IκB/NF-κB interaction (Baeuerle 1998). IκBα and IκBβ have a proline, glutamic acid/aspartic acid, serine and threonine residues rich domain (PEST domain) in their carboxyl-terminal which may interact directly with the DNA-binding region of one of the dimers of NF-κB subunits and is required for inhibition of DNA binding by the NF-κB dimers (Ernst et al. 1995) **(Figure 13).** The most frequent expressed form of NF-κB inhibitors in the nervous system (NS) is IκBα.

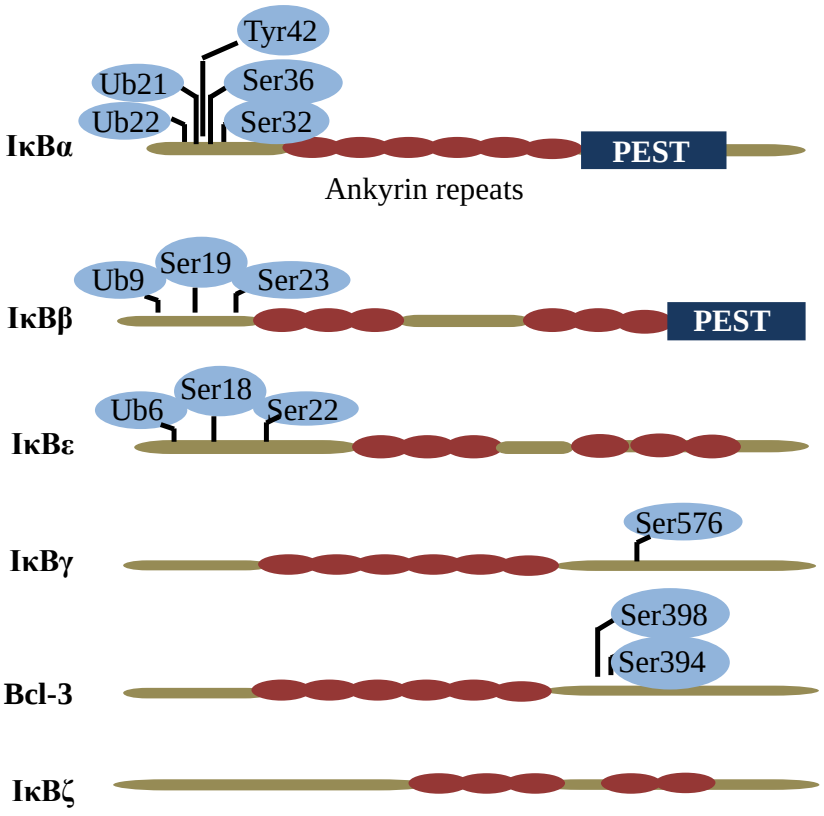

Figure 13. Members of the NF-kB inhibitors: The inhibitory κB (IκB) family consists of six members: IκBα, IκBβ, IκBε, IκBγ, IκBδ, and Bcl-3. They share the conserved ankyrin repeat motifs. IκBα and IκBβ have in their carboxyl-terminal end a PEST domain (Mincheva-Tasheva & Soler 2012).

The activation of NF-κB induces IκBα phosphorylation at Ser32 and Ser 36 residues of the ankyrin repeats which leads to the IκB release of the complex, polyubiquitination at Lys21 and Lys22 residues, and its degradation by the 26S proteasome (Chen et al. 1995). NLS in IκBα permits the localization of newly synthesized IκBα into the nucleus where IκBα binds with RelA/p50 dimers removing them from the κB DNA sequences. Later, the nuclear export sequence (NES) in IκBα promotes the translocation of this inactive complex from the nucleus to the cytoplasm (Tam et al. 2000). The reduction of the IκBα protein level in the cytoplasm is temporary since the reduced level of IκBα protein after the activation of the pathway increases one hour later in response to the newly synthesized IκBα (Place et al. 2001). This suggests that IκBα prevents the excessive activation of the pathway because it is one of the earliest genes transcribed after the entry of NF-κB to the nucleus (Ito et al. 1994). In some cell types, IκBα can be detached from NF-κB by phosphorylation at Tyr42, located in the ankyrin motifs (Takada et al. 2003). This phosphorylation induces the removal of IκBα from the complex, but, not its degradation by the proteasome. Thus, the IκBα protein level does not decrease early after Tyr42 phosphorylation (Imbert et al. 1996). The p100 and p105 precursors also contain several ankyrin repeats. When the pathway is activated, these ankyrin

repeats undergo proteasomal processing, and thus, these immature forms of p52 and p50 may also be considered as inhibitors of the NF-κB signaling pathway (May & Ghosh 1998).

1.3.3. IκB Kinases (IKKs)

Catalytic activation of a serine-threonine kinase complex called IκB kinases (IKKs) is required for the phosphorylation IκB. The IKK complex is composed of three subunits: two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO (also called IKKγ). IKKα and IKKβ contain a helix-loop-helix domain and a leucine-zipper domain (Woronicz 1997; Zandi et al. 1997; Häcker & Karin 2006). The helix-loop-helix domain is responsible for IKK homo- or hetero-dimerization and the leucine zipper domain is involved in the modulation of the kinase activity (Zandi et al. 1997) **(Figure 14)**. IKKα/IKKβ heterodimers activate the NF-κB pathway more efficiently than the IKK homodimers (Huynh et al. 2000). The IKKα and IKKβ catalytic domains show 65% homology, and their kinase activation is induced by the phosphorylation at two serine residues, i) Ser177/Ser181 of IKKβ, and ii) Ser176/Ser180 of IKKα (Kwak et al. 2000). IKKα and IKKβ contribute differently to IKK complex activation. Studies using IKKβ mutants demonstrated that this kinase is responsible for NF-κB activation after tumor necrosis factor-α (TNFα), interleukin-1 (IL-1) or lipopolysaccharide (LPS) stimulation (Delhase 1999).

The IKKs have different cellular distribution. IKKβ is distributed mainly in the cytoplasm, whereas, IKKα shows both cytoplasmic and nuclear localization due to the NLS sequence in the kinase domain. The cytoplasmic localization of IKKs is related to their ability to phosphorylate IκBα and the consequent RelA/p50 translocation to the nucleus. The presence of IKKα in the nucleus is related to the expression of NF-κB responsive genes through the phosphorylation and acetylation of histone 3 (Yamamoto et al. 2003). This suggests that IKKα and IKKβ kinases have different functions based on their cellular distribution.

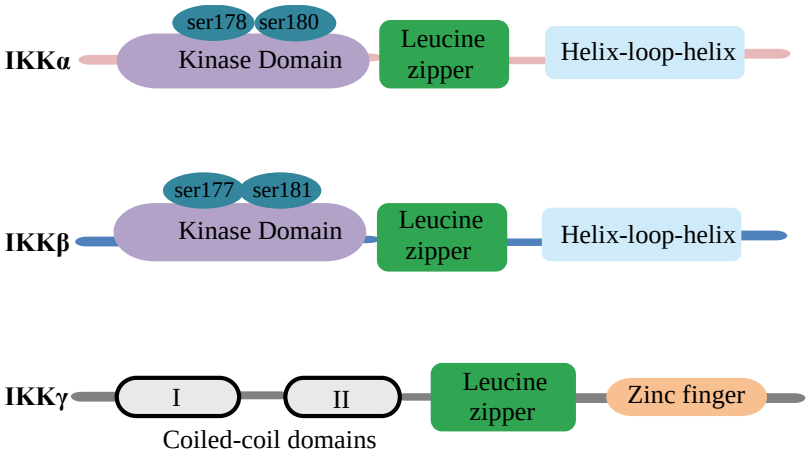

Figure 14. Members of the IκB kinases: The IKK complex is composed of three subunits: two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO (also called IKKγ). IKKα and IKKβ contain a kinase domain, a leucine zipper domain, and a helix-loop-helix domain. IKKα kinase domain contains the NLS responsible for its nuclear localization. The regulatory subunit IKKγ contains four domains—two coiled-coil, a leucine zipper, and a zinc finger domain (Mincheva-Tasheva & Soler 2012).

1.3.4. Activation of NF-κB Signaling Pathway

The NF-κB signaling pathway can be activated by three ways; i) Canonical (classical), ii) Non-canonical (alternative), and iii) Atypical pathways. Canonical and non-canonical pathways are IKK-dependent, but, atypical pathway is IKK-independent (Bender et al. 1998; Kato et al. 2003). Two main characteristics usually distinguish canonical and noncanonical pathways: i) the NF-κB dimer translocated to the nucleus (RelA/p50, and RelB/p52, respectively), and ii) the IκB contribution to their activation (IκB dependent and IκB independent, respectively) (Heissmeyer et al. 1999). However, both of them require the presence of the IKK complex for their activation. In contrast, the atypical pathway is IKK-independent, but IκB dependent, and induces RelA/p50 nuclear translocation (Perkins 2007).

1.3.4.1. Activation via Canonical Pathway

The canonical pathway is the most common form of NF-κB activation pathway in all cell types. This type is characterized by the activation of dimers composed of p50, and RelA or c-Rel. The canonical pathway is activated in response to stimuli such as pro-inflammatory cytokines (TNF-α, TNF-β, IL-1, CNTF, or CT-1) (Barger et al. 1995; Middleton et al. 2001; Sparacio et al. 1992), neurotrophins (Burke & Bothwell 2003), or oxygen-glucose deprivation (Sarnico, Lanzillotta, Boroni, et al. 2009). The activation of this pathway depends on the phosphorylation of IKKα, and IKKβ kinases at residues Ser181, and Ser180, respectively. Once activated, the IKK-complex phosphorylates IκBα (Ser32 and/or Ser36), promoting its degradation by the proteasome 26S, and also induces p105 phosphorylation. Phosphorylation of p105 promotes the generation of the mature form, p50 (Heissmeyer et al. 1999). RelA/p50 heterodimers are released from the IκBα inhibitor and

translocated to the nucleus, where they bind to the DNA κB sites and induce the activation or repression of specific genes **(Figure 15)**.

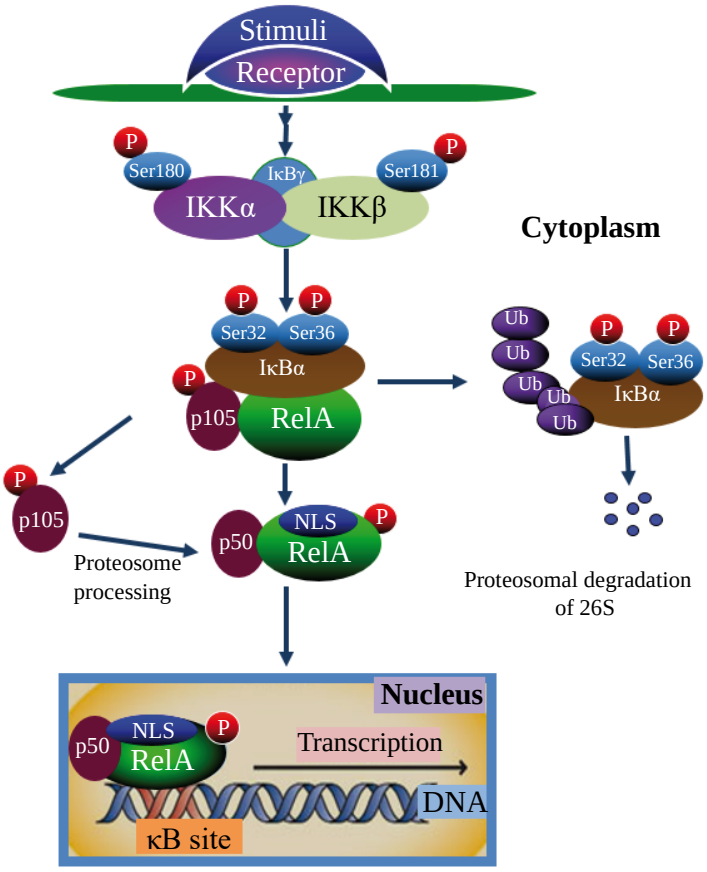

Figure 15. Schematic representation of Canonical pathway: The canonical pathway is IKK dependent, and the activation is mediated by IκB phosphorylation at serine or tyrosine residues, inducing IκB release of the complex (Ser293 or Tyr42). IκB phosphorylated at Ser293 is degraded by the proteasome or by the protease calpain, and Tyr42 phosphorylation induces IκB dissociation of the complex without protein degradation. IκBα release unmasks RelA nuclear localization sequence (NLS), promoting its translocation to the nucleus. Canonical activation induces gene expression or repression by the nuclear translocation of the RelA/p50 heterodimer (Mincheva-Tasheva & Soler 2012).

1.3.4.2Activation via Non-canonical Pathway

The non-canonical pathway is also known as the IκB independent pathway and is characterized by the translocation of the heterodimers, RelB/p52. This pathway is activated by a limited number of stimuli, including lymphotoxin B, CD40 ligand, LPS, and NTFs (Müller & Siebenlist 2003; Bhattacharyya et al. 2010). The activation of this pathway is mainly mediated by IKKα activity induced by the NF-κB-inducing kinase (NIK) (Bonizzi et al. 2004). IKKα phosphorylation leads to a polyubiquitination- dependent degradation of the p100 precursor to p52. The heterodimers RelB/p52 translocate to the nucleus, where, they bind to the DNA κB sites, and induce the activation or repression of specific genes which includes Cox-2, Cycline D, Mn-SOD, and Bcl-xL (Holley 2011; Jacque et al. 2005; Maehara et al. 2000; Zhang et al. 2007) **(Figure 16)**.

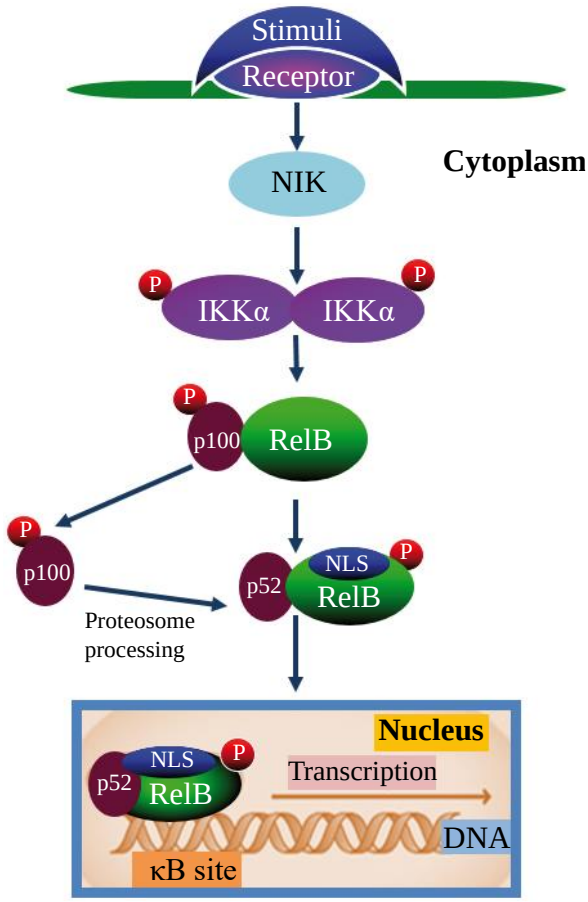

Figure 16. Schematic representation of Non-canonical pathway: The non-canonical pathway is IKK dependent, and IκB independent. The activation of this pathway is due to p100 phosphorylation induced by NF-κB-inducing kinase (NIK), and IKKα activity. The proteasome processing of p100 unmasks RelB nuclear localization sequence (NLS), promoting their translocation to the nucleus. Non-canonical activation induces gene expression or repression by the nuclear translocation of the RelB/p52 heterodimer (Mincheva-Tasheva & Soler 2012).

1.3.4.3. Activation via Atypical Pathway

The atypical pathway is IKK independent, but, IκBα dependent, and promotes RelA/p50 nuclear translocation which is initiated by tyrosine (Tyr42) phosphorylation at the N-terminus of the IκBα inhibitor or by serine phosphorylation at its PEST domain (Bender et al. 1998; Kato et al. 2003; Schwarz et al. 1996). In the nervous system, various stimuli cause the activation of this pathway such as hydrogen peroxide, erythropoietin, or neurotrophic factors. The atypical NF-κB pathway activated by IκBα phosphorylation at Tyr42 is mediated by Syk (spleen tyrosine kinase) in response to CNTF or NGF (Bui et al. 2001; Gallagher et al. 2007; Takada et al. 2003) or by members of the Src family of tyrosine kinases in response to BDNF stimulation (Gavaldà et al. 2004). This phosphorylation leads to the release of IκBα from the RelA/p50 dimer. IκBα can also be serine-phosphorylated by CKII (casein kinase II) at Ser293 located in the PEST domain (Schwarz et al. 1996). In contrast to the tyrosine phosphorylation of IκBα, serine phosphorylation promotes calpain-

mediated IκBα degradation (Wei et al. 2009). Released IκBα is not degraded by the proteasome as it occurs during canonical pathway activation (Bui et al. 2001; Takada et al. 2003) **(Figure 17).**

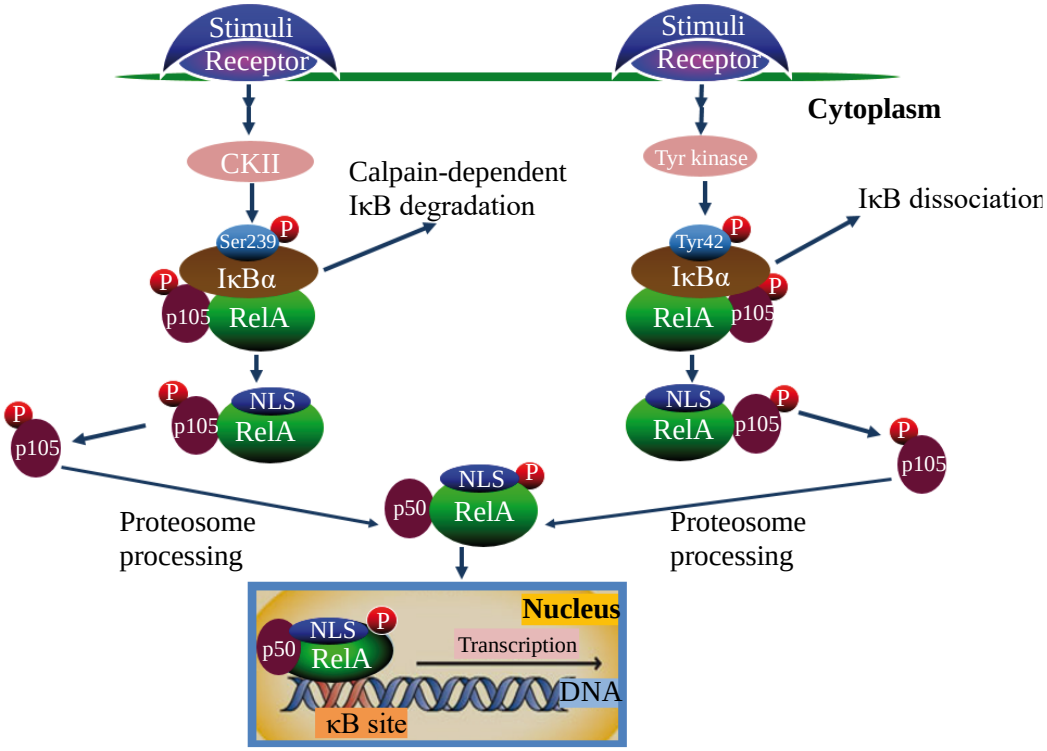

Figure 17. Schematic representation of Atypical pathway: Atypical pathway is IKK independent, and depends on casein kinase II (CKII) or tyrosine kinases. The activation is mediated by IκB phosphorylation at serine or tyrosine residues, inducing IκB release of the complex (Ser293 or Tyr42). IκB phosphorylated at Ser293 is degraded by the proteasome or by the protease calpain, and Tyr42 phosphorylation induces IκB dissociation of the complex without protein degradation. IκBα release unmasks RelA nuclear localization sequence (NLS), promoting its translocation to the nucleus. Atypical activation induces gene expression or repression by the nuclear translocation of the RelA/p50 heterodimer (Mincheva-Tasheva & Soler 2012).

1.3.5. Regulation of the Transcriptional Activity of NF-κB

In addition to IκBα inhibitor degradation, several steps are required to control the NF-κB-mediated gene expression. Several studies have described post-translational modifications of the NF-κB members can modulate the activity of NF-κB pathway. These amendments can be directly induced by the members of the pathway or by different signaling pathways (Hayden & Ghosh 2008). Post-translational modifications of the NF-κB members RelA, RelB, and c-Rel that contains TAD domain affects both RHD and TAD domains.

The RelA can be phosphorylated on different serine residues by different kinases. In RHD domain, Ser276 is phosphorylated by PKA, MSK1, and MSK2 (mitogen- and stress-activated protein kinase 1 and 2) and Ser311 by PKCξ. Phosphorylation at Ser276 promotes RelA interaction with the transcriptional co-activators CBP (CREB binding protein) and

p300 (Olson et al. 2007; Zhong et al. 1998). PKA and MSK kinases are in turn regulated by the ERK/MAPK signaling pathway in response to different stimuli (Vermeulen et al. 2003).

In TAD domain, Ser468 is phosphorylated by the GSK-3B kinase predominantly within the nucleus (Buss et al. 2004; Schwabe & Brenner 2002). When RelA is liberated from the IκBα inhibitor in response to IL-1 or TNF-α, Ser529 is phosphorylated by the CKII kinase (D. Wang et al. 2000). In response to cytokines and mediated by the PI3-K/Akt pathway Ser536 is phosphorylated by IKKs (Gutierrez et al. 2008a; Sizemore et al. 2002). CAMKIV phosphorylates Ser535 (Bae et al. 2003).

The RelA can be acetylated at different lysine residues which are reversible. This acetylation regulates different biological activities of the NF-κB complex. Acetylation at Lys310, Lys314, and Lys315 is essential for full transcriptional RelA activity (Chen et al. 2005; Rothgiesser, Erener, et al. 2010; Rothgiesser, Fey, et al. 2010), whereas, acetylation at Lys122 and Lys123 exerts adverse effects on NF-κB mediated transcription (Kiernan et al. 2003) **(Figure 18)**.

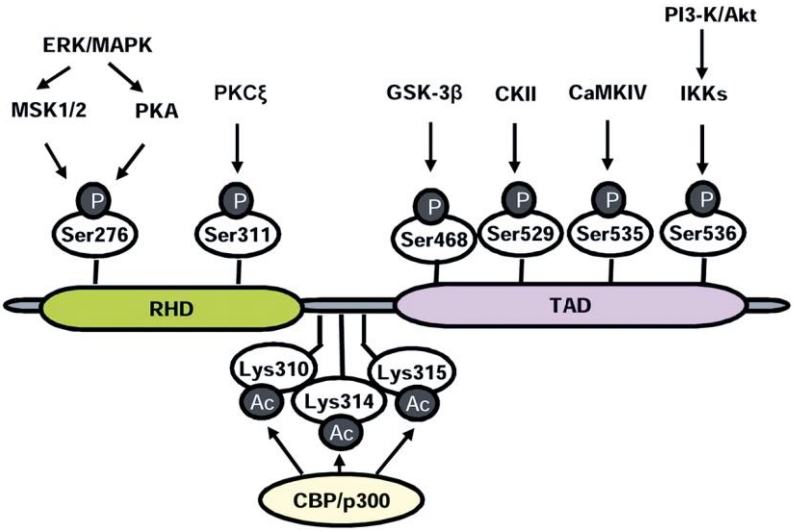

Figure 18. Post-translational modifications of RelA: Several posttranslational modifications induce increased RelA transcriptional activity. Phosphorylation at different serine residues located in Rel homology domain (RHD) or in transcriptional activation domain (TAD) is mediated by protein kinases of various families (Mincheva-Tasheva & Soler 2012)

1.3.5.1. Crosstalk between NF-κB and other Transcription factors

IKKs and/or NF-κB can regulate or can be regulated by some transcription factors. Tumor suppressor p53 can promote NF-κB activation (Bohuslav et al. 2004), and RelA can also activate p53 promoter in response to stress and suppresses cell growth (Lozanos 1994).

In microglial cells, during oxygen-glucose deprivation (OGD) interaction between NF-κB, and FOXO3a has been documented (Shang et al. 2010), and by knockdown strategies, these studies pointed out that FoxO3a reduction enables p65 translocation to the nucleus. Also, NF-κB controls PKA expression, and consequently CREB activation (Kaltschmidt et al. 2006).

1.3.6. Knockout Mice for Different Members of NF-κB Signaling Pathway

Knockout mice studies for various genes of the NF-κB pathway allows us to understand the role of these proteins in the physiological processes related to development. Some major knockout genotypes and their phenotypes are explained below, and rest of the genotypes and phenotypes are listed in **Table 2.**

RelA-/-

RelA knockout mice die during embryonic development (E15-16) due to massive liver apoptosis (Beg et al. 1995). The lack of RelA also show defects in the nervous system, like reduced survival of nodose neurons in response to CNTF, and CT-1 cytokines (Middleton et al. 2000), compromises peripheral myelin formation by Schwann cells (Nickols et al. 2003), and promotes the loss of axonal growth in cortical neurons at E15 (Sole et al. 2004). This suggests the role of RelA in peripheral nervous system development, affecting both neuronal, and non-neuronal cells. Different knockdown strategies have been generated using embryonic neuronal models to determine the function of RelA during nervous system development. Subsequent studies have demonstrated that RelA is also involved in neuronal survival of sensory neurons, and spinal cord MNs in response to NTFs (Hamanoue et al. 1999; Mincheva et al. 2011).

IKKα-/-

IKKα knockout mice die shortly after birth by abnormalities in the skin, and skeletal muscles, caused by blocking the differentiation of keratinocytes (Qiutang Li et al. 1999a). Detailed studies demonstrated that inactivation of the NLS sequence in the kinase domain of IKKα inhibits differentiation, indicating the primary function of IKKα in keratinocytes (Sil et al. 2004). No defects during nervous system development have been described in these mice.

IKKβ-/-

IKKβ knockout embryos die between E12.5, and E13.5 days because of a massive liver apoptosis due to the reduction of RelA activation in the liver cells (Q Li et al. 1999b). In the nervous system, the specific deletion of IKKβ in sensory neurons of the dorsal root

ganglia demonstrated that IKKβ is a negative modulator of sensory neuron excitability (Bockhart et al. 2009).

IKKγ-/-

IKKγ knockout mice die during embryogenesis, characterized by a massive hepatic apoptosis induced by TNF stimulation (Rudolph et al. 2000), and impaired B-cells development (Kim et al. 2003). These mice show no defects in nervous system development.

Knockout mice	Phenotype/Alterations	References
RelB-/-	Multiple pathological lesions; defects in the development of T-cell death	(Weih et al. 1995; Yilmaz et al. 2003)
c-Rel-/-	Defects in lymphocyte proliferation, humoral immunity, neuronal survival, and synaptic plasticity	(Ahn et al. 2008; Grumont et al. 1998; Kontgen et al. 1995; Pizzi et al. 2002; Deenick et al. 2010)
p50-/-	Defects in the immune response; neuronal degeneration	(Lu et al. 2006; Sha et al. 1995; Yu et al. 1999; Yu et al. 2000)
p52-/-	Lymphatic nodes abnormality and defects in T-cell response	(Beinke & Ley 2004; Franzoso et al. 1998)
IKKα-/-/**IKKβ**-/-	excessive apoptosis in the neural tube, spinal cord and dorsal root ganglia, abnormalities in neural tube closure	(Li et al. 2000)
RelA-/-/**p50**-/-	Death at E13; defects in B-cell development	(Franzoso et al. 1997; Franzoso et al. 1998; Grossmann et al. 2000; Horwitz et al. 1997; Ahn et al. 2008; Lo et al. 2006; Weih et al. 1995)
p50-/-/**p52**-/-	Growth retardation and craniofacial abnormalities; serious defects in the development of secondary lymphoid organs	(Franzoso et al. 1997; Lo et al. 2006)
RelB-/-/**p50**-/-	The phenotype resembles RelB knockout mice	(Weih et al. 1997)
RelA-/-/**c-Rel**-/-	Death at E13; reduction in the number of B-cell, and monocytes; increase in granulocitos, and increase in cell cycle by inhibiting T-cells	(Grossmann et al. 2000)
c-Rel-/-/**p50**-/-	Severe defects in the immune system	(Pohl et al. 2002)

Table 2. Phenotype Characteristics of Knockout Mice of NF-κB Pathway Members (Mincheva-Tasheva & Soler 2012)

1.3.7. NF-κB in the Nervous system

The presence of RelA/p50 heterodimers in astrocytes (Sparacio et al. 1992), Schwann cells (Carter et al. 1996), microglia (Nakajima & Kohsaka 1998), and neurons (Kaltschmidt et al. 1993; Meffert et al. 2003; Schmidt-Ullrich et al. 1996) in several regions

of the developing, and adult nervous system suggests the involvement of NF-κB in physiological processes of the nervous system during development and during adult life.

1.3.7.1. Activators of the NF-κB Signaling Pathway in Nervous system

A wide range of molecules can activate the NF-κB pathway in both neuronal, and non-neuronal cells. The physiological response of these cells depends on the stimulus and the cellular origin. The stimulus can cause positive effects like survival, neurite outgrowth, differentiation, proliferation, and plasticity on neuronal, and non-neuronal cells or can also induce "negative effects" such as cell death, and toxicity (Akama et al. 1998; Bauer et al. 1997; Grilli & Memo 1999; Kitaoka et al. 2004; Koo et al. 2010; Mir et al. 2008; Shou et al. 2002; Stephenson et al. 2000; Vollgraf et al. 1999; Xu et al. 2001) **(Table 3).**

Activators	Cell type	Effects	Reference
CNTF	Sensory neurons	Survival and neurite growth	(Gallagher et al. 2007)
CT-1	Sensory neurons	Survival	(Middleton et al. 2000)
GDNF	Astrocytes	Neuroprotection in cerebral ischemia	(Chu et al. 2008)
BDNF	Neurons	Survival and neurite growth	(Gutierrez et al. 2005)
	PC12	Neurite outgrowth	(Sole et al. 2004)
NGF	Neurons	Survival	(Maggirwar et al. 1998)
	Schwann cells	Myelin formation	(Carter et al. 1996)
IGF-1	Neurons	Survival	(Heck et al. 1999)
NTFs cocktail	Neurons	Survival	(Mincheva et al. 2011)
TNF-α	Neurons	Synaptic plasticity	(Snow et al. 2014)
cAMP	Schwann cell	Myelin formation	(Yoon et al. 2008)
TNF-α	Neurons; glia	Neuronal degeneration and death	(Mir et al. 2008)
Focal cerebral ischemia	Neurons	Neuronal death	(Stephenson et al. 2000)
Amyloid	Astrocytes	NO production increased	(Akama et al. 1998)
	oligodendrocytes	apoptosis	(Xu et al. 2001)
NMDA	Neurons	Apoptotic death	(Kitaoka et al. 2004)
LPS	Microglia	Cox-2 expression	(Bauer et al. 1997)
Kainic acid	Neurons	Apoptotic death	(Nakai et al. 2000)
Oxidative stress	Neurons	Neuronal death	(Shou et al. 2002)
Glutamate	Neurons	Apoptotic death	(Grilli & Memo 1999)
IL-1β	Neurons	Depressive-like behaviors	(Koo et al. 2010)
H_2O_2	Oligodendrocytes	Cell death	(Vollgraf et al. 1999)

Table 3. Neuroprotection and Neuronal Degeneration Induced by NF-κB Pathway Activation in the Nervous System (Mincheva-Tasheva & Soler 2012).

1.3.7.2. Genes and Proteins Regulated by NF-κB Signaling Pathway in the Nervous system

The survival of neurons can be controlled by NF-κB signaling pathway. As we discussed before, the activation or inhibition of several pro- or anti-apoptotic genes depends

on the nature of the activator of this pathway **(Table 4).** For example, Bcl-2 and Bcl-x_L promoters have binding sites for NF-κB dimers (Tamatani et al. 1999) or NF-κB positively regulates cIAP gene expression (Baud et al. 2001; Criollo et al. 2010). When NF-κB is activated, the expression level of TNFR1/2 adaptor proteins TRAF1 and TRAF2 is elevated. TRAF1 and TRAF2 themselves can induce IKK phosphorylation, which in turn activates the NF-κB pathway (Häcker & Karin 2006; Wang et al. 1998). NF-κB pathway can also regulate the expression level of Survival motor neuron (Smn) protein which is essential for MN physiology (Mincheva et al. 2011).

Target Genes	Pathway	Cellular Function	References
Bcl-2, Bcl-x	Canonical pathway	Anti-apoptotic	(Tamatani et al. 1999)
Bcl-xL	Atypical,Canonical,Non-canonical pathway	Anti-apoptotic	(Bui et al. 2001)
c-FLIP	Unknown information	Anti-apoptotic	(Chu et al. 1997)
TRAF1,2	Unknown information	Anti-apoptotic	(Wang et al. 1998)
IAPs	Canonical pathway	Anti-apoptotic	(Chu et al. 1997)
Bax,Bcl-xs	Unknown information	Pro-apoptotic	(Shou et al. 2002)
Bim, Nova	Canonical pathway	Pro-apoptotic	(Inta et al. 2006)
Smn	Canonical pathway	Survival Motor Neuron	(Mincheva et al. 2011)
CREB	Canonical pathway	Transcription factor	(Mincheva et al. 2011)
p53	Canonical pathway	Transcription factor	(Lozanos 1994)
STAT2	Unknown information	Transcription factor	(Khorooshi et al. 2008)
c-Myc	Canonical pathway	Transcription factor	(Qin et al. 1999)
Mn-SOD	Canonical pathway	Antioxidant enzyme	(Maehara et al. 2000)
Cu/Zn-SOD	Canonical pathway	Antioxidant enzyme	(Rojo et al. 2004)
Cox-2	Non-canonical pathway	Enzyme responsible for inflammation	(Kaltschmidt et al. 2002)
iNOS	Canonical pathway	Nitric oxide synthesis	(Xie et al. 1994)
IkBα	Canonical pathway	NF-κB inhibitor	(Bui et al. 2001)
BDNF	Canonical pathway	Neurotrophic factor	(Saha et al. 2006)
NCAM	Canonical pathway	Neural adhesion molecule	(Simpson 2000)
Cyclin D1	Non-canonical pathway	Cell cycle regulator	(Liang et al. 2007)

Table 4. Target Genes Regulated by NF-κB Pathways, and their Cellular Effects in the Nervous System (Mincheva-Tasheva & Soler 2012)

NF-κB signaling pathway can also promote apoptosis, and cell death in the nervous system (Kaltschmidt et al. 2000; Pizzi et al. 2002; Kaltschmidt et al. 2002). In response to oxidative stress, NMDA receptor activation or apoptotic stimuli, NF-κB pathway increases the expression of the pro-apoptotic genes, Bax and Bcl-xl in cortical neurons (Shou et al. 2002). In some diseases, such as cerebral ischemia, RelA increases the expression of Bim and Noxa pro-apoptotic genes (Inta et al. 2006). Even though nitric oxide participation in neuronal apoptosis is still under debate, the transcriptional control of the inducible nitric oxide synthase (iNOS) is also regulated by NF-κB (Xie et al. 1994). The cell cycle regulator

Cyclin D is also controlled by NF-κB and has been related to neuronal apoptosis in striate, and cortical neurons (Liang et al. 2008).

The NF-κB regulates the neuronal homeostasis by controlling the transcription of antioxidant enzymes (Maehara et al. 2000; Rojo et al. 2004), adhesion molecules (Simpson 2000), transcription factors (Khorooshi et al. 2008; Qin et al. 1999; Lozanos 1994), and NTFs (Saha et al. 2006).

1.3.7.3. Role of NF-κB Signaling Pathway in the Nervous System

In the nervous system, NF-κB pathway regulates neuronal survival, neurite outgrowth, and synaptic plasticity. NF-κB signaling pathway plays a vital role in the regulation of apoptotic cell death during neuronal development. In CNS, NF-κB transcriptional activity is detected during embryogenesis particularly in the spinal cord, and in the brain from day E12.5 (Schmidt-Ullrich et al. 1996). Inhibition of different members NF-κB pathway causes embryonic death at various stages of embryonic development. IKKα and IKKβ double knockout cause defects in neural tube closure due to massive apoptosis of the neuroepithelium (Li et al. 2000). NF-κB activity in the spinal cord is first observed at the beginning of MN-NTF dependence and PCD (Schmidt-Ullrich et al. 1996; Yeo & Gautier 2004). In embryonic spinal cord MNs, the reduction of some NF-κB pathway members causes apoptotic cell death even in the presence of NTFs (Mincheva et al. 2011). Studies using different neuronal models including PC12 cells (Azoitei et al. 2005; Sole et al. 2004), sensory neurons (Gallagher et al. 2007; Gutierrez et al. 2005; Gutierrez et al. 2008a), and hippocampal neurons (Sanchez-Ponce et al. 2011), have reported that blockade of NF-κB reduces neurite length and branching.

In the mature nervous system, NF-κB plays a vital role in synaptic signaling and learning (Kaltschmidt & Kaltschmidt 2009). Independent studies have shown that RelA, c-Rel, and p50 involves the formation of long-term memory (Meffert et al. 2003; Mattson 2005). RelA/TNFR1, double knockout mice, have defects in spatial learning memory; c-RelA knockout mice have impaired hippocampus-dependent memory formation (Ahn et al. 2008), and p50 knockout presents deficit in short-term memory (Denis-Donini et al. 2008). In these processes, the importance of NF-κB may be related to the presence and function of NF-κB family members in the pre- and post-synaptic space, which contributes to transduction of synaptic signals to transcriptional changes (Meffert et al. 2003). NF-κB controls synaptic plasticity by regulating the expression of the catalytic subunit of PKA, an essential memory regulator, and phosphorylation of CREB (Kaltschmidt et al. 2006).

1.3.7.4. NF-κB Signaling Pathway and Neurodegenerative Disorders

During development, many signal transduction processes regulate neuronal survival and differentiation. To know the molecular mechanisms involved in the pathology of neurodegenerative diseases, it is important to understand these signal transduction processes. The NF-κB signaling cascade is one among them. Several studies indicate an alteration in NF-κB activity in neurodegenerative diseases such as AD, PD, HD, and ALS, and in processes related to injury or ischemia (Mémet 2006). However, some authors suggest that the activation of NF-κB exerts a neuroprotective role in neurodegenerative diseases (Barkett & Gilmore 1999; Cardoso & Oliveira 2003; Fridmacher et al. 2003; Smith Darrell 2009), while, others indicate that it would be a cause of neuronal death (Mémet 2006; Meunier et al. 2007; Julius et al. 2008).

1.3.8. Hypothesis behind the Controversial Role of NF-κB

Two hypotheses have been generated to illustrate the different roles of NF-κB pathway in neuronal damage, and disorders. Kaltschmidt proposed the model for NF-κB homeostasis (Kaltschmidt et al. 2005). This model defends the principle that the maintenance of low or high levels of NF-κB activation over an extended duration of time (weeks to months) cause neuronal death. Under physiological conditions, nuclear RelA promotes survival by activating the transcription of anti-apoptotic genes through its binding to co-activators. The maintenance of physiological NF-κB activation depends on IκB protein expression that is responsible for bringing RelA back to the cytoplasm and deactivating the pathway. However, in pathological conditions dysregulation is observed in the activation of the NF-κB pathway which results in the interaction of RelA with histone co-repressors deacetylases (HDAC1, and HDAC2), and downregulating the expression of anti-apoptotic genes (Kaltschmidt et al. 2005; Ashburner et al. 2001; Kaltschmidt et al. 2000).

In 2001, Mattson and Camandola proposed another hypothesis suggesting that NF-κB activation in neurons induces anti-apoptotic genes that mediate cell survival, whereas, NF-κB activation in glial cells results in the production of proinflammatory cytokines that mediate neuronal death (Mattson & Camandola 2001). Thus, the same stimulus produces different responses by activating NF-κB in various cell types. One of the cytokines that can be generated by glial cells is TNFα. Binding of TNFα to TNFR1 and TNFR2 receptor activates the NF-κB pathway. TNFR1 activation in glial cells induces the production of nitric oxide, which may lead to neuronal death, whereas, TNFR2 activation in neurons increases the expression of anti-apoptotic genes.

1.4. Spinal Muscular Atrophy (SMA)

Spinal Muscular Atrophy (SMA) is a severe fatal autosomal recessive neurodegenerative neuromuscular disease characterized by the degeneration of α-MNs in the ventral horn of the spinal cord, resulting in progressive proximal muscle weakness, and paralysis. SMA is the leading cause of infantile mortality after cystic fibrosis with an estimated incidence of 1 in 6,000 to 1 in 10,000 live births, with a carrier frequency of 1/40-1/60 (Pearn 1978b; Ogino et al. 2002; Feldkötter et al. 2002). SMA is caused by mutations in the *Survival Motor Neuron 1* (*SMN1*) gene and decreased SMN protein. SMA was first described by Werdnig (Werdnig 1981), and by Hoffmann (Hoffmann 1893).

1.4.1. Genetics of SMA

SMN1 was found to be highly conserved among all organisms (Miguel-Aliaga et al. 1999; Paushkin et al. 2000) which lie within the telomeric part on chromosome 5q13. The SMA locus is characterized by a 500 kb inverted duplication genomic region (Brzustowicz et al. 1990) **(Figure 19)**. *SMN1* gene consists of a 28 kb genomic region and comprises nine exons (1, 2a, 2b, 3, 4, 5, 6, 7, 8). The coding sequence of *SMN1* is 882 bp and produces a ~1.7 kb transcript (together with 5´, and 3´ UTR) which is ubiquitously expressed, but, unusually high in the spinal cord. The smallest deletions involving the telomeric copy of *SMN1*, and the presence of intragenic mutations of *SMN1* in patients, including missense, non-sense or splice site mutations have determined *SMN1* as the SMA disease causative gene (Lefebvre 1995).

Figure 19. 500 kb telomeric, and the centromeric region containing *SMN1*, and its duplicated version *SMN2*, respectively.

In the centromeric region, *SMN1* is duplicated with a nearly identical copy, termed *SMN2*. *SMN2* lies within a highly repetitive genomic region prone to rearrangements. Therefore, the *SMN2* copy number is variable among the population. The *SMN2* gene is present in all SMA patients but unable to compensate the defects for the *SMN1* gene. *SMN2* differs from *SMN1* by only five nucleotides without any effect on the amino acid sequence; A synonymous mutation in exon 7 (c.*C*280*T* in *SMN2*), exon 8 (nt 27869 *G*>*A*), intron 6 (nt 27092 *G*>*A*) and another two in Intron 7 (nt 27289 *A*>*G* and 27404 *A*>*G*) (Lefebvre 1995; Burglen, L. et al., 1996).

SMN1 and *SMN2* have distinct splicing patterns. Full-length transcript (*FL-SMN*) containing exon 7 are almost exclusively produced by *SMN1*, whereas, the additional transcript lacking exon 7 (Δ7SMN2) is found in humans carrying both *SMN1*, and *SMN2*, (Lefebvre 1995). The nucleotides located in exon 7 are responsible for the alternative splicing of exon 7 which is peculiar to the *SMN2* transcripts. The c.*C*280*T* transition in exon 7 of *SMN2* causes the disruption of an exonic splicing enhancer (ESE) (Lorson et al. 1999; Cartegni & Krainer 2002) resulting in exon 7 skipping in about 90 % of the total transcripts. The skipping of exon 7 leads to a frame shift and an alternative stop codon in exon 8. This skipping leads to synthesize a truncated protein (Δ7SMN2) of reduced oligomerization capacity, and stability (Lorson et al. 1998) **(Figure 20A)**. The 10% of *SMN2* transcripts are still full length. Due to this 10% full-length *SMN2* transcripts, the *SMN2* copy number is inversely correlated with the disease phenotype, and prognosis (Burghes 1997; Brahe 2000; Feldkötter et al. 2002).

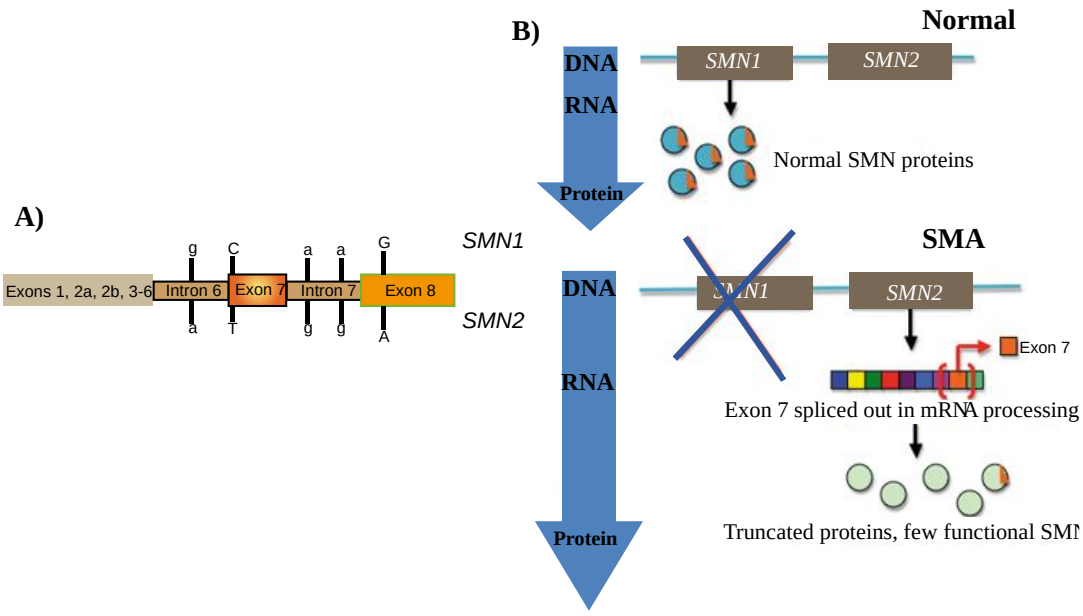

Figure 20. A) A human chromosome consists of two identical *SMN* gene, one copy of *SMN1*, and multiple copies of *SMN2*. *SMN1* differ from *SMN2*; *C* to *T* transition in the sixth position of exon 7. Due to this, change exon 7 is spliced during pre-mRNA transcription, and *SMN2* is unable to synthesis fully functional protein (Wirth 2000). **B)** *SMN1*, and *SMN2* in normal and with SMA.

1.4.2. **Classification of SMA**

Clinically SMA is classified into five types **(Table 5).**

Type	Age of Onset	Highest Function	Natural Age of Death
Type 0 (very severe)	Neonatal with prenatal signs	Never sits or walks	Without any intervention, no survival beyond the first month of life
Type1 (severe)	0–6 months	Never sits or walks	If untreated, life expectancy < 2 years
Type 2 (intermediate)	7–18 months	Sits, some stands, but, never walks	Survival into adulthood
Type 3 (mild)	>18 months	Stands, and walks	Survival into adulthood
Type 4 (mild)	Second or third decade	Walks during adult years	Survival into adulthood

Table 5. Classification of SMA (Castro & Iannaccone 2014).

Type 0 SMA

Type 0 SMA is the most severe form of SMA. Reduced fetal movements in the later stages of pregnancy, little ability to move, lack of respiratory efforts, and difficulty in swallowing independently are the marked symptoms. Infants with Type 0 SMA often die before the age of 6 months (Brachial 2014).

Type I SMA (Werdnig-Hoffmann disease)

Type I SMA is the severe type of SMA, and it accounts for ~50 % of all SMA cases (Werdnig 1981; Pearn 1978a; Markowitz 2004). The onset of the disease is between the birth, and before the sixth postnatal month. Children will never be able to sit independently and shows strong hypotonia of the musculature. The survival of patients with disease onset in the first three months was determined to ~7 months. The survival rate of patients with the disease onset of more than two years is ~6 % (Cobben et al. 2008; Rudnik-Schöneborn et al. 2009).

Type II SMA (Intermediate form)

The onset of Type II SMA is typically diagnosed between six to eighteen months of age. These patients can sit, but, may never become independently ambulatory. The prognosis is extremely variable in SMA Type II patients and depends on to a large extent on the degree of respiratory muscle involvement, and the problems associated with the development of kyphoscoliosis (Talbot 1999). The patients with SMA Type II survive until age four or to the adult. The survival rate is 98.5 % at five years, and 68.5 % at 25 years in SMA Type II patients (Zerres et al. 1997).

Type III SMA (Kugelberg-Welander)

Type III SMA is a very different form of SMA with first mild symptoms occurring after the first 18-24 months of life (Kugelberg & Welander 1956). Walking, and running as well as climbing stairs are possible, but, become increasingly difficult with age. The prognosis for SMA Type III patients is good since life expectancy is almost normal. Type III

SMA is further sub classified into Type IIIa, with onset before the age of 3 years, and Type IIIb SMA, with onset after the 3rd year.

Type IV SMA (Adult form)

SMA Type IV is a very mild form of SMA with late onset beyond 30 years of age (Pearn et al. 1978; Zerres et al. 1995). As in all SMA types, weakness is progressive but affects mostly proximal muscle while distal muscle function is preserved, and does not affect life span.

1.4.3. SMN protein

SMN is a ubiquitously expressed protein of 294 aa protein of about 38 kDa in size, encoded by eight exons (Lefebvre 1995), and not sharing significant homology to any other protein (the molecular basis of SMA). The SMN protein has several functional domains, including an N-terminal RNA-binding-domain (RBD, exon 2b, and 3), a Tudor domain involved in Sm protein interaction (exon 3), a proline-rich stretch (exon 4, and 5), which can interact with profilin as well as a C-terminal YG-box (exon 6, and 2) that is necessary for self-oligomerization (Lorson et al. 1998; Bertrandy et al. 1999; Selenko et al. 2001; Bowerman et al. 2009; Bowerman et al. 2010) **(Figure 21)**.

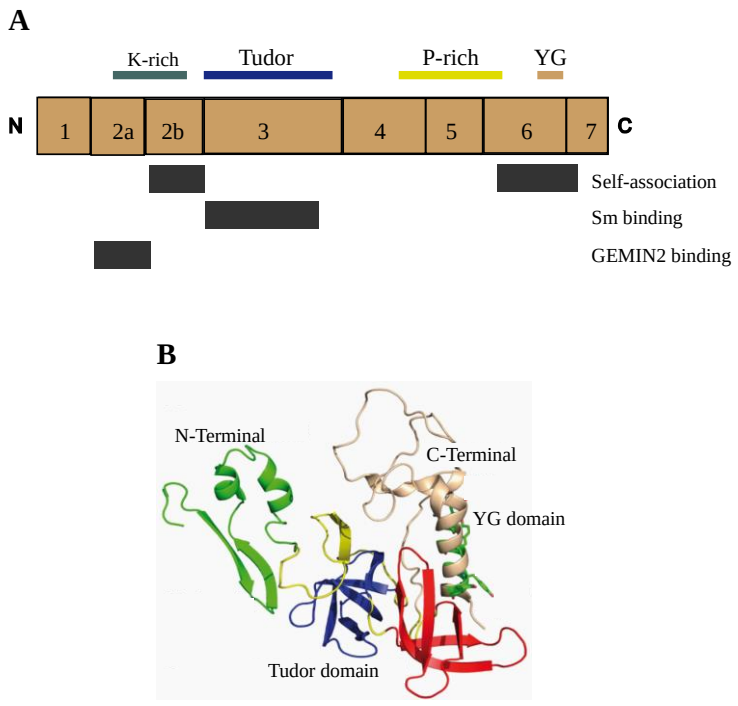

Figure 21. A) Exon 2B encodes a domain that is necessary for GEMIN2 binding and self-association. The K domain is rich in lysine (K-rich), and exon 3 encodes for a Tudor domain that binds Sm proteins. Exon 5, and part of exon 6 contain a proline-rich (P-rich) domain that may influence profilin binding. The domain of exon 6 contains the conserved YG box, and is important for self-association. Exon 8 is not displayed (Burghes & Beattie 2009). **B)** Three-dimensional structure of SMN obtained by single crystal X-ray (Seng et al. 2015).

The SMN is highly expressed in CNS and the liver (Liu & Dreyfuss 1996; Carvalho et al. 1999; Young et al. 2000; Young et al. 2001). SMN shows diffused distribution in the cytoplasm. In the nucleus, it is present in concentrated or punctate structures known as Gems (`Gemini of Coiled bodies´(CBs)) (Liu & Dreyfuss 1996). Gems are often associated with or coincident to Cajal bodies (coiled bodies) to form SMN-complexes that are implicated in the nuclear biogenesis of spliceosomal small nuclear ribonucleoproteins, snRNPs (Darzacq et al. 2002; Jády et al. 2003).

1.4.3.1. Functions of SMN

SMN has multiple roles in many cellular processes and pathways. In the cytoplasm, SMN is involved in the assembly of snRNP into the spliceosome. In the nucleus, SMN is directly involved in pre-mRNA splicing, transcription and metabolism of ribosomal RNA (Liu et al. 1997; Charroux et al. 1999; Campbell et al. 2000; Gubitz et al. 2002; Fischer et al. 1997; Jones et al. 2001; Pellizzoni et al. 2001; Hebert et al. 2001; Friesen et al. 2001; Charroux et al. 2000; Pellizzoni, Baccon, et al. 2002; Pellizzoni et al. 1998). In MNs, SMN is involved in survival, axonal transport, and axonal growth as well as in neuromuscular junction (NMJ) maturation. The functions of SMN in muscle and other non-neuronal tissues in SMA disease will also be discussed here.

1.4.3.1.1. The SMN complex and its function in snRNP assembly

SMN has been shown to play a significant role in the assembly of snRNPs, within the spliceosome (Fischer et al. 1997; Meister et al. 2001; Pellizzoni, Yong, et al. 2002; Gubitz et al. 2004; Eggert et al. 2006; Burghes & Beattie 2009). snRNPs are important in splice site(s) recognition, and catalytic removal of introns from pre-mRNA and consist of i) a U small nuclear RNA (snRNA) (U1, U2, U4, U5, U11 or U12), and ii) a heptameric ring of Sm proteins (D1, D2, E, F, G, and D3, B) (Raker et al. 1999). In the SMN-complex, the SMN-interacting proteins include; Gemin 2 (formerly SIP1 for ‘SMN interacting protein 1’), Gemin 3 (a DEAD box putative RNA helicase previously known as dp103), Gemin 5, the spliceosomal small nuclear ribonucleoprotein (RNP) Sm, and Lsm protein, the small nucleolar RNP proteins including fibrillarin and GAR1, heterogeneous nuclear RNP-Q, and coilin (Liu et al. 1997; Charroux et al. 1999; Campbell et al. 2000; Gubitz et al. 2002; Fischer et al. 1997; Jones et al. 2001; Pellizzoni et al. 2001; Hebert et al. 2001). These proteins are characterized by the presence of arginine- and glycine-rich domains. These arginine residues are symmetrically di-methylated, which promotes interactions with SMN. This modification may modulate uridine-rich snRNP Sm core assembly (Friesen et al. 2001). The SMN complex is also composed of Gemin 4, and 6, the two novel proteins of unknown function

(Charroux et al. 2000; Pellizzoni, Baccon, et al. 2002). The Sm proteins can self-assemble on snRNAs *in vitro*, however, *in vivo* this process requires the SMN complex (Meister et al. 2001; Meister et al. 2002; Pellizzoni, Yong, et al. 2002; Pellizzoni 2007). SMN is believed to act as the core backbone of the complex; however, the exact stoichiometry of SMN and its binding partners are still unknown.

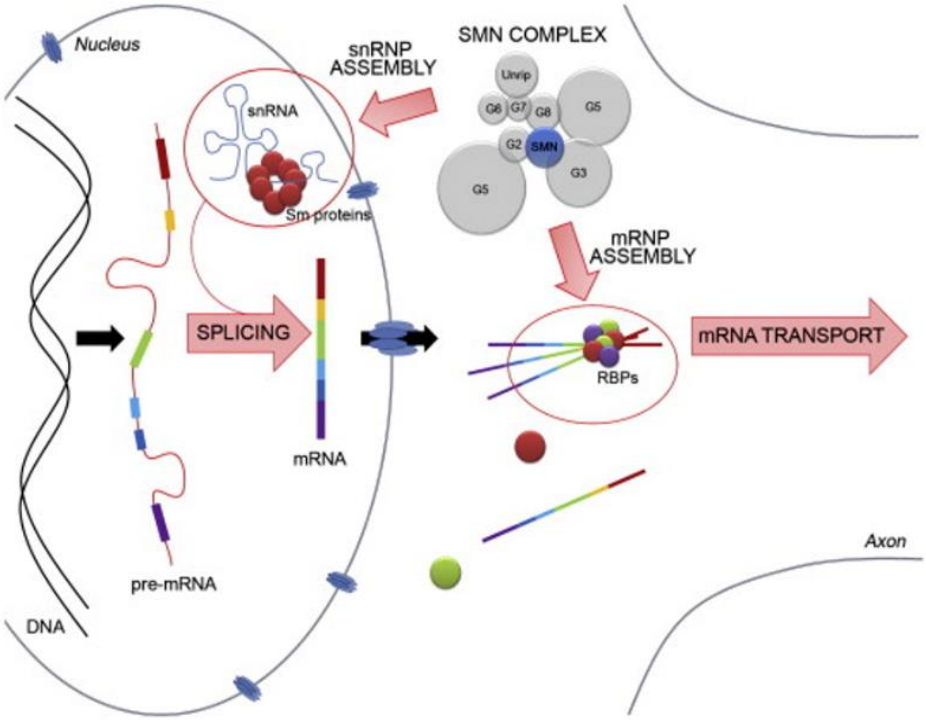

Figure 22. SMN protein, the primary role in the assembly of snRNP biogenesis, and pre-mRNA splicing requirement. SMN protein forms complexes with other co-protein (Unrip, Gemin2-8) in the cytoplasm, and inside nucleus, it forms Cajal bodies that involve in splicing of intron during the pre-mRNA transcription process. SMN also engage in the binding of specific mRNA with its target protein that helps in axon transport (Fallini et al. 2012)**.**

With this framework, the SMN complex, containing the assembled snRNPs, is then transferred into the nucleus. In the nucleus, the complex play an essential role in the biogenesis during mRNA processing (Fischer et al. 1997; Meister et al. 2002; Paushkin et al. 2000). The snRNPs are further processed, and matured in the gems while the SMN complex translocates out of the nucleus again (Chari et al. 2009). Based on these findings, it is assumed that *SMN1*-deletion impacts on correct splicing **(Figure 22)**.

1.4.3.1.2. SMN function in translational regulation, and axonal transport

SMN is localized in granules that are transported along the axons of neurons, and that show colocalization with ribosomal RNAs suggesting the translational regulation function of SMN (Zhang et al. 2003). In some models, MNs derived from SMA mice showed

the same survival as wildtype MNs, the axon length was significantly reduced (Rossoll et al. 2003). Electron microscopic (EM) analyses indicate that the Smn protein is associated with axonal microtubules (Bechade 1999; Pagliardini et al. 2000; Rossoll et al. 2003). In murine MNs, Smn protein is colocalized with hnRNP-R, one of the members of the heterogeneous nuclear ribonuclear protein (hnRNPs) family (Rossoll et al. 2002), which in turn was shown to associate with β-Actin mRNA. The hnRNP-R is involved in the processing of pre-mRNAs (Liu & Dreyfuss 1996; Mourelatos et al. 2001).

Interaction of SMN with hnRNP-R mediates delivery of β-actin to the presynaptic nerve terminal where it promotes axonal elongation, and determine the size of the growth cone. Decreased level of SMN correlate with a reduced amount of β-actin in synaptic terminals, and minimize the size of the growth cone, and elongation (Rossoll et al. 2003). Besides hnRNP-R, many other interaction partners of SMN were identified in the past that have important functions in RNA binding or translational regulation, e.g.: hnRNP-Q, KH-type splicing regulatory protein (KSRP), Hu antigen D (HuD) and fragile X mental retardation protein (FMRP) (Rossoll & Bassell 2009; Fallini et al. 2011).

1.4.3.1.3. SMN function in embryonic development

Increased SMN expression has been observed during embryonic, and fetal stages compared to the postnatal period (Burlet et al. 1998), indicating the essential function of SMN during development. The 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). SMN is necessary for blastocyst development (Hsieh-Li et al. 2000). In zebrafish MNs, a mutation in smn shows abnormal development. Deletion of *SMN* gene shows a decrease in the number of progeny and locomotive defects. Also 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 involved in particular gene expression events at very early developmental stage (Miguel-Aliaga et al. 1999).

1.4.3.1.4. The role of SMN in axonal growth and neuromuscular junction (NMJ) maturation

Studies of expression analyses revealed the presence of SMN *in vivo* in neuritis, and growth cones of MNs in mouse and rat spinal cord, and embryonic chick forebrain, *in vitro* in primary rat cortical neurons transfected with recombinant SMN as well as in differentiating, neuron-like mouse P19 cells (Bechade 1999; Pagliardini et al. 2000; Dodds, E. et al., 2001; Jablonka et al. 2001; Fan & Simard 2002; Zhang et al. 2003; Briese et al.

2005). In PC12 cells, increased SMN protein levels were documented during neuronal differentiation under continuous NGF treatment (Bergeijk et al. 2007).

Knockdown of SMN significantly reduced neurite length in PC12 cells while overexpression of SMN led to extensive sprouting (Bergeijk et al. 2007). SMN interact with Profilin IIa, a protein that plays a significant role in the axonal growth, and branching, and influences the sequestering ability of Profilin IIa negatively, thereby, triggering polymerization (Rossoll et al. 2002; Sharma et al. 2005) indicating the involvement of SMN in axonal growth via the regulation of actin dynamics. Several SMA animal models show axonal growth defects upon SMN depletion (Briese et al. 2009; Chan et al. 2003; Ymlahi-Ouazzani et al. 2010; McWhorter et al. 2003; Carrel et al. 2006; Oprea et al. 2008; Hao et al. 2011). However, axonogenesis defect was observed *in vivo* in SMA mouse models whereas their respective MNs *in vitro* exhibit reduced axon elongation and growth cone size (Mcgovern et al. 2008; Torres-Benito et al. 2012).

Electrophysiological combined with morphological analysis revealed reduced endplate Acetyl Choline Receptor (AChR) cluster currents and endplate disorganization (Chan et al. 2003) suggesting primary defects at the SMA NMJ. Severe SMA mice show a significant reduction of axonal inputs per endplate during synapse elimination process, also termed axonal pruning, in proximal *Transversus abdominis* (TVA) muscle (Murray et al. 2008). The electrophysiological recordings of synaptic activity at the NMJ shows some abnormalities in Smn depleted mice (Torres-Benito et al. 2012). In the proximal slow twitch muscle (TVA), the quantal content (QC, amount of neurotransmitter released per action potential) was reduced up to ~50 % in the SMNΔ7 mouse model, followed by ~40 % reduction in the distal and fast twitch muscles *Tibialis anterior* (TA), and ~25 % reduction in *Extensor digitorum longus* (EDL) (Ruiz et al. 2010). SMN reduction shows a two-fold decrease in the amplitude of evoked endplate current, abnormality in the neurotransmission, and NMJs cause weakness in the muscle of SMA model mouse and cardiac defects (Kong et al. 2009; Kariya et al. 2008; Shababi et al. 2010; Heier et al. 2010).

1.4.3.1.5. SMN function in muscle and other non-neuronal tissues

Smn and its complex partners are localized into the Sarcomeric Z-disc of skeletal, and cardiac muscles in the mouse. Furthermore, Smn was proven to be a direct target of calpain, a protease with an important function in Z-disc turnover (Walker et al. 2008; Fuentes et al. 2010; Anderton et al. 2011). In this context, myofibers from SMA mice display morphological defects that are consistent with a Z-disc deficiency (Walker et al. 2008). Besides the neuronal functions of Smn, these findings point to an additional role of Smn in

the muscle. SMN depletion has often been reported to affect other non-neuronal tissues, such as heart, bone, blood vessels, and liver (Finsterer & Stöllberger 1999; Kelly et al. 1999; Hsieh-Li et al. 2000; Hachiya et al. 2005; Arai et al. 2007; Shanmugarajan et al. 2009b; Khatri et al. 2008; Rudnik-Schöneborn et al. 2008; Araujo et al. 2009; Meyer et al. 2009; Bevan et al. 2010; Gogliotti et al. 2010; Heier et al. 2010; Hua et al. 2010; Michaud et al. 2010; Riessland et al. 2010; Shababi et al. 2010; Shanmugarajan et al. 2009a; Felderhoff-Mueser et al. 2002).

1.4.4. SMA models

In spite of broad understanding of the genetic cause of SMA, the cellular and molecular mechanisms through which SMN reduction leads to selective MN degeneration and clinical symptoms is still unknown. There is the only limited availability of suitable human material, either from biopsy or post-mortem. For this reason, they are many animal models provided to examine the disease progression.

1.4.4.1. Yeast

For the genetic study of basic eukaryotic cell biology, yeast model systems are considered as an important model system. *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* are the two most commonly used species. *S. cerevisiae* has no SMN ortholog, whereas, *S. pombe* has a protein Yab8 or ySMN as an ortholog of the human *SMN* gene. Protein Yab8 shares significant homology with SMN at the amino and carboxy termini, but, exhibits little internal homology by lacking the ribonucleic acid (RNA)–binding TUDOR domain (Talbot et al. 1997)**.** Yab8 interacts with a protein YIP1p which is an ortholog of SMN-interacting protein 1 (SIP1), 20% identical, and 45% similar to SIP1 at the amino acid level (Talbot et al. 1997; Paushkin et al. 2000). *S. cerevisiae* appears to contain a YIP1p ortholog, known as Brr1p, but, it remains unclear whether YIP1p and Brr1p can interact with human SIP1 orthologs (Talbot et al. 1997; Paushkin et al. 2000). Immunocytochemistry and expression studies confirmed the ySMN protein is localized both in the nucleus, and the cytoplasm (Paushkin et al. 2000), whereas, the smaller size of the nuclei makes it difficult to characterize the subnuclear localization of ySMN with conventional microscopy. ySMN and hSIP1 interactions do not appear to be conserved while other binding interactions described for hSMN are conserved in *S pombe.* ySMN protein is capable of binding to human orthologs of SMN itself and hSmB (RNA-binding protein). But, these interactions are less robust. Loss of ySMN is lethal, and the exogenously supplied human SMN (hSMN) cannot rescue the loss of ySMN function. Overexpression of ySMN is also damaging, but, not lethal, and

produces a slow growth phenotype with slower growing cells that form smaller colonies (Paushkin et al. 2000).

1.4.4.2. Caenorhabditis elegans

The documentation of the complete cell lineage makes the nematode *Caenorhabditis elegans* as an efficient model for studying various disease-related gene functions. Because the acquisition of each cell's fate has been thoroughly documented, the worm combines the advantages of a complex organism with single cell biology. It is an extremely simple organism, with fewer than 100 MNs in a male adult (Sulston et al. 1983). The *C.elegans* genome contains a single SMN ortholog, CeSMN (C41g7.1) (Talbot et al. 1997), that encodes an SMN protein 36% identical to the human ortholog (Lehmeier et al. 1994). It consists of 5 exons, and four small introns, and encodes a 745 nucleotide transcript, and a 207 amino acid protein. It also contains a TUDOR domain (Miguel-Aliaga et al. 1999). A *C. elegans* ortholog of SIP1 (Gemin 2), known as survival motor neuron-interacting protein 1 (SMI-1), is also present (Burt et al. 2006). Studies revealed that the CeSMN is transcribed throughout development, and the protein localizes to the nucleus and is most strongly expressed in the nervous system. In neurons, and excretory cells CeSMN protein is also localized in the cytoplasm in addition to the nucleus.

Reducing the expression of smn-1 by RNAi causes larval lethality, suggesting that smn-1 is essential for the embryonic survival of *C. elegans* animals. The egg-laying defective mutant, egl-32, contains a short genomic deletion in the promoter of CeSMN, (Miguel-Aliaga et al. 1999). A null mutation of *smn-1* (ok355) that deletes most of the smn-1 coding region causes developmental arrest, reduced lifespan, and progressive loss of MN function. A point mutation in *smn-1* that mimics a human SMA disease mutation causes weak motor defects and a slightly reduced lifespan. This mutant was used to screen a library of chemicals for potential drugs that could ameliorate the mild defects, (Sleigh et al. 2011). *Smn-1* transgene partially rescues the developmental arrest and motor defects. Overexpression of CeSMN resulted in sterility and embryonic lethality, but, the viability is not significantly affected (Miguel-Aliaga et al. 1999).

1.4.4.3. Drosophila

The Drosophila genome contains a single copy of SMN ortholog at cytological map position 73A7-9 with 41% sequence homolog to human SMN1. It generates a transcript with a single exon, 828 bp in size and encodes a 226 aa protein. Expression studies suggest that SMN is expressed in both neuronal, and muscle tissues (Rajendra et al. 2007; Chan et al. 2003; Miguel-Aliaga et al. 2000). Mutants with point mutations in 73A7-9 showed defects

in late developmental stages, and a late larval lethality (Chan et al. 2003). The production of female germ line clones producing oocytes that lacked maternal transcript was also reported. A small number of eggs were produced, and they were not viable, suggesting an essential role for the maternal transcript in both egg production, and embryonic viability (Chan et al. 2003). The survival of Smn zygotic mutant embryos, due to maternal wild-type Smn activity, and mutant larvae show severe motor abnormality.

Drosophila Smn mutants have been used in screens for genes that interact with Smn. The members of the BMP signaling pathways (Chang et al. 2008), and genes involved in endocytosis and RNA processing (Dimitriadi et al. 2010) interact with SMN. Many of candidates Smn-interacting genes primarily a group of RNA splicing factors (Sen et al. 2013) were identified as candidate SMN-interacting genes. Drosophila Smn is required for the integrity of the germline U body-P body pathway (Lee et al. 2009), the expression of FGF signaling components (Sen et al. 2011), stem cell division, and differentiation (Grice & Liu 2011), sensory–motor circuit function (Imlach et al. 2012), and chronic glutamate receptor-dependent developmental homeostasis (Timmerman & Sanyal 2012).

1.4.4.4. Zebrafish

Zebrafish *(Danio rerio)* have a rapid life cycle and external transparent embryonic development. The well-characterized stereotypical neuromuscular system at the cellular level makes them as an ideal model system to study MN development and their axonal projections. This model organism serves to bridge the gap between worm/fly, and mouse/human genetics. The zebrafish spinal cord primary MNs are commonly used to study changes in axon morphology, protein aggregation, and neuromuscular junction formation, which are more relevant to SMA pathogenesis. Antisense morpholino (MO) knockdown of *smn* in zebrafish during early stages of embryogenesis showed many aspects of MN defects in SMA disease (McWhorter et al. 2003). The optical transparency of zebrafish embryos offers accessibility to MNs, and the effects can be thoroughly assessed *in vivo* for imaging, electrophysiology, and motor behavioral studies (Cantu et al. 2013; McLean et al. 2007; McLean & Fetcho 2011; McWhorter et al. 2003). The knockdown of smn recapitulates MN defects in SMA disease. Fifty-five percent of embryos died within 24 h after fertilization. In the surviving embryos, there is no difference in the number of MNs present at 24 h, but specifically, possess short or excessively branched motor axons or both. These results are consistent with cultured MNs from severe SMA mice, which also have short aberrant axons. Smn knockdown within MNs didn't affect muscle development, but, resulted in an autonomous cell phenotype of motor axons indicating that Smn is required within MNs. It also reveals that the motor axon development is the first phenotypic consequence of reduced Smn protein.

Three smn mutants: two stop mutations (smnY267stop and smnL265stop), and one exon 7 missense mutation (smnG264D), which was found to correspond to a human SMA mutation (SMNG279V) have been screened (Boon et al. 2009). Characterization of these mutants shows a decrease in the synaptic vesicle protein, SV2 (Boon et al. 2009). New zebrafish SMA models by expressing a human SMN2 transgene in zebrafish carrying endogenous smn mutations have been generated (Hao et al. 2011). This model reveals that *SMN2* is similarly spliced in fish as in humans, producing low levels of full-length SMN protein, and comparatively high levels of exon 7 excluded protein. The manipulation of *SMN2* transcripts via an antisense oligonucleotide sequence directed against an intronic splicing silencer site in intron 7 promotes exon 7 inclusion, thus increasing full-length SMN levels (Hao et al. 2011). Thus, this model will prove invaluable in building the knowledge base of SMA disease development and progression, as well as serve an *in vivo* entry point into drug testing and therapy development.

1.4.4.5. SMA Mouse Models

Mouse models of human diseases are essential tools for studying their physiopathology. SMA modeling in mice is complicated despite the conserved nature of mouse and human nervous system. Unlike humans, mice only harbor one Smn gene. Homozygous deletion of *Smn* exon 2 or exon 7 (*Smn-/-*), generates inactive Smn protein, and results in lethality at the 8-cell stage of development (Schrank et al. 1997). This demonstrated that SMN is required for survival of all cells. But, the heterozygous Smn mice (*Smn*±) had a complete lifespan, and appear totally normal (Jablonka et al. 2000). This thin line of difference between lethality, and an average phenotype forced to adapt the transgenic methods for the manipulating dosage of SMN by supplementing human SMN protein to a *Smn* null background. The embryonic lethality of *Smn-/-* genotype was rescued by inserting 2 (Line 89) or 8 (Line 566) copies of the human *SMN2*. Irrespective of the number of *SMN2* copies, *Smn-/-SMN2+/+* mice appear normal at birth. However, when only two copies of *SMN2* are added, the mice exhibit an average life expectancy of 5 days and mimic the severe Type I SMA seen in humans. Currently, it is the most utilized model for studying severe SMA. The high-copy *Smn-/-SMN2+/+* mice live a normal life and do not show any obvious phenotype (Monani et al. 2000). Incorporation of four copies of *SMN2* have a full lifespan, but, mimics the mild human SMA by showing peripheral necrosis of the ears, and tail. These mice are referred as the ‘‘Hsieh-Li’’ mice or “Taiwanese” SMA mouse model and are commonly used as a model to study mild SMA (Hsieh-Li et al. 2000).

A transgenic mouse model was created by harboring a 3.4-kb portion of the human *SMN* promoter followed by a truncated *SMN2* cDNA (*SMNΔ7*) clone introduced into the severe SMA mouse model. The resulting *Smn-/-SMN2+/+SMNΔ7+/+* ("Delta7") mice show a prolonged survival (with a mean survival of 13 days) compared with the severe *Smn-/-SMN2+/+* mice (Le et al. 2005).

Several different *SMN* genes harboring human patient mutations were incorporated into the severe Line89 mouse model via transgenesis. Heterozygous expression of *SMN1 A2G* patient mutation transgene in Line89 extends survival from 5 to 227 days (Monani et al. 2003). At 3.5 months of age, 29% reduction of spinal cord MNs and 19% reduction of the facial nucleus was observed. At 5 months, 25% reduction in the spinal motor axon, muscle atrophy, and gastroc/triceps axon sprouting was noticed. Another mutation *SMN1 A111G* was studied in Line89 background (Workman et al. 2009). The heterozygous expression of this mutation rescues the SMA features of Line89 mice and leads to a normal lifespan. At ten months, it exhibits gastrocnemius hypertrophy. The transcript from this *A111G* mutation rescues the deficits in snRNP assembly seen in the Line89 mice.

To increase the lifespan of severe SMA mouse models, a null *Smn* mouse line with three copies of *SMN2* was created. This line was developed by crossing two transgenic mouse lines: N46 mice that contain two copies of the transgene and N11 mice that contain one *SMN2* transgene. The average of life expectancy is 15 days, with severe SMA characteristics (Michaud et al. 2010).

A mouse exhibiting a single disease locus was created. The mouse *Smn* gene mutated to resemble the human *SMN2* gene. This mouse contains a *C>T* mutation in the mouse *Smn* gene, at the position corresponding to the nucleotide transition between human *SMN1* and *SMN2*. This mutation induces exon 7 skipping, and reduces Smn protein levels, and results in a phenotype resembling mild, or Type IV SMA at 60 days with impaired motor function, and activity. These mice exhibit a normal lifespan (Gladman et al. 2010).

When the splicing enhancer *Tra2beta (2B)* is disrupted, even in the absence of a *C>T* mutation (as in *SMN1* and *wt Smn*), large amounts of skipped product are observed (DiDonato et al. 2001). The mutated 2B site in mouse (*Smn2B/-*) leads to low Smn protein levels and a lifespan of one month with MN loss during disease progression (Bowerman et al. 2009; Bowerman et al. 2010).

Tissue-specific models have been developed to determine the effect of SMN depletion in specific cell types. These mouse lines were generated by crossing mice

containing a *Cre-recombinase* gene downstream of a tissue-specific promoter with mice containing a *Smn* allele with two loxP sites flanking exon 7 (*SmnF7*). Double-transgenic mice from this combination contain both the tissue-specific *Cre* expression and *SmnF7* genes. Resulting mice exhibit Cre-mediated recombination and exclusion of exon 7 in specific tissues which result in the reduction of the levels of full-length protein. *SmnF7/Δ7; NSE-Cre* mice were generated to analyze the reduction of Smn in neural cells. This SMN deficiency results in mice that exhibit an SMA phenotype with a mean survival of 25 days (Cifuentes-Diaz et al. 2002; Frugier et al. 2000). *SmnF7/Δ7; HSA-Cre* line was created targeting muscle cells, was created. Muscle cell death was observed, and average life expectancy was approximated 33 days (Cifuentes-Diaz et al. 2001). *SmnF7/F7; Alfp-Cre* line targets hepatocyte, and this genotype was embryonic lethal at E18.5 (Vitte et al. 2004). *Oligo-2 Cre* line targets neural progenitor cells. This line involves a *Cre* cassette knocked into the *Oligo-2* gene, which is expressed embryonically in the spinal cord. These mice exhibit spinal cord MN loss, reduced innervations in the dorsal root ganglion, and muscular atrophy. Seventy percent of these mice survived to 12 months of age (Park et al. 2010).

1.4.5. Therapeutic strategies

Although much research has been invested in the development of therapy for SMA, till date, there is no effective treatment for SMA. Symptomatic treatments like physical exercise or respiratory drainage to remove tracheal mucus help to improve patient´s life quality and the introduction of several non-targeting strategies, like neurotrophic agents, reduces α-MN degeneration. But they do not do not target the underlying genetic cause of SMA. The loss of SMN protein due to the absence of *SMN1* gene could be compensated by increasing the levels of functional protein levels by the *SMN2* gene. The general treatment strategies target the *SMN2* gene locus for SMA treatment. The gene and stem cell therapies are also discussed here.

1.4.5.1. *SMN2* promoter induction

Histone deacetylase inhibitor (HDACi) class of substances block the activity of histone deacetylases (HDAC). Therefore, these drugs activate gene transcription by shifting the modification of histones towards an increased acetylation state. Various HDAC inhibitors like Sodium butyrate, Valproic acid (VPA) phenylbutyrate, LBH589, Trichostatin A (TSA) and Suberoylanilide hydroxamic acid (SAHA) activates *SMN2* expression in cell culture or murine SMA (Chang et al. 2001; Brichta et al. 2003; Sumner et al. 2003; Andreassi et al. 2004; Brahe et al. 2005; Riessland et al. 2006; Avila & Burnett 2007; Mercuri et al. 2007; Tsai et al. 2008; Garbes et al. 2009; Hauke et al. 2009; Brichta et al. 2006). The β-

adrenoreceptor agonist Salbutamol rapidly increases *FL-SMN2* levels, and subsequently, elevates SMN protein in SMA fibroblasts (Angelozzi et al. 2008). Based on the identification of an interferon-response-element (IRE) in the *SMN2* promoter, it has been shown that treatment with either the interferon IFN-β or IFN–γ, elevates SMN levels (Baron-Delage et al. 2000). A compound screening campaign resulted in the identification of quinazolines which doubled *SMN2* promoter activity (Jarecki et al. 2005).

1.4.5.2. Correction of the *SMN2* splicing pattern

Another therapeutic strategy is the correction of the *SMN2* splicing pattern towards increased levels of *FL-SMN* transcripts. HDAC inhibitors such as VPA, TSA, and sodium butyrate have dual effects on *SMN* mRNA expression; i) they open the chromatin structure and therefore increase the rate of transcription, but ii) they also affect the splicing process (Chang et al. 2001; Sumner et al. 2003; Brichta et al. 2003; Avila & Burnett 2007). Antisense oligonucleotides (ASOs) such as morpholinos or RNAs (Lim & Hertel 2001; Cartegni & Krainer 2003; Dickson et al. 2008) elevate SMN levels in cell culture, and even in SMA mice (Passini et al. 2011). Their inability to cross the blood-brain barrier is the major difficulty about using ASOs for SMA therapeutics **(Figure 23)**.

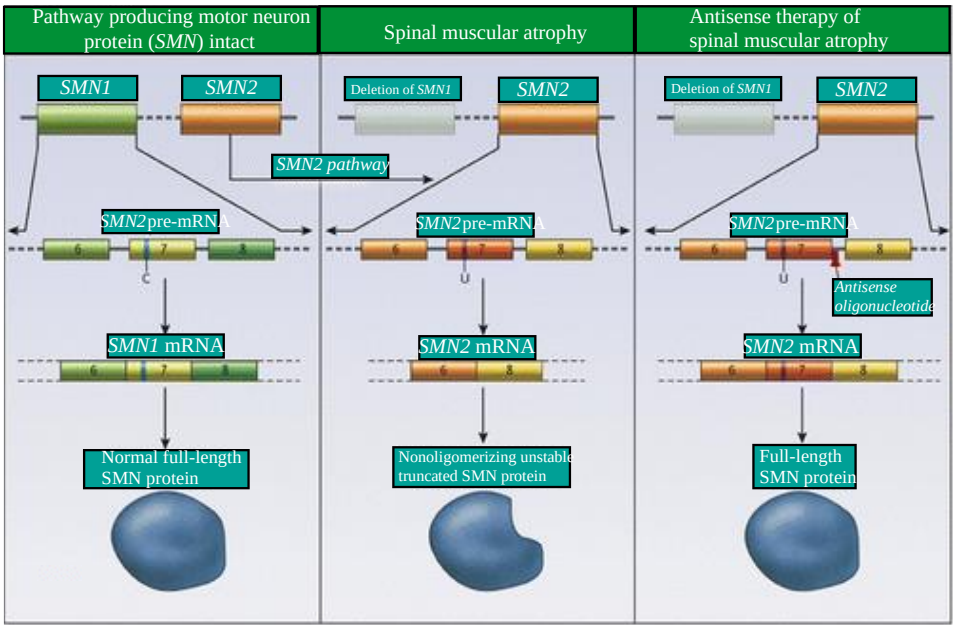

Figure 23. Antisense therapy of SMA (MacKenzie 2012).

The antibiotic aclarubicin increases full-length SMN transcript by altering the splicing process *in vitro* (Andreassi et al. 2001). The lactation hormone prolactin (PRL) increases *SMN2* gene transcription in genetically engineered Δ7 SMA mouse model (Farooq et al. 2011). The phosphatase inhibitor Na-Vanadate has been shown to increase exon 7 inclusion *in vitro* (Ting et al. 2007; Zhang et al. 2001). The cytostatic drug hydroxyurea (HU)

increases *FL-SMN2* in B-lymphoblastoid cells from SMA patients (Grzeschik et al. 2005), but, when it was tested in SMA patients, only slight improvements were observed (Liang et al. 2008).

1.4.5.3. Stabilizing the full-length *SMN* transcript

A relatively new approach to stabilizing the full-length *SMN* transcript has been developed. Decapping Scavenger enzyme (DcpS), an integral part of the RNA degradation machinery, was targeted by C5-substituted quinazolines which bind with the enzyme, and potently inhibit DcpS decapping activity. Thus, full-length *SMN* mRNA decay is blocked, resulting in an increase of SMN protein in cell culture (Singh et al. 2009). *SMN* mRNA has a specific AU-rich element (ARE) region in its 3' UTR which marks the mRNA for degradation. The activation of the p38 pathway results in the accumulation of RNA binding protein HuR in the cytoplasm which then binds to the ARE in 3'UTR region of *SMN* mRNA and stabilizes the transcript (Farooq et al. 2009).

1.4.5.4. Full-Length SMN protein stabilization

Aminoglycosides like tobramycin, geneticin, and amikacin have been used to post-transcriptionally elevate SMN levels by inducing read-through of the first stop codon in exon 8 of *SMN2Δ7* (Heier & DiDonato 2009; Mattis et al. 2006; Wolstencroft et al. 2005). Read-through leads to an elongated C-terminus of the SMNΔ7 protein, thus increasing its stability (Wolstencroft et al. 2005). Aminoglycosides TC007 *in vivo* improves motor function and increases survival significantly by 30% (Virginia B. Mattis et al. 2009). It has been suggested that inhibition of SMN turnover by MG-132 may be beneficial for SMA patients, but, this has not yet been tested *in vivo* (Chang et al. 2004). The proteasome inhibitor, bortezomib increases SMN both *in vitro* and *in vivo* by blocking proteolysis of SMN protein (Kwon et al. 2011).

1.4.5.5. Gene Therapy

Another approach to treating SMA is the gene therapy in which *SMN* is delivered into the CNS of SMA mice using adeno-associated vectors (AAV) to complement the lack of *Smn*. The self-complementary adeno-associated virus 9 (scAAV9) was used for gene delivery into MNs of SMA mice by intravenous injection. The scAAV9-SMN was able to cross the blood-brain barrier, and highly efficiently transduced MNs in the lumbar region of the spinal cord as well as muscle tissue were observed. The survival was extended from 15 to more than 100 days (Foust et al. 2010; Valori et al. 2010; Dominguez et al. 2011; Bevan et al. 2010) **(Figure 24)**.

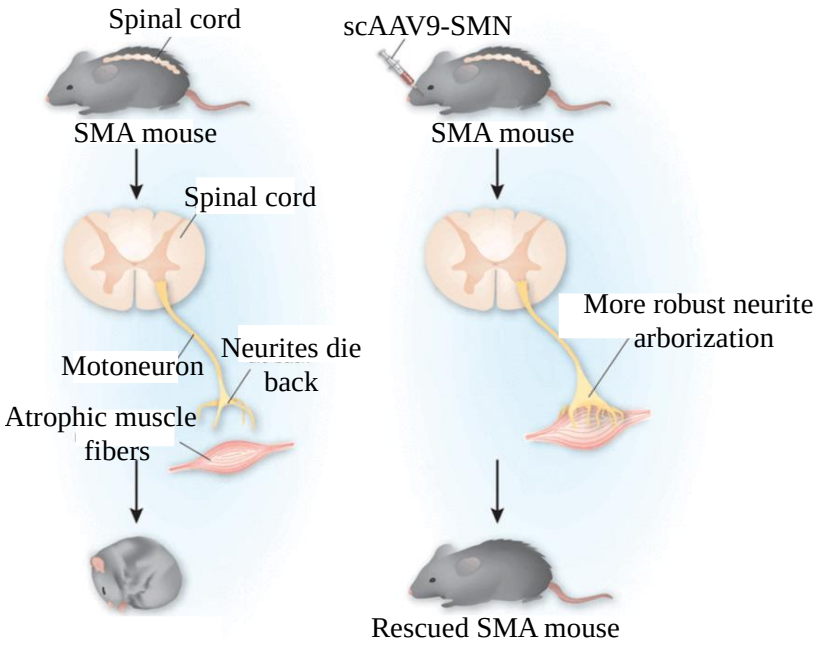

Figure 24. Gene therapy of SMA (MacKenzie 2010).

1.4.5.6. Stem cell therapy

Stem cell therapy has generated much attention as a treatment for SMA. The idea of using stem cells in SMA therapy is to either replace the degenerated MNs or to support the remaining cells by the addition of neural stem cells (NSC). When transplanted into the spinal cord of the SMNΔ7 mouse model, NSC differentiated into MN-like cells, protected host MNs from degeneration and significantly improved motor ability as well as survival (Corti et al. 2010). The use of embryonic stem cell-derived NSC, which led to a similar amelioration of the phenotype, exhibited markedly increased levels of secreted NTFs (Corti et al. 2008).

Although induced pluripotent stem cells (iPSCs) from an SMA patient have been differentiated into MNs (Dimos et al. 2008; Ebert et al. 2009), it remains elusive whether injection of (pre-differentiated) iPS-cells into an SMA patient is indeed a therapeutic option due to the ectopic over-expression of several oncogenes **(Figure 25)**.

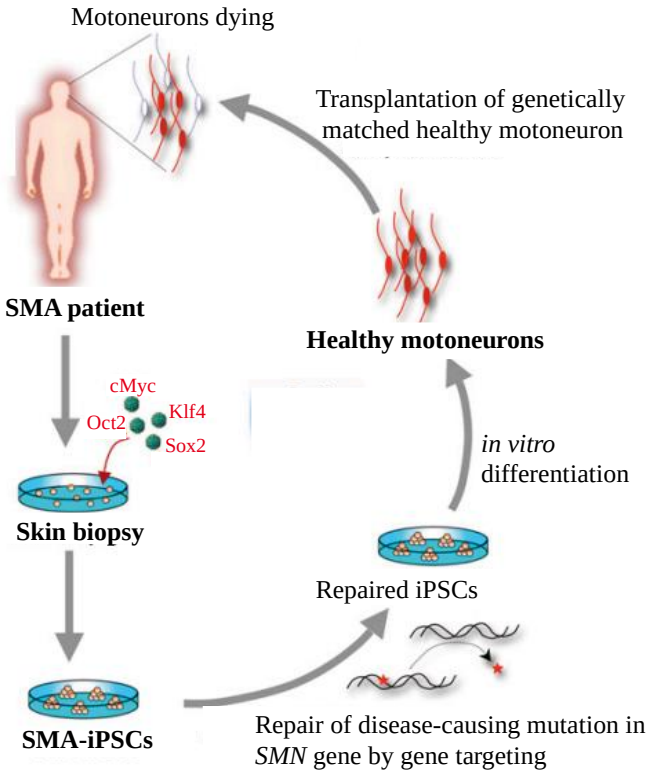

Figure 25. Stem cell therapy of SMA (Stadtfeld & Hochedlinger 2010).

1.4.5.7. Other Therapeutic Approaches

Riluzole, a small benzothiazole which antagonizes the effects of the neurotransmitter glutamate, improved survival, and NMJ architecture in SMA-like mice (Haddad et al. 2003), but, no effect was detected in Riluzole-treated SMA patients (Russman et al. 2003). SMA-like mice were treated with NMDA (N-Methyl-D-Aspartate) to activate the glutamatergic NMDA-receptors (NMDAR). NMDA improved NMJ maturation, induced *SMN2* expression in the spinal cord via CREB activation, and reduced motor neuron apoptosis (Biondi et al. 2010). The pharmacological Rho-kinase inhibitor increases the life span of a mild SMA mouse model and improves disease phenotype. This improvement in the disease phenotype is through making NMJ better, larger, and more mature (Bowerman et al. 2010), and not due to SMN increase leading to the idea of approaching SMN independent novel avenues as the therapeutical target for SMA.

In summary, different therapeutic strategies are currently ongoing or under investigation. Olesoxime (TRO-19622), a cholesterol-like neuroprotectant (Bordet et al. 2007) that targets the mitochondrial permeability transition pore putatively, possibly preventing the release of apoptotic factors or occluding other cell death mechanisms, promotes survival of MNs in culture. Trophos SA conducted a Pivotal efficacy, and safety study of olesoxime in 24-month, Phase II, multicenter, randomized, adaptive, double-blind,

placebo-controlled study in non-ambulant SMA patients (Type II and Type III) aged 3 to 25 years was performed (NCT01302600)**.** As a result, Trophos has been granted orphan drug designation for olesoxime for the treatment of SMA by the US Food and Drug Administration (FDA). In the European Union, olesoxime has been granted Orphan Medicinal Product designation for SMA by the European Commission (Zanetta et al. 2014).

Hypothesis & Objectives

Hypothesis & Objectives

Precedent

In our previous work (Mincheva et al. 2011), we demonstrated that the canonical NF-κB pathway mediates NTFs-promoted cell survival in a developmental model of spinal cord MNs. Even in the presence of NTFs, selective reduction of IKKα, IKKβ, or RelA proteins induced apoptotic cell death. In the same work, we showed that the Smn protein level was decreased in shRelA-transduced MNs.

Hypothesis

I. Members of the NF-κB signaling pathway can regulate Smn protein level, and

II. The NF-κB pathway has a significant role in the pathogenesis of SMA.

Objectives

I. To measure the Smn levels in RelA, RelB, IKKα and/or IKKβ knockdown embryonic spinal MNs.

II. To measure the levels of IKKα, IKKβ and RelA phosphorylation in Smn-deficient MNs.

Chapter-II Materials & Methods

2.1. Cell culture

2.1.1. Human Embryonic Kidney Cell line (HEK-293T)

HEK-293 cell line is established from primary embryonic human kidney cells transformed with sheared human adenovirus type 5 DNA (Graham et al. 1977; Harrison et al. 1977). The E1A adenovirus gene is expressed in these cells and participates in transactivation of some viral promoters, allowing these cells to produce very high levels of protein. HEK-293T cell line is a highly transfectable derivative of 293 cells and contains the SV40 T-antigen. This cell line is competent to replicate vectors carrying the SV40 region of replication. It gives high titers when used to produce retroviruses. It has been widely used for retroviral production, gene expression, and protein production.

2.1.1.1. Thawing and Maintenance

The cryovial was removed from liquid nitrogen, and thawed quickly in a 37°C water bath. Once thawed, the cells were collected into a conical bottom centrifuge tube. DMEM medium **(Table 6)** was added to reach a final volume of 5 ml and centrifuged 5 min at 1000 rpm. The pellets were resuspended in the appropriate medium and grown in 100 mm tissue culture plate in the incubator with controlled CO_2 atmosphere.

When the 100 mm plate was ~80-90% confluence, the cells were removed with 4 ml of the medium, and centrifuged at 1000 rpm for 3 min and the supernatant was discarded. The cell pellet was then re-suspended in 1 ml of the medium and seeded in the plates (1:3 or 1:4). The medium was usually changed every 2-3 days, depending on the rate of growth.

Medium	Dulbecco's Modified Eagle's Medium (DMEM), high glucose, pyruvate, 10% Fetal Bovine Serum (FBS), (Heat inactivated (HI) at 55°C), 20 µg/ml Penicillin and Streptomycin (P/S)
Culture condition	Incubated at 37°C in controlled atmosphere (5% CO_2) incubator

Table 6. HEK-293T cells; culture medium composition and maintenance conditions

2.1.2. Neuroblastoma X Spinal Cord Hybrid Cell Lines (NSC-34)

NSC-34 is a hybrid cell line, produced by fusion of MN- enriched embryonic mouse spinal cord cells with mouse neuroblastoma (Cashman et al. 1992). Cultures contain two populations of cells; i) small, undifferentiated cells that have the capacity to undergo cell division, and ii) larger, multi-nucleate cells. These cells express many properties of MNs, including generation of action potentials, neurofilament triplet proteins, choline acetyltransferase, and acetylcholine synthesis; storage and release. Also, similar to neuronal development, these cell lines induce acetylcholine receptor clusters on co-cultured myotubes, and undergo a vimentin-neurofilament switch with maturation in culture. The NSC-34 cell lines are a good model for neuronal studies that require a large number of MNs.

2.1.2.1. Thawing and Maintenance

The cryovial was removed from liquid nitrogen and thawed quickly in a 37°C water bath. The cells were initially incubated in a 60 mm tissue culture plate in growth medium **(Table 7)**. The cells were diluted five fold (5 ml medium) for the initial plating is not recommended to centrifuge the cells before plating. The same day, after the cells have attached to the plate (approximately 4-6 h), the medium was refreshed to remove the Dimethyl sulfoxide (DMSO).

The cells grow right on the surface of the conventional tissue culture plastic; no special coating is required. Cell density is not a concern for the first few days after thawing because it is viewed more as a recovery period. Once they are growing properly, they can be plated at whatever density is most appropriate for the experiment being done**.** Cultures were split at ~80% Confluency. The cells were removed from 60 mm plate using 0.25% trypsin-EDTA solution (approx 2 ml, 3 min at 37°C) and were diluted with 10 ml medium in 100 mm plate (without centrifuge)**.** When the 100 mm plate was ~80% confluence, the cells were removed with 4 ml of the medium, and centrifuged at 1000 rpm for 3 min, and the supernatant was removed. The cell pellet was then re-suspended in 1 ml of the medium and plated in the plates (1:3 or 1:4). The medium was typically changed every 2-3 days, depending on the rate of growth.

Medium	Dulbecco's Modified Eagle's Medium (DMEM), high glucose, without pyruvate, 10% Fetal Bovine Serum (FBS), (Heat inactivated (HI) at 55°C), 2 mM L-Glutamine, 20 µg/ml Penicillin, and Streptomycin (P/S)
Culture conditions	Incubated at 37°C in controlled atmosphere (5% CO_2) incubator

Table 7. NSC-34 cells culture medium composition and maintenance conditions

2.1.3. Cryopreservation of the cell lines (HEK-293T and NSC-34)

Cells were detached using 4 ml of the corresponding culture medium, and collected into a conical bottom centrifuge tube; centrifuged at 1000 rpm for 3 min. The medium was aspirated, and the cell pellet was re-suspended in a freezing medium **(Table 8)** at the ratio of $2x10^6$ cells/ml in a 2 ml cryogenic vial. The vials were kept at -20°C for 4 h, later in -80°C for 24 h to get a slow freezing process, and then frozen in liquid nitrogen for future use.

Freezing Medium	90% Fetal Bovine Serum (FBS), (Heat inactivated (HI) at 55°C), 10% Dimethyl sulfoxide (DMSO)

Table 8. Freezing medium composition

2.2. Primary culture of spinal cord MNs

2.2.1. Preparation of culture plates for MNs

2.2.1.1 Poly-DL-ornithine coating

A 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. After incubation for 4 h at room temperature, the plates were washed with sterile water for three times and air-dried at room temperature for 30 min. The plates can be stored at 4°C up to 15 days.

Boric-borate: pH 8.3	150 mM Sodium tetraborate, 150 mM Boric acid

2.2.1.2. Laminin coating

Laminin, 3.8 µg/ml in the L15 medium was added to the poly-DL-ornithine treated plates with a suitable volume and kept in the CO_2 incubator at 37°C for minimum 2 h. Laminin solution was removed immediately before cell plating. Laminin-coated plates could be used during one week.

2.2.2. Isolation of Spinal cord MNs

2.2.2.1. Dissection

Spinal cords were dissected from E12.5 mouse embryos. 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 steps for obtaining spinal cord from mouse embryos **(Figure 26)** are as follows;

- Embryos from the uterus were removed and transferred to a plate with the Glucose-HEPES Buffer Saline (GHEBS) solution.
- Head of each embryo was removed in the presence of GHEBS.
- After fixing the embryo upside down on a silicon support, the tail was removed, and the column was opened from downwards.
- The dorsal parts were removed on both sides, and discarded to obtain MNs from the ventral side of the spinal cord.
- By holding head-side carefully, 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 (4 spinal cord/tube) with the conical bottom in the presence of GHEBS.

GHEBS: pH 7.4	137 mM NaCl, 2.6 mM KCl, 25 mM Glucose, 25 mM HEPES, 20 mg/ml Streptomycin, 20 IU/ml Penicillin

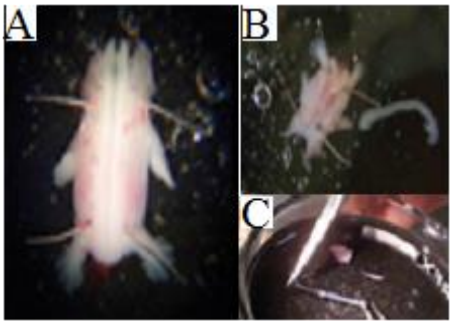

Figure 26. Extraction of spinal cords from E12.5 mouse embryos. Embryos were removed from the uterus of CD1 pregnant female; they were kept on a plate with GHEBS **(A and B)**. Heads were removed by placing on silicon support and fixing the four corners with mini-needle, and the dorsal part of the spinal cord was carefully dissected, meninges were detached, and the spinal cord was cut into pieces **(C)**.

2.2.2.2. Purification

The purification process must be done as quickly as possible, but, always treating the sample with smoothness, and care. To remove traces of red blood cells or other cells, the obtained spinal cord pieces were washed twice with GHEBS.

2.2.2.2.1. Enzymatic dissociation

To perform an enzymatic dissociation, the spinal cord fragments were treated with Trypsin without EDTA (0.25%) (900 µl GHEBS+100 µl Trypsin) for 9 min at 37°C, shaking frequently.

2.2.2.2.2. Mechanical dissociation

- Using a Pasteur pipette, the spinal cord pieces were collected and placed into another tube containing **Solution A** (800 µl L15c+100 µl BSA 4%).
- One hundred µl DNAse was added to **Solution A** and was shaken gently for 2 min.
- First, mechanical dissociation was performed by passing the trypsinized pieces of spinal cord tissue four times through a 1 ml pipette tip (with filter).
- The tubes are left upright and incubated for 2 min without stirring for the non-dissociated tissue pieces to sink to the bottom of the tube, and the supernatant was collected, and placed in a new tube.
- **Solution B (**900 ul L15c+100ul BSA 4%+20 ul DNAse) was added to tubes that contain the remaining non-dissociated spinal cord fragments.
- Second mechanical dissociation was performed by passing the non-dissociated spinal cord fragments for nine times through a 1 ml pipette tip (with filter), and the tubes are incubated for 2 min without stirring.
- The supernatant obtained was placed in the tube containing the supernatant from the first mechanical dissociation (if there are remaining fragments it is possible to repeat the dissociation a third time with **Solution B)**.

2.2.2.2.3. BSA Gradient

- 4 ml of 4% BSA solution was added (take BSA with the Pasteur pipette, place pipette touching the bottom of the tube, and leave BSA slowly, and softly) to the tubes where the dissociated cells are placed, forming two phases, and centrifuged for 5 min at 140 g (1000 rpm).
- The pellet was re-suspended in 1 ml of the L15c medium by passing 5-6 times through 1 ml pipette tip.

2.2.2.2.4. Optiprep Gradient

- 12.5% Iodixanol (Optiprep) solution was prepared in GHEBS to perform the density gradient. The density gradient is carried out to purify the MNs from other cells of the tissue.

- Cells were settled carefully on the Optiprep solution, and centrifuged at 520 g (1700 rpm) for 10 min.
- After centrifugation, the interface band corresponding to MNs was collected (0.5 ml or 1 ml) in a sterile new tube.

Motoneuron Culture Medium & Solutions	Components
L15 complete (L-15c)	Leibovitz's L-15 Medium (L-15), 18 mM D-glucose, 1% N-2, 20 mg/ml streptomycin, 20 IU/ml of penicillin
Bovine Serum Albumin (BSA) 4%	BSA 7.5%, in L-15
Optiprep	12.5% Iodixanol in GHEBS
Neurobasal medium complete (NBMc)	Neurobasal medium (NBM), 2% B-27, 2% Horse Serum (Heat Inactivated), 125 nM L-glutamine, 25 mM β-mercaptoethanol
NTFs Cocktail	NBMc, 1 ng/ml BDNF, 10 ng/ml of CNTF, GDNF, and HGF
Aphidicolin	1.5 µg/ml (4.5 mM) in NBMc

Table 9. Compositions of Motoneuron culture medium & solutions.

2.2.2.3. MN culture

Plating cell number is vital for maintaining cell cultures, preparing cells for transfection experiments, preparing cells for downstream experiments that require an accurate and consistent number of cells. We performed cell counting manually using a hemocytometer. The hemocytometer has an “H” shape engraved in the middle that encloses two separate mirror-like polished grid surfaces and provides the cover slip mounting area.

Both the hemocytometer, and its coverslip were cleaned by removing any dust particles with lens paper. The coverslip was placed on the counting surface before loading the cell suspension. Then the pipette tip was placed with the sample into the wells. The area under the coverslip fills by capillary action. Enough liquid was introduced so that the mirrored surface is just covered, usually around 10 µl, but care was taken not to overfill the surface. The loaded hemocytometer was then placed on the microscope stage.

Total cells = Average number of cell in one large square X 10^4 (conversion factor)

The appropriate number of MNs were seeded in culture plates pre-treated with poly-DL-ornithine, and Laminin. In MNs, the maximal MN survival was obtained when the culture medium was supplemented with the following NTFs cocktail: 1ng/ml BDNF, 10 ng/ml of HGF, 10 ng/ml CNTF, and 10 ng/ml GDNF. We, therefore, supplemented our culture medium with the above mentioned NTFs cocktail for our experiments. Cells were plated in NBMc medium with the cocktail of NTFs and were kept in CO_2 incubator. MNs

were maintained between 12-15 days in culture. Aphidicolin (APH) was added to culture medium to eliminate the mitotic cell division of non-neuronal cells. The efficacy of the purification is evaluated by using an antibody against Islet 1/2; a transcription factor expressed only in MNs, and Hoechst dye for nuclear localization **(Figure 27).**

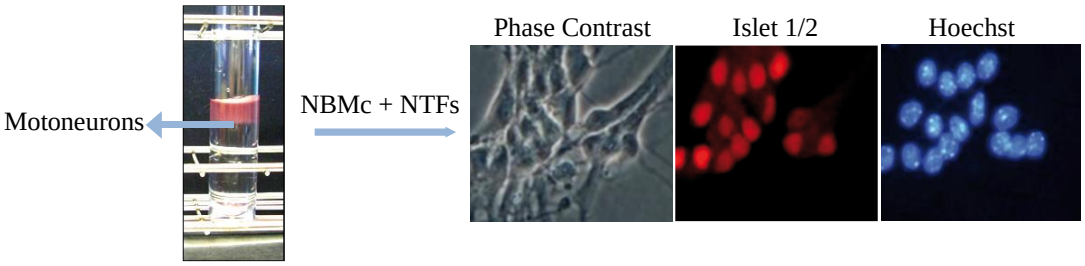

Figure 27. MNs are collected from Optiprep density gradient and seeded in NBM complete medium supplemented with NTFs. Image showing phase contrast, immunofluorescence microscope image stained with Islet ½, and Hoechst dye.

2.2.3. 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+/+* (mutSMA). The littermates *Smn+/+; SMN2+/+* (WT) were used as controls.

2.2.3.1. Genotyping

For MN purification E12.5 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.

2.2.3.1.1. DNA extraction protocol

Fifty µl DNA extraction mix (Three volumes (75 µl) of extraction buffer was added to one volume (25 µl) of Tissue Prep) was added to each sample and incubated for 10 min at room temperature (RT). Later, the samples were incubated at 95°C for three min (using thermomixer). Fifty µl of neutralization buffer was added to each sample and centrifuged at 13000 rpm for 10 min. Then, the supernatant was collected 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.

2.2.3.1.2. PCR DNA amplification

The reaction mixture for PCR was prepared following the protocol.

Mix	Volume (µl)
Buffer red extraction	4.65
Sterile H_2O 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 10. PCR mix preparation**.**

The 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'

2.2.3.1.3. PCR cycles

$$\underset{35\ \text{cycles}}{94°C\ 5'//94°C\ 1'/62°C\ 1'30''/72°C\ 1'30''//72°C\ 7'//4°C\infty}$$

2.2.3.1.4. 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.

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

2.3.1. RNA Interference Technique

Ribonucleic acid (RNA) interference (RNAi) is a naturally occurring intracellular mechanism, which causes sequence specific post-transcriptional gene silencing. The reaction is triggered by the introduction of double-stranded (*ds*) RNA into the cytoplasm of the cell, and results in the specifically targeted destruction of mRNA, and a subsequent reduction in protein production (Elbashir et al. 2001). RNAi takes place predominantly within the cytoplasm of the cell and is triggered by the introduction of a *ds* oligonucleotide into the cell cytoplasm. The mechanism is mediated by the activation of two major molecules; the initial activity of the endonuclease Dicer (an RNAse III family enzyme), followed by the activity

of the RNA interfering silencing complex (RISC) (Chiu & Rana 2002). An adenosine triphosphate (ATP) dependent reaction involving the endonuclease Dicer is responsible for cleaving the long *ds* nucleotide into short interfering (si) *ds* RNAs, 21–23 nucleotides (nt) in length. RISC then unwinds the double-stranded siRNA, using a helicase, and subsequently binds to the free antisense strand. This complex can identify the specific complementary strand of mRNA and degrades it with the help of one of its primary components, Argonaute 2 protein (Song et al. 2004; Liu et al. 2004). The result is the destruction of the mRNA that is complementary to the antisense strand of the original *ds*RNA introduced into the cytoplasm, and prevention of translation and protein production.

RNAi can be initiated by transient transfection of small interfering RNAs (siRNAs) or stable expression of small hairpin RNAs (shRNAs). The latter are integrated into the genome and intracellularly processed to siRNAs. siRNA expression by transfection leads to a transient knockdown, and due to limitations in transfection methods, is limited in its application to a small range of cell lines. Stable expression of shRNAs allows a constitutive mRNA knockdown. Using a lentiviral transfer approach, the expression range of shRNAs can be extended to non-replicative primary cells, and animals. In the present study, we have used shRNA (small hairpin RNA), an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNAi to reduce the levels of NF-κB pathway family member proteins, and Smn protein in cultured spinal cord MNs. Expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors. We used lentiviral vectors to deliver our shRNA constructs.

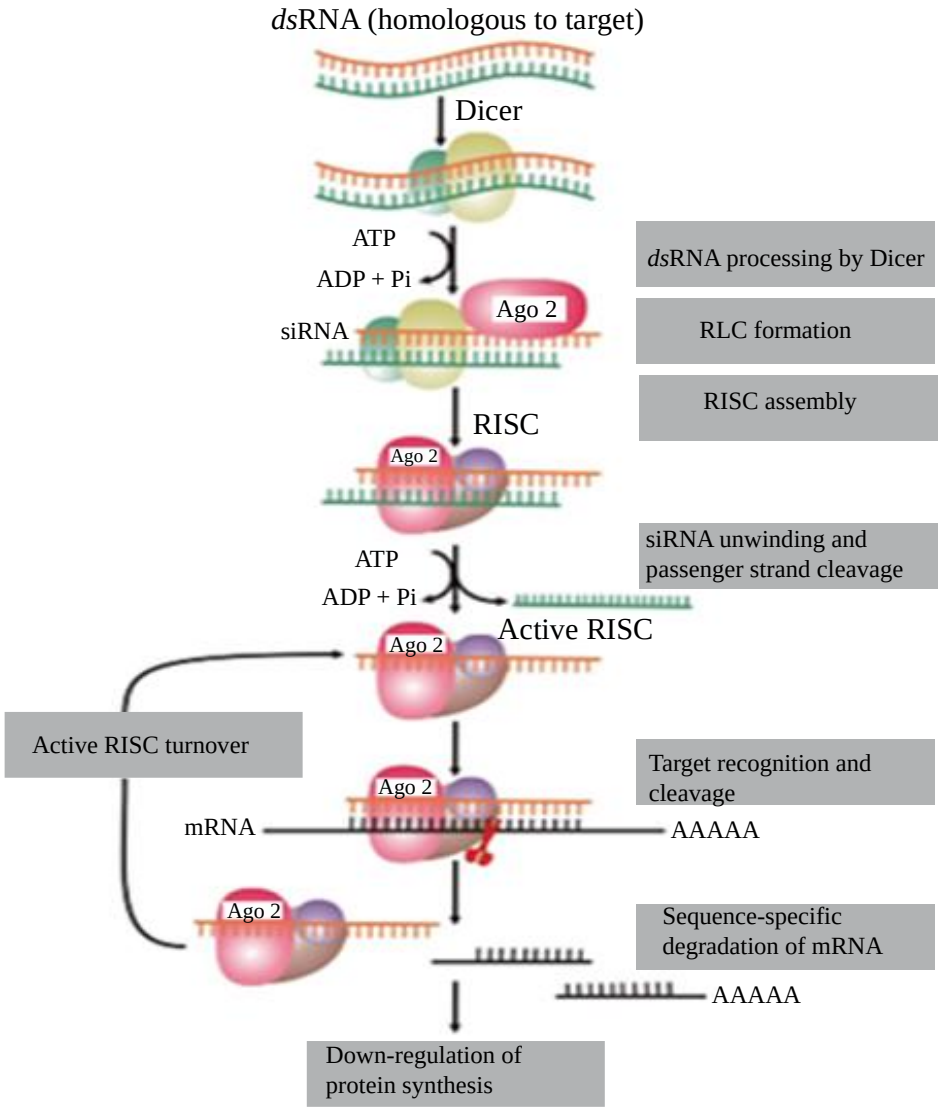

Figure 28. The RNAi process and biochemical machinery involved. *ds*RNA is processed into short pieces (siRNA) by the endonuclease Dicer. The siRNA is loaded into the RNA-induced silencing complex (RISC) via the RISC loading complex (RLC), followed by cleavage and release of the passenger strand. The guide strand then associates with a homologous mRNA strand by conventional base pairing, and the mRNA strand is cleaved by RISC and released for further degradation within the cytoplasm (Barnard et al. 2012).

Lentiviruses belong to the class of retroviruses, which means that they integrate their genome into the host cell's DNA. Commonly used lentiviral vector systems belong to the 2nd or 3rd generation, ensuring safe application, as these viruses are unable to self-replicate, since the spontaneous self-assembly is prevented by distributing the least necessary number of virus elements on three and four plasmids, respectively. Directed virus assembly takes place via transient transfection of the viral plasmids into helper cells, which secrete the virus into the supernatant, from which it can be collected. Commonly available vector systems differ only in their generation and their 'application' plasmid. We have used the 2nd generation lentiviral system which comprises three plasmids.

2.3.1.1. Transfer plasmid/lentiviral vector

Lentiviral transfer plasmid encodes the insert of interest. This sequence is flanked by long terminal repeats (LTRs) that facilitate host genome integration. To improve safety, transfer vectors are all replication incompetent and may additionally contain a deletion in the 3'LTR, rendering the virus "self-inactivating" (SIN) after integration.

RNA sequences for silencing the genes, IKKα, IKKβ, RelA, and RelB were cloned in FSV vector. FSV consisted of a U6 promoter for expression of short-hairpin RNAs (shRNAs) and the Venus variant of green fluorescent protein (GFP) under the control of an SV40 promoter for monitoring transduction efficiency (Dolcet et al. 2006).

For Smn RNAi experiments, constructs were generated in pSUPER.retro.puro (OligoEngine, Seattle, WA, USA). Oligonucleotides were obtained from Invitrogen. Adaptors to clone the oligonucleotides into the BglII/HindIII sites of pSUPER.retro.puro were added as required. Lentiviral constructs were generated by digesting pSUPER-sh with EcoRI and ClaI to replace the H1 promoter with the H1-short hairpin RNA (shRNA) cassette in pLVTHM. pLVTHM vector contains the GFP under the control of an EF-1α promoter for monitoring transduction efficiency. Vector pLVTHM was a gift from Didier Trono (Addgene plasmid # 12247).

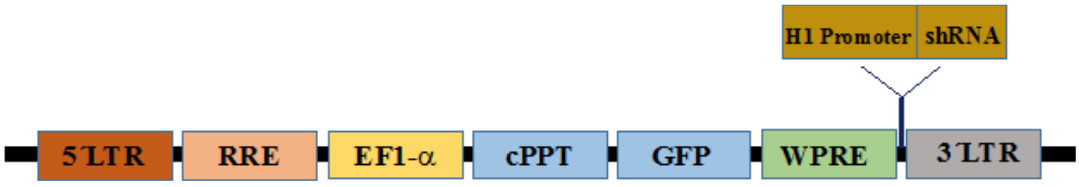

Figure 29. Vector pLVTHM (**http://tcf.epfl.ch/page-20849-en.html**).

2.3.1.2. Packaging plasmid

We have used psPAX2 vector, an 2nd generation lentiviral packaging plasmid which include all the important packaging components **CAG** (promoter), **Gag** (gene coding for different proteins of the nucleocapsid and matrix), **Pol** (gene coding for reverse transcriptase, protease and for integrase), **Rev** (gene encoding a protein necessary for the regulation of the transport and processing of RNA), **Tat** (gene encoding a protein required for transactivation) for producing viral particle, **RRE** ("Rev-responsive element" needed for action Rev). Vector psPAX2 was a gift from Didier Trono (Addgene Plasmid #12260).

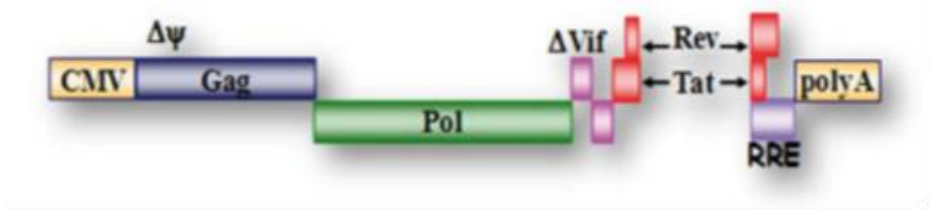

Figure 30. Vector psPAX2 (**http://tcf.epfl.ch/page-20849-en.html**).

2.3.1.3. Envelope plasmid

We have used pMD2G, the **VSV-G** (The vesicular stomatitis virus (***VSV***) ***G protein***) expressing envelope plasmid, which is flanked by a cytomegalovirus (CMV) and a **polyA** tail sequence. VSV-G protein facilitates the infection of infection of a wide variety of tissues and cell lines. Vector pM2 was a gift from Didier Trono´s Lab.

Figure 31. Vector pM2 (http://tcf.epfl.ch/page-20849-en.html).

2.3.2. Lentivirus production

2.3.2.1. Preparation of collagen plates

For virus production, Collagen was used at working concentration of 100 µg/ml in acetic acid 0.02N. An adequate volume of solution was added in each 100 mm plate and left overnight at RT to dry. Once the plates were dry, they were stored at 4°C (up to 15 days).

Transfer Plasmid	Target Protein	RNAi Sequence	Precedence
pSUPER pLTVHM	SMN	5'-TGACAAGTGTTCTGCTGTT-3'	Dr.A. Garcera
FSV	IKKα	5′-CAGGCTCTTTCAGGGCAT-3′	Dr.M. Encinas
FSV	IKKβ1	5′-GCTGCACATTTGAATCTGTAT-3′	Dr.M. Encinas
FSV	IKKβ2	5′-GCTCTTAGATACCTTCATGAA-3′	Dr.M. Encinas
FSV	RelA	5′-AGATCTTGAGCTCGGCAGTG-3′	Dr. X.Dolcet
FSV	RelB	5′-GCATGCGCTTCCGCTACGAGT-3′	Dr. X.Dolcet

Table 11. Lentiviral vectors and the RNAi sequence cloned

To produce the lentiviral particles carrying the siRNA or over-expression sequences, we used the HEK-293T cell line. HEK-293T cells are transfected with plasmids required for

producing lentivirus using polyethyleneimine (PEI). The PEI is obtained by polymerization of aziridine (ethyleneimine) forming a highly branched network with a high potential to bind with cationic DNA. PEI has been used commonly as a polymeric agent for condensing the plasmid DNA that could strongly promote the arrival of DNA into the nucleus of mammalian cells. It has been shown that cationic lipid-DNA complexes can bind and interact with proteoglycans expressed on the surface of mammalian cells, promoting their intracellular incorporation.

To produce lentiviral particles, HEK-293T cells were seeded in collagen coated 100 mm plates at a density of $1.5x10^6$-$2x10^6$ cells, 24 h before transfection. For PEI transfection, the DNA was mixed as follows:

Lentiviral Vector	20 µg
pSPAX2	13 µg
pM2	7 µg

Table 12. The mixing ratio of plasmids.

2.3.2.2. Protocol for preparing one plate lentivirus

The plate was washed with 5 ml opti-MEM and left in the incubator with 4 ml opti-MEM. Two different mixes, PEI-optiMEM mix, and DNA-optiMEM mix, were prepared.

DNA-optiMEM	20 µg Lentiviral vector + 13 µg pSPAX2 + 7 µg pM2 + 1500 µl opti-MEM
PEI-optiMEM	240 µl PEI +1260 µl opti-MEM

Table 13. DNA-optiMEM and PEI-optiMEM mix.

PEI-optiMEM mix was added to the DNA-optiMEM mix (dropwise), and vortexed at 1800 rpm for 2-5 s, and let at RT for 10 min. Then the mix was added to the plate with HEK-293T cells (dropwise) and incubated at 37°C. After 1.5 h to 4 h, the medium was changed to DMEM medium. The cells were analyzed via fluorescence microscopy to determine the efficiency of transfection. The percentage of GFP^+ cells should be between 70-100%. After 48-72 h, the medium was collected, centrifuged at 2500 rpm for 10 min, and the supernatant was filtered with a 0.45 µm diameter filter. Aliquots of the medium containing the virus particles in 2 ml cryogenic vials were stored at 4°C for immediate use, and at -80°C for long term usage.

Biological titers of the viral preparations were expressed as the number of transducing units per ml (TU/ml). For titration, three wells of 20,000 HEK-293T cells/well were seeded in a 24 well plate (2 cm2/well) for each virus to titer. Two hours later, each one

of the three wells 50 µl, 100 µl, and 150 µl of the medium containing virus was added. After 72 h photographs were made using an inverted microscope to assess the GFP^+ cells of the total number of cells. The titer corresponds to a number of cells seeded, and is calculated as follows:

$$\frac{\%GFP^+ \text{ cells X } 2x10^4 \text{ seeded cells}}{\text{Volume of virus added}}$$

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

2.3.4. Molecular Biology Techniques

2.3.4.1. Transformation in *E. coli*

We have performed the transformation in *E. coli* cells to amplify the number of the plasmid of our interest. We have used competent cells from strain DH5α. To allow entry of the DNA plasmid, these cells were submitted to heat shock. Competent *E.coli* were cultured in 20 ml LB-medium at 37°C overnight at 200 rpm in a shaking incubator. Four ml of this culture was used to inoculate 400 ml culture; cultured at 37°C at 200 rpm until the optical density (OD): 0.375. Bacteria were incubated for 10 min on ice and then centrifuged at 4°C, for 10 min at 4000 rpm. All further steps were performed at 4°C. The pellets were re-suspended in 40 ml cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% Glycerol, 10 mM HEPES), and again centrifuged for 5 min at 4000 rpm at 4°C. The pellet was then re-suspended in 40 ml of cold $CaCl_2$ solution, incubated for 30 min on ice, and again centrifuged for 5 min at 4000 rpm at 4°C. The pellet was re-suspended in 8 ml of cold $CaCl_2$ solution, incubated on ice for 4 h. Aliquots of 400 µl were prepared, shock frozen in liquid Nitrogen, and stored at -80°C.

One hundred µl of competent *E.coli* cells were thawed on ice and incubated 20 min on ice with a variable amount of DNA. After a 45 s heat shock at 42°C, and 2 min incubation on ice, 1 ml pre-warmed LB-medium was added, and the mixture was incubated at 37°C, shaking for 60 min. Later, the mixture was centrifuged for 2 min at 5000 rpm. Six hundred µl of the supernatant was collected and discarded. The other half of cells remaining in the Eppendorf tube were re-suspended, and 150 µl was finally plated on LB plates with ampicillin (50 µg/ml) for the plasmid DNA selection, and incubated at 37°C, overnight. Next

day, a colony was taken, and cultured in 5-10 ml of LB medium containing ampicillin at 37°C with shaking for 15-20 h.

The selection of the bacteria which have incorporated the plasmid was made by adding ampicillin, as the plasmids of our interest is ampicillin resistant. Whenever we make this process, is very important to use a negative control.

2.3.4.1.1. a) Glycerol Stock

Nine hundred ul of medium with grown colony was taken in a frozen tube, and 600 ul of 50% glycerol (to make it to a final concentration of 30%) was added, mixed well, and frozen quickly with liquid nitrogen. The tubes were stored at -80°C.

2.3.4.1.2. b) Plasmid Extraction

In an Erlenmeyer flask, 1-2 ml of the colony was grown in 250 ml LB with ampicillin for 15-20 h. To extract the plasmid DNA of E. coli, we have used two commercial kits: NucleoBond Xtra Maxi Plasmid DNA purification (Macherey-Nagel), and Qiagen Maxi plasmid purification (Qiagen) following the protocols indicated by the corresponding manuals.

In Xtra Maxi plasmid purification kit, the extraction process involves the first step, wherein, the bacterial cells are re-suspended, and plasmid DNA is liberated by alkaline SDS. Then, plasmid DNA is bound to a silica membrane, to remove all contaminants and finally the DNA is eluted.

The Qiagen Maxi plasmid purification kit is based on an alkaline lysis followed by binding of plasmid DNA to an ion exchange resin. Plasmid DNA was eluted in a buffer with high salt content. The eluting solution was discarded, and the DNA was concentrated by precipitation with isopropanol and resuspended with milliQ H2O. The purified DNA was quantified by NanoDrop spectrophotometer.

2.3.5. Inhibitors

In this study we have used two types of inhibitors; NF-κB signaling pathway activation inhibitor and the proteasome inhibitor.

i) NF-κB signaling pathway activation inhibitor

We have inhibited the NF-kB pathway by using the SN50 (30 μM) inhibitor peptide, a cell-permeable peptide that blocks the nuclear localization sequence of p50, and inhibits

translocation of NF-kB active complex to the nucleus (Lin 1995). SN50M (30 μM) was used as its negative control.

ii) The proteasome inhibitor

We have used MG-132 (5 μM), as a proteasome inhibitor, to investigate, whether Smn protein is degraded by ubiquitin/proteasome pathway.

2.3.6. Immunoblot Technique

Immunoblot (Western blot) is an analytical method to separate, and identify proteins, wherein a mixture of proteins are separated based on molecular weight, and thus by type, through gel electrophoresis. The separated proteins are electro-transferred onto a membrane. The transferred proteins were detected using a specific primary antibody, and secondary enzyme labeled antibody, and substrate.

2.3.6.1. Cell lysates

We have obtained the total cell lysates after different treatments or desired growing time to analyze the proteins by immunoblot under denaturing conditions. To this end, samples were collected with lysis buffer containing the denaturing detergent Sodium dodecyl sulphate (SDS).

The medium of the plate was aspirated, and the MNs were rinsed four times with cold PBS to stop the intracellular reactions. The lysis buffer was added, and the cells were scraped from the bottom of plates using pipette tips. The samples were collected in an eppendorf tube and were heated at 95°C for 5 min to denature the protein. The samples were stored at -20°C for later use or submitted to gel electrophoresis. In the case of cell lines, samples were well sonicated to break the intracellular structures before heating.

Lysis Buffer	123 mM Tris-HCl, 2% SDS (pH 6.8)

2.3.6.2. Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Analytical electrophoresis of proteins was carried out in polyacrylamide gels under conditions that ensure the dissociation of the proteins into their individual polypeptide subunits, and that minimize aggregation. Polyacrylamide gels were prepared by free radical induced polymerization of acrylamide with N, N'-methylene bisacrylamide as cross-linker. Ammonium per sulphate (APS) and N, N, N, N'-tetramethylethylenediamine (TEMED) were added as a provider, and stabilizer of free

radicals, respectively. The strong anionic detergent SDS was used in combination with heat to dissociate proteins before they were loaded on the gel. SDS is an amphipathic detergent. It has an anionic head group and a lipophilic tail. It binds non-covalently to proteins, with a stoichiometry of around one SDS molecule per two amino acids. SDS causes proteins to denature, and disassociate from each other (excluding covalent cross-linking). It also confers negative charge. In the presence of SDS, the intrinsic charge of a protein is masked. During SDS-PAGE, all proteins migrate toward the anode (the positively charged electrode). SDS-treated proteins have very similar charge-to-mass ratios and similar shapes. During PAGE, the rate of migration of SDS-treated proteins is effectively determined by molecular weight. β-Mercaptoethanol was added to the loading buffer to reduce disulphide bonds.

2.3.6.2.1. Sample Preparation

We have quantified the protein by NanoDrop spectrophotometer which requires only 2 µl of sample. The samples were adjusted to equal protein concentrations through dilution in the corresponding lysis buffer. Protein samples received one-fourth of their volume loading buffer (5x). Five µl of loading buffer (5X) was added to 20 µl of cell lysates (10-50 µg of protein). The samples were heated at 95°C for 5 min, and centrifuged (10000 rpm for 5 min).

Loading Bufffer (5X)	10% SDS, 50% Glycerol, 375 mM Tris-HCl (pH 6.8), 25% β-Mercaptoethanol, 1% Bromophenol blue

2.3.6.2.2. Protocol

The running gel and stacking gel solutions were prepared according to the table given below **(Table 15)**. Polymerization was started by adding TEMED and ammonium persulphate (APS). The solution was carefully mixed and poured into the gap between two glass plates of a slab gel unit. Then the running gel was cautiously overlaid with isopropanol. After completion of the polymerization (30-45 min), the overlay was poured off preceding complete removal by washing with milliQ H_2O, and dried with the edge of filter paper. The stacking gel solution was then poured directly onto the surface of the polymerized running gel, and a clean plastic comb was inserted immediately, avoiding the formation of air bubbles. After complete polymerization, the comb was removed, and the slab gel unit was inserted into the electrophoresis chamber filled with electrophoresis buffer.

The molecular marker was loaded to the gel followed by the protein samples. Electrophoresis was performed at 15 mA/gel till the sample reaches the separating gel and

then increased to 20 mA/gel. When the bromophenol blue had attained the bottom of the running gel, electrophoresis was stopped. The gel slab units were disassembled and prepared for immunoblotting.

Stacking gel buffer (1X)	0.5 M Tris-HCl (pH 6.5)
Running gel buffer (1X)	1.5 M Tris-HCl (pH 8.6)
Electrophoresis buffer (1X)	25 mM Tris-HCl + 192 mM Glycine + 0.1% SDS (pH 8.3)

Table 14. Immunoblot Buffers

Components	Running gel		Stacking gel
	7%	8%	3%
milliQ H_2O	4.98 ml	4.64 ml	3.8 ml
Acri:Bis 30%-0.8%	2.32 ml	2.66 ml	0.6 ml
Stacking gel buffer (1X)	-	-	1.5 ml
Running gel buffer (1X)	2.5 ml	2.5 ml	-
10% SDS	100 µl	100 µl	60 µl
APS	80 µl	80 µl	40 µl
TEMED	10 µl	10 µl	10 µl

Table 15. Composition for gel preparation

2.3.6.2.3. Immunoblotting

Proteins separated by SDS-PAGE can be identified by binding specific antibodies. An antigen-antibody reaction cannot properly occur inside the polyacrylamide gel so that the proteins have to be first transferred to a membrane before exposure to the antibodies. We have used polyvinylidene difluoride (PVDF) membranes from with 0.45 µm pore size (Millipore). The transfer of proteins to the PVDF membrane was performed directly after electrophoresis. The proteins were negatively charged at the transfer pH, due to the bound SDS so that they would migrate from the cathode to the anode.

In our laboratory, we have used Semi-dry transfer method using Amersham Biosciences semidry Trans-Blot. The PVDF membranes were activated in methanol for 5 min 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 a shaker. Air bubbles were removed by rolling a glass pipette over the sandwich. The sandwich of transfer was made using blotting paper, membrane, gel, and blotting paper, as shown in the figure. Transfer of the proteins was performed at a constant amperage of 60 mA/gel for 60 min.

Transfer Buffer (1X)	48 mM TRIS-HCl, 39 mM Glycine, 0.0375% SDS, 10% Methanol

2.3.6.2.3.1. Immunodetection of blotted proteins

Membranes were incubated with the respective primary antibodies. Subsequently, they were incubated with the secondary antibodies, specific for the common structure of the primary antibodies. The secondary antibodies were coupled to peroxidase to produce a chemiluminescent signal, and can be detected on X-ray films or by using imaging instruments.

2.3.6.2.3.2. Blocking of membranes

Once proteins were transferred to PVDF membrane, the membrane was blocked for 2 h at RT with TBST 1X + 5% fat-free milk powder to prevent the membrane from non-specific binding of antibodies. Then the membrane was washed for three times of 5 min for each wash with TBST to remove the excess of blocking solution.

TBST Buffer (1X)	20mM TRIS-HCl + 125 mM NaCl + 0.1% TWEEN 20

Primary antibody incubation

The antibody is specific for the protein of our interest. The primary antibody was diluted in TBST and 0.02% sodium azide. The membrane was incubated with primary antibody for 2 h at RT or overnight at 4°C temperature. After overnight incubation, the membrane was continuously incubated in primary antibody for 1 h at RT. Then, the membrane was washed three times for 5 min with TBST to remove residual sodium azide residues. The list of primary antibodies used is given below **(Table 16)**.

Secondary antibody incubation

After rinsing the membrane to remove unbound primary antibody, the membrane was exposed to horseradish peroxidase (HRP) conjugated secondary antibodies, and the secondary antibody binds to the primary antibody which has reacted with the target protein. We have used anti-mouse and anti-rabbit immunoglobulin as secondary antibodies, as the host species for primary antibodies are mainly mouse and rabbit **(Table 17)**. The secondary antibody was prepared with 5% milk powder and incubated for 1 h at RT. The membranes were finally rinsed several times with TBST to eliminate the excess of secondary antibody.

Primary Antibodies	Species	Dilution	Commercial Brand
anti-SMN	Mouse	1:5000	BD Transduction Laboratories
anti-IKKα	Mouse	1:1000	Calbiochem
anti-IKKβ	Rabbit	1:1000	Calbiochem
anti-RelA	Rabbit	1:1000	Cell Signaling TECHNOLOGY
anti-CREB	Rabbit	1:1000	Cell Signaling TECHNOLOGY
anti-phospho- RelA (Ser536)	Rabbit	1:1000	Cell Signaling TECHNOLOGY
anti-RelB (C-19)	Rabbit	1:1000	Santa Cruz Biotechnology
anti-α-Tubulin	Mouse	1:50000	Sigma

Table 16. List of Primary Antibodies.

Secondary Antibody	Species	Dilution	Commercial Brand
anti-Mouse IgG-HRP	Mouse	1:20000	Sigma
anti-Rabbit IgG-HRP	Mouse	1:20000	Amersham

Table 17. List of Secondary Antibodies.

Blots were developed using the chemiluminescent kits **(Table 18)**, according to the manufacture's recommendation. In the chemiluminescence reaction, HRP catalyzes the oxidation of luminol into a reagent which emits light when it decays. As the oxidation of luminol is catalyzed by horseradish peroxidase, and the HRP is complexed with the protein of interest on the membrane, the amount and location of light that HRP catalyzes the emission of, is directly correlated with the site and amount of protein on the membrane.

Chemiluminescent substrates	Commercial Brand
Super Signal chemiluminescent substrate	Pierce
ECL Advance Western Blotting Detection Kit	Amersham Biosciences
Luminata ™ Forte Western HRP substrate	Millipore Chemicals
EZ-ECL Chemiluminescence Detection Kit	Biological Industries

Table 18. List of Chemiluminescent Kits**.**

Acquisition and image analysis

The images were acquired by using ChemiDoc XRS (Bio-Rad) machine. The band intensity of target proteins was analyzed using Quantity One or ImageJ software.

2.3.7. RNA preparation

RNA was prepared by using "NucleoSpin® RNA" columns (Macherey-Nagel, Düren, Germany), and eluted in a volume of 40 µl RNase-free water. The concentration of

the RNA was determined spectrometrically, and adjusted to 0.1µg/µl. In short, cultured MNs are lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases which are present in virtually all biological materials and creates appropriate binding conditions which favor adsorption of RNA to the silica membrane. Contaminating DNA, which is also bound to the silica membrane, is removed by an rDNase solution which is directly applied onto the silica membrane during the preparation. Simple washing steps with two different buffers remove salts, metabolites, and macromolecular cellular components. Pure RNA is finally eluted under low ionic strength conditions with RNasefree H_2O. The RNA preparation using NucleoSpin® RNA kits can be performed at room temperature. To maintain RNA stability, isolated RNA was kept frozen at -20°C for short-term or -80°C for long-term storage. To avoid RNAse contamination, disposable plastic ware, and RNAse-free water was used whenever possible.

2.3.7. 1. Reverse transcription

Total RNA was reverse transcribed with Reverse Transcriptase in 10X polymerase chain reaction (PCR) buffer, 10 mM dNTPs, 25mM $MgCl_2$, and Random hexanucleotide primers.

2.3.7.2. The first strand cDNA synthesis from total RNA

One thousand ng of total RNA was used as a template for the reverse transcription in 40 µl volume. RNA sample was boiled at 90°C for 2 min and kept immediately on ice before performing the reaction mix. The reaction mix and the PCR conditions are given below **(Table 19 & Table 20)** and the synthesized cDNA was stored at -20°C.

PCR Reaction Mix	
RNA	10 µl
H_2O	6 µl
PCR Buffer (10X)	4 µl
10 mM dNTPs	8 µl
25 mM $MgCl_2$	9 µl
Random Hexanucleotide Primers	2 µl
Reverse Transcriptase	1 µl

Table 19. PCR Mix for cDNA Synthesis.

Denaturation	90°C	2 min
Annealing	25°C	5 min
Extension	42°C	60 min
Inactivation	92°C	5 min
Storage	4°C	Hold

Table 20. PCR Conditions for cDNA Synthesis.

2.3.7.3. PCR amplification of cDNA

The cDNA transcribed from the total RNA was further amplified in a polymerase chain reaction. This is a thermal cycling technique that is used for direct amplification of specific regions of DNA. One amplification cycle includes three steps; a denaturation step where double-stranded DNA is separated, a hybridization step where primers hybridize to the target sequence, and an elongation step where a DNA polymerase replicates the single stranded DNA from the 3'end of the annealed primers to the complementary sequence. The number of cycles used depends on the concentration of the product of interests within the sample.

Gene	Primer Sequences
Smn	Forward: 5′ CCGCCACCCCCTCCCATCTCT 3′ Reverse: 5′ CATCTCCTGAGACAGAGCTGA 3′
Gapdh	Forward: 5′ TGCACCACCAACTGCTTAG 3′ Reverse: 5′ GGATGCAGGGATGATGTTC 3′
RelA	Forward: 5′ CGAGCTCAGTGAGCCCATG 3′ Reverse: 5′ GGCACAGCAATACGCCGGG 3′
IKKα	Forward: 5′ CACCGTGCAGAGTCAGGAC 3′ Reverse: 5′ CTCCATCTTGAGGAGTTAC 3′
IKKβ	Forward: 5′ GGAGAAGCGGCAGAAGGAAC 3′ Reverse: 5′ CTTCACAGTGTCCTGCAGCG 3′

Table 21. Sequences used in DNA Amplification.

Eight µl of reverse transcribed cDNA was used for PCR amplification with specific primer pairs **(Table 22)**. The reaction volume was 40 µl.

The final PCR products were electrophoresed on 3% agarose gels containing *SYBR® Safe* along with DNA markers using TAE buffer. The intensity of the PCR product bands was determined by using the image analysis software, Gel DocTM EZ imager.

TAE Buffer (1X) pH 8.0	**20 mM TRIS-HCl, 1 mM EDTA, 1.14 ml Acetic acid**

PCR Mix for cDNA Amplification	
cDNA	8 µl
H_2O	14.48 µl
PCR buffer (10X)	4 µl
10 mM dNTPs	8 µl
25 mM $MgCl_2$	2 µl
Reverse Primer	1.6 µl
Forward Primer	1.6 µl
Taq polymerase	0.32 µl
Total Volume	40 µl

Table 22. PCR Mix for DNA Amplification.

PCR conditions for DNA Amplification			
Pre-denaturation	94°C	5 min	
Denaturation	94°C	2 s	
Annealing	55°C	30 s	30 Cycles
Extension	72°C	30 s	
Storage	4°C	Hold	

Table 23. PCR Conditions for DNA Amplification.

2.3.8. Quantitative real-time PCR

In contrast to traditional PCR, the quantitative real-time PCR (qPCR) is both qualitative and quantitative. The principle of qPCR is the same as for the conventional PCR, but, the amplified DNA is detected in real time (between each round of amplification) instead of a detection of the amplified DNA only at the end of the reaction. In this study, the iTaq™ Universal SYBR® Green Supermix was used according to the manufacturer's instructions.

Components	20 µl Reaction
iTaq Universal SYBR Green supermix (2x)	10 µl
Forward Primer (10 µM)	0.8 µl
Reverse Primer (10 µM)	0.8 µl
cDNA template	1 µl (24 ng/µl)
RNAse free milliQ H_2O	7.4 µl
Total Volume	20 µl

Table 24. Mix for Real-Time PCR.

PCR reaction was done with 96 well plates (Hard-Shell 96-well plates, clear well, white shell) purchased from BioRad (Ref. HSP-9601), covered with optical micro seal adhesive covers from BioRad (Ref. MSB-1001). The instrument used was CFX96 Real-Time System (BioRad). All genes of interest and the reference gene (*Gapdh*) were run in

triplicates, and three independent experiments were performed for each assay. CFX Manager Software v. 3.1 (BioRad) was used to analyze the data, giving the value of the Relative Gene Expression in each case, through normalization by *Gapdh*, and by reference to the Control situation.

Step	Temperature	Duration	Cycles
Polymerase Activation/DNA Denaturation	95°C	30 s	45 Cycles
Denaturation	95°C	5 s	
Annealing/Extension	60°C	30 s	
Melt curve	Instrument Default Settings		

Table 25. Thermal Cycling Conditions for Real-Time PCR.

2.3.9. Statistics

All experiments have 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

Reagent setup

Boric Borate Buffer (pH 8.3)

- Dissolve separately
  - Solution (i): 3.04 g Sodium tetraborate in 100 ml milli-Q H_2O.
  - Solution (ii): 4.6 g Boric acid in 300 ml milli-Q H_2O, and then, another 200 ml milli-Q H_2O was added.
- Mix Solution (i), and Solution (ii).
- The pH was adjusted to pH 8.3.
- The solution was filtered through 0.22 µm filter in the hood and stored at 4ºC.

GHEBS

- 8 g NaCl, 0.2 g KCl, and 4 g Glucose was dissolved in 750 ml milli-Q H_2O.
- 25 ml of 1M HEPES, 2 ml of Penicillin / Streptomycin was added.
- The pH was adjusted to 7.4 with NaOH (never with KCl).
- The final volume was made up to 1 L with milli-Q H_2O.
- The solution was filtered through 0.22 µm filter and stored at 4ºC.

Poly-DL-ornithine

A stock solution of 10 mg/ml poly-DL-ornithine (Sigma) was prepared with Boric-borate buffer (pH 8.3). The stock solution was made into 1 ml aliquots and stored at -80ºC. Aliquots of 35µl were stored at -20 ºC. The working concentration is 35 µg/ml in the same Boric-borate buffer (35 µl stock solution of poly-DL-ornithine in 10 ml Boric-borate buffer).

Laminin

Aliquots of 38 µl of the stock solution of 1 mg/ml were stored at -80ºC. The working concentration of 3.8 µg/ml was prepared with serum-free basal L-15 medium (38 µl Laminin in 10 ml L-15 medium).

Glucose

A stock of 3.6 g of D-glucose in 50 ml L-15 medium was prepared, and the aliquots of 1.25 ml were stored at -20ºC.

L-glutamine

Aliquots of 125 µl (200 mM) were stored at -20ºC.

β-mercaptoethanol

Aliquots of 50 µ1 (25 mM) of β-mercaptoethanol (19 µl of β-mercaptoethanol dissolved in 10 ml L-15 medium) were stored at -20ºC.

Horse serum

Aliquots of 1 ml, and 500 µl heat inactivated (at 55°C) horse serum were stored at -20°C.

N-2 supplement

Aliquots of 250 µl N-2 supplement were stored at -20°C.

B-27 supplement

Aliquots of 1 ml B-27 supplement were stored at -20°C.

Neurotrophic factors

CNTF, CT-1, GDNF, and HGF

L-15 medium with 10% HS was prepared and filtered using 0.22 µm filter. NTFs (CNTF, CT-1, GDNF, and HGF) of each 10 µg was dissolved in 1 ml of the prepared L-15 medium with 10% HS. Aliquots of 30 µl were stored at -80°C. This 30 µl of NTFs were added to 30 ml NBMc medium to obtain the working concentration of 10 ng/ml.

BDNF

L-15 medium with 10% HS was prepared and filtered using 0.22 µm filter. BDNF (10 µg) was dissolved in 1 ml of the prepared L-15 medium with 10% HS. Aliquots of 50 µl (Intermediate concentration) were stored at -80°C. In the 50 µl aliquot, 450 µl of was added, and aliquoted as 30 µl and were stored at -80°C. This 30 µl of NTFs were added to 30 ml NBMc medium to obtain the working concentration of 1 ng/ml.

BSA 4%.

8 ml of BSA 7.5% was dissolved in 7 ml L-15 medium.

Optiprep

For 2 tubes; 12% optiprep was prepared by dissolving 1 ml of optiprep in 7 ml GHEBS.

Aphidicolin

One mg of Aphidicolin was dissolved in 166.6 µl DMSO (17.73 mM), and the aliquots of 10 µl were stored at -20°C. Each aliquot was dissolved in 390 µl NBMc+NTFs medium (443.25 µM) and stored. The working concentration of 1.5 µg/ml (4.43 µM) was obtained by dissolving 10 µl of the intermediate stock (443.25 µM) in 1 ml of NBMc+NTS medium.

DNase

A stock solution of 20 mg/ml was prepared by mixing 5 ml GHEBS in 100 mg DNase, and the aliquots of 1 ml were stored at -20°C. To work, 1 ml of the stock solution was mixed with 19 ml L-15 medium, and the aliquots of 600 µl were stored at -20°C.

Trypsin

Trypsin without EDTA (T4424-sigma) was stored at -20°C as 1 ml aliquots.

Acrylamide/Bis-acrylamide	BioRAd (1610158)
Agarose	Sigma (A5093)
Ammonium Per Sulphate	Sigma (A3678)
Aphidicolin	Sigma (A0781)
B27	Gibco (17504-04)
Boric acid	Merck (1.00165.0500)
Bovine serum albumin	Sigma (A8412)
Bromophenol blue	Sigma (B0146)
Calcium Chloride	Sigma (22.350-6)
D-Glucose	Sigma (G7021)
DMEM, high glucose, pyruvate	Gibco (41966-029)
DMEM, high glucose, without pyruvate	Gibco (41965-039)
Dnase	Roche (10104159001)
EDTA	Sigma (E5134)
Fetal Bovine Serum	Gibco (10270-106)
Glutamin	Gibco (25030-024)
Glycine	Sigma (G7126)
HEPES	Sigma (H3375)
HEPES (1M)	Gibco (15630-056)
Horse Serum	Gibco (16050-122)
Human BDNF	Bionova (450-02-10)
Human HGF	Bionova (100-39-10)
Isopropanol	Fisher Scientific (102842)
KH_2PO_4	Merck (1.04873.1000)
L-15 Medium	Gibco (11415-049)
Laminin	Sigma (L2020)
Methanol	Fisher Scientific (1067-5112)
Murine Cardiotrophin	Bionova (250-25-10)
Murine GDNF	Bionova (450-44-10)
N-2	Gibco (17502-048)
$Na_2HPO_4.7H_2O$	Sigma (S9390)
Neurobasal Medium	Gibco (21103-049)
Optiprep	Axisshield (1114542)
PBS-10X	Gibco (10010-031)
Penicillin and Streptomycin	Gibco (15140-122)
poly-DL-ornithine	Sigma (P8638)
Potassium Chloride	Merck (1.04936.0250)
Sodium Chloride	Sigma (71376)
Sodium tetraborate	Merck (1.06306.0250)
SYBR safe	Life technologies (S33102)
TEMED	Sigma (T9281)
Trizma Base	Sigma (T1503)
Trypsin	Gibco (25200-056)
Trypsin without EDTA	Sigma (T4424-sigma)
Tween 20	Sigma (P1379)

β-Mercaptoethanol	Sigma (M7522)
β-Mercaptoethanol	Sigma (M3148)
DMSO	Sigma (D2650)
Collagen, Type I from Rat tail	Sigma (C7661)
Acetic acid, glacial	Fisher (10394970)
PEI	Sigma (408727)
Optimem	Life Technology (31985047)
Super Script II Reverse Transcriptase (10,000U)	Life Technology (18064-014)
Taq Polymerase, (5U/µl), 1000U	Biotools (10048)
d'NTPs	Sigma (D7295)
Magnesium Chloride	Sigma (63069)
Extraction kit SIGMA: RED Extract-N-Amp Tissue PCR kit.	Sigma (XNAT-100RXN)
Magnesium chloride	Sigma (M8266-100G)
Protein ladder	BenchMark pre-stained protein ladder
	Biorad (All Blue) (161-0373)
	Biorad (Kaleidoscope) (161-0375)
DNA ladder, 1 kb	Bioron, (305105)
TAE, 10X	Sigma (T9650)

Table 26. List of Chemicals Used.

100 mm plates	BD Falcon Bioscience Corning (353003)
60 mm plates	BD Falcon Bioscience Corning (353004)
6-well plates	BD Falcon Bioscience-Corning (353046)
12-well plates	BD Falcon Bioscience Corning (353043)
4-well tissue culture dish	Nunc, Thermo Fisher Scientific (10507591)
Tubes 50ml	Fisher (6537X)
Polypropylene centrifuge tubes, 15 ml	Fisherbrand (05-539-12)
Green cap tubes, 12ml	Deltalab (301402)
White cap tubes	Fisher (144AS)
Polypropylene Cryogenic Vial, 2 ml	Corning (430488)
PCR Tubes, 0.2 ml Flat Cap (Natural)	Fisher, (14230225)
Pasteur pipettes (150mm)	Quimega (6085779)
Syringe, 50 ml	Fisher (1640M)
Syringe, 20 ml	Fisher (1640H)
Syringe 10 ml	BD Plastipak (302188)
0.22 µm pore size (PES) membrane.filter	Merck Millipore (SLGP033RB)
Steritop-GP, 0.22 µm, polyethersulfone, 150 mL 45 mm	Merck Millipore (SCGPT01RE)
Ultracentrifuge	Optima L-100XP, Beckman Coulter
	Avanti J-26 XP, Beckman Coulter

Centrifuge	5804, Eppendorf
Microcentrifuge	MiniSpin, Eppendorf
Water bath	Precisterm, Selecta
	Clifton
	Lauda Alpha
Stereo Microscope	SZX7, Olympus
	ACH 1X, Olympus
	SZ, Olympus
Inverted Microscope	IX71, Olympus
Halogen Lamp Power Supply Unit	TH4-200, Olympus
Power supply unit	U-RFL-T, Olympus
Magnetic Stirrers	Big squid, IKA
	Agimatic-E, Selecta
Vortex mixer	ZX3, VELP Scientifica
Roller mixers	SRT6, Stuart
Thermomixer	Thermomixer comfort, Eppendorf
Orbital Platform Shaker	Unimax 1010, Heidolph
Mini Shaker	IKA MS2
Shaker	Unimax 1010, Heidolph
	Heidolph Reax 2000
Incubator shaker	Unitron
Incubators	Thermo Forma
	Forma Scientific, Inc.
	Memmert
Vertical Laminar Flow Benches	AV-100, Telstar
Biological Safety Cabinet	Bio II A, Telstar
Fume Cupboards	Burdinola
Steam sterilizer	Raypa
Drying and sterilization ovens	Selecta
	Thermo fisher scientific
Block heater	Stuart Scientific
Water Purification System	Advantage A10
	Millipore
Cryopreservation tank	170 L, Arpege
Imaging System	ChemiDoc MP TM system, Bio-rad
	Gel Doc EZ imager, Bio-rad
Electrophoresis Power Supply Unit	Bio-Rad
	EPS 300 Electrophoresis, Pharmacia Biotech
	EPS 301, GE Healthcare, Life Sciences
Immunoblot Transfer unit	ECL TE 70 Semi-Dry Transfer Unit, GE Healthcare Amersham
Spectrophotometer	ND-1000, NanoDrop
Ice Machine	Manitowoc
Ultrasonic Disintegrator	Soniprep 150
Vacufuge Concentrator	Concentrator plus, Eppendorf

pH Meter	pH 1500, Eutech
Thermal cycler	T100, Bio-rad
Precision Weighing Balances	Acculab Atilon ALT 220-4NM, Kern
Electrophoresis unit	Bio-rad
PVDF membrane	Merck-Millipore (PVH00010)
Blot paper	ACEFESA (10426994)
Tally Counter	
Haemocytometer	

Table 27. List of Materials & Instruments Used.

Chapter III Results

Part 1: Members of NF-kB pathway regulate Smn protein level in cultured spinal cord motoneurons

3.1.1. Effect of IKKα or IKKβ knockdown in NSC-34 cell line

RNAi represents a robust method to inhibit gene expression at the post-transcriptional level. To substantiate the role of IKKα or IKKβ in regulating Smn protein level, we performed lentiviral-based knockdown of IKKα or IKKβ in NSC-34 cell line; a good model for neuronal studies. To this end, we have generated the shRNA sequences targeting the specific sites of mouse IKKα, and IKKβ, and produced lentiviral particles containing the shRNAs.

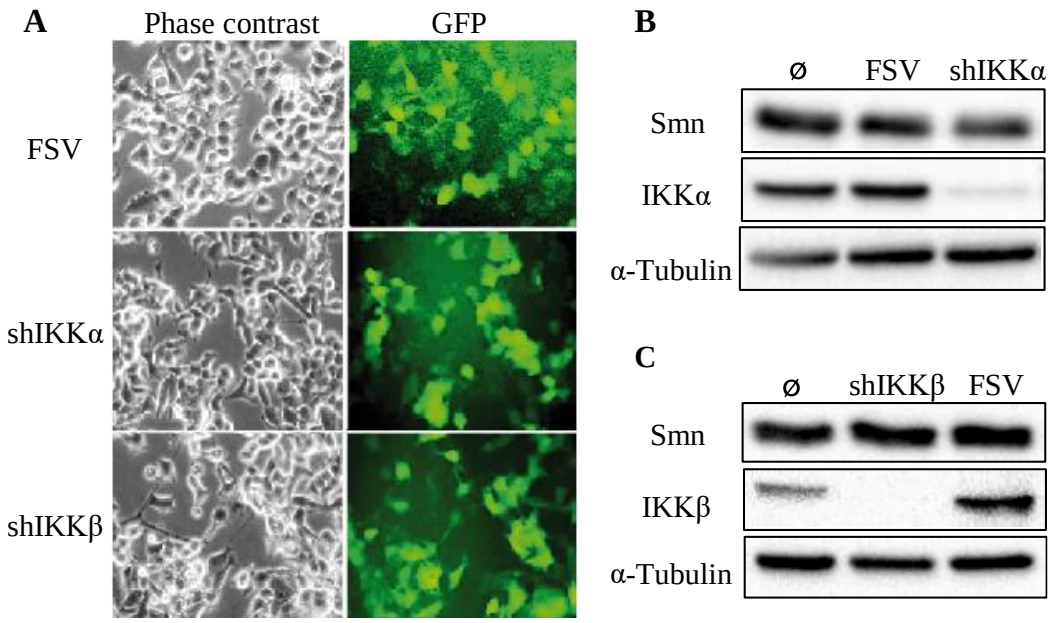

Figure 32. NSC-34 cells were transduced with FSV or shIKKα or shIKKβ lentiviral constructs. **A)** Representative microscopy images of 72 h FSV- (control) or shIKKα- or shIKKβ-transduced NSC-34 cells - phase contrast (left) and GFP (right). GFP indicates green fluorescent protein-expressing cells in the same microscopic field. **B)** Representative immunoblot for shIKKα-transduced cells – After 72 h of transduction, the protein extracts of NSC-34 cells transduced with shIKKα or FSV or non-transduced cells (Ø) were collected, and immunoblot analysis was performed. The membrane was probed with anti-Smn, and anti-shIKKα antibodies. All the membranes were reprobed with an antibody against α-Tubulin as a loading control. **C)** Representative immunoblot for shIKKβ-transduced cells - NSC-34 cell extracts were collected after 72 h of transduction with shIKKβ lentiviral particles. The extracts were processed and submitted to immunoblot analysis against Smn, and IKKβ antibodies. Non-transduced cells (Ø) were used as an additional control. The same membranes were reprobed against α-Tubulin as a loading control.

NSC-34 cells were transduced with shIKKα or shIKKβ or shRNA empty vector control (FSV) with an appropriate amount of lentiviral particles. Twenty hours later, the medium containing lentiviral particles was substituted with DMEM containing 10% FBS. The transduction efficacy was visualized 72 h after lentiviral treatment. The GFP^+ cells were monitored by fluorescence microscopy **(Figure 32).** The assessment of IKKα or IKKβ protein inhibition was performed by immunoblot analysis. A significant decrease in the level of

respective protein was observed IKKα or IKKβ inhibited cells relative to their controls **(Figure 32B & Figure 32C).** In shIKKα-transduced cells, Smn was decreased compared to the non-transduced cells (ø) or to the FSV-transduced cells **(Figure 32B)** whereas, Smn protein level was increased in IKKβ-inhibited cells compared to the controls **(Figure 32C)**.

Our initial idea was to check the performance of the lentiviral particles. Therefore, we initially performed our experiments in NSC-34 cell line. But we found difficulty in maintaining the number of NSC-34 cells due to its capacity to undergo rapid cell division. Due to this reason, they lost their MN-like morphological character. These cells have to

be plated in appropriate cell density to maintain their MN-like characteristics. Meanwhile, our lentiviral system started working in the spinal cord MNs culture. Hence, we decided to continue our experiments using isolated spinal cord MNs cultures.

3.1.2. NF-kB inhibitor decreases the Smn in NTF-stimulated spinal cord MNs

To investigate the role of NF-kB signaling pathway in regulating Smn protein level, we pharmacologically inhibited the NF-kB pathway by using the SN50 inhibitor peptide. Purified spinal cord MNs from E12.5 CD1 mouse embryos were cultured (50000 cells/well) in NBMc medium. After 2 h, the NBMc medium was replaced with the medium containing NTFs cocktail with or without SN50 (30 μM) or its inactive control peptide SN50M (30 μM).

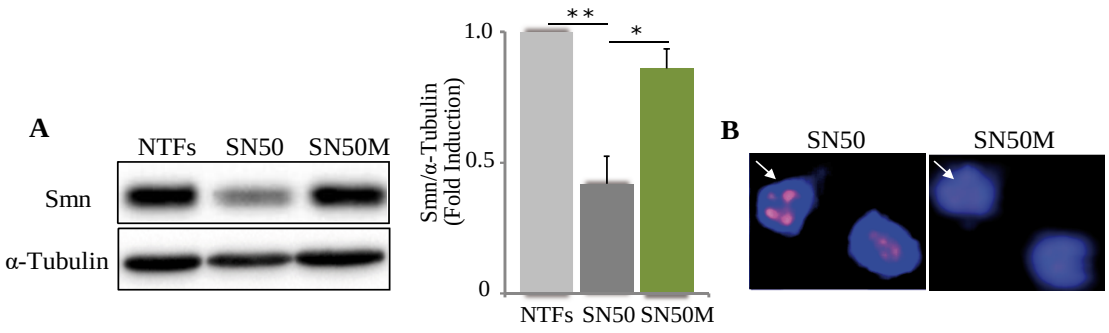

Figure 33. NF-kB inhibitor decreases Smn protein: A) Representative immunoblot for Smn protein from the MN cell lysates cultured in the presence of NTFs cocktail or the NF-kB inhibitor, SN50 (30 μM) or its inactive control SN50M (30 μM). Cell lysates were collected after 72 h and were analyzed by immunoblot technique. The membrane was probed with anti-Smn antibody and was reprobed with an antibody against α-Tubulin, as a loading control. The graph represents the Smn protein level after treated with NTFs, and the NF-kB inhibitor, SN50 (30 μM) or its inactive control SN50M (30 μM). Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using one-way ANOVA test and Bonferroni post-hoc multiple comparisons (*p<0.05; **p<0.005). **B)** Representative microscopy images of Hoechst staining of MNs cultured in the presence of SN50, and SN50M. The arrow shows the apoptotic nuclear morphology.

The presence of the NF-kB inhibitor, SN50 in the culture medium significantly decreased Smn protein level in the MNs when compared to its negative control, SN50M or the MNs cultured in the presence of NTFs **(Figure 33A)**. Hoechst staining of MNs showed that the presence of NF-κB inhibitor in the NTFs-supplemented culture medium induces apoptosis in MNs **(Figure 33B)**. This result shows that the NF-κB pathway regulates the Smn protein level in cultured spinal cord MNs.

3.1.3. RelA, but not RelB, inhibition decreases the Smn in spinal cord MNs

To determine which of the NF-κB activation pathway is involved in the regulation of Smn protein in spinal cord MNs, we have analyzed the effect of RelA or RelB knockdown on Smn protein level. RelA/p50 is the most prominent, and extensively studied NF-kB dimer activated in the canonical pathway, whereas, RelB/p52 heterodimer participates in the non-canonical scheme (Perkins 2007). We have generated shRNA targeting specific sites of mouse RelA (shRelA) or RelB (shRelB) sequences. Spinal cord MNs were isolated, and purified from mouse embryos, and maintained in the presence of NTFs (50000 cells/well). Two hours after plating, lentiviral particles of shRelA or shRelB or FSV were added. Twenty hours later, the medium was washed, and replaced with fresh medium supplemented with NTFs, and APH.

As shown in **Figure 34A & Figure 35A**, the frequency of transduction was near 100% of the cells present in the culture (GFP^+ cells, monitored by fluorescence microscopy). MN cell extracts from shRelA-transduced cells were collected after five days, whereas MN cell extracts from shRelB-transduced cells were collected after seven days, and analyzed by immunoblot method. The level of RelA or RelB proteins in shRelA or shRelB-transduced cultures, respectively, was clearly reduced compared to the FSV-transduced cells (**Figure 34B & Figure 35B)**. Reduction of endogenous RelA significantly decreased Smn protein level **(Figure 34B)**. However, the knockdown of RelB had no effect on Smn protein level **(Figure 35B)**. This result concludes that the Smn protein expression is regulated only by NF-κB canonical pathway and not by the non-canonical pathway.

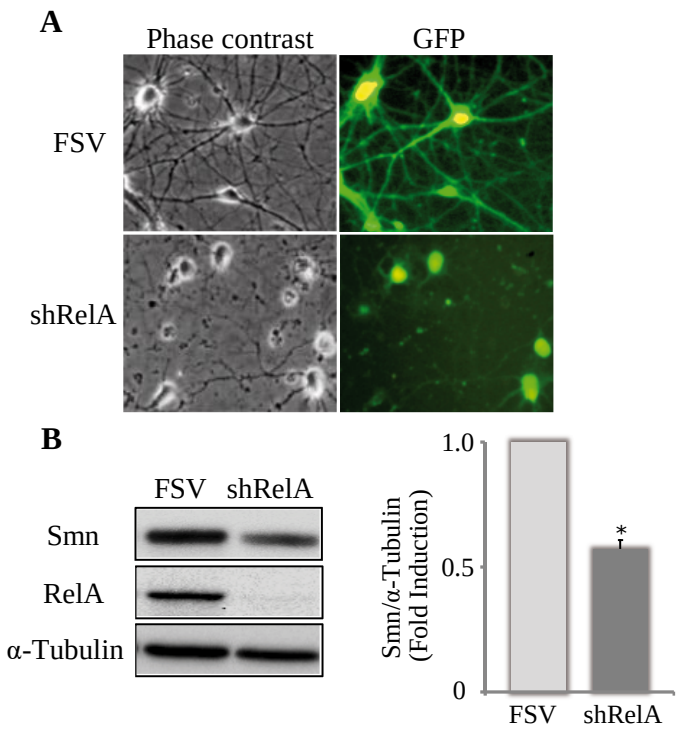

Figure 34. RelA inhibition decreases Smn protein level: A) Representative microscopy images of 5 days FSV- (control) or shRelA-transduced MNs: phase contrast (left), and GFP (right) in the same microscopic field. **B) Immunoblot analysis of Smn protein level in shRelA transduction -** The protein extracts of MNs transduced with shRelA or FSV were probed with anti-Smn, anti-RelA antibodies. All the membranes were reprobed with an antibody against α-Tubulin as a loading control. The graph represents the Smn protein level after FSV or shRelA transduction. Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using student´s t-test (*p<0.05).

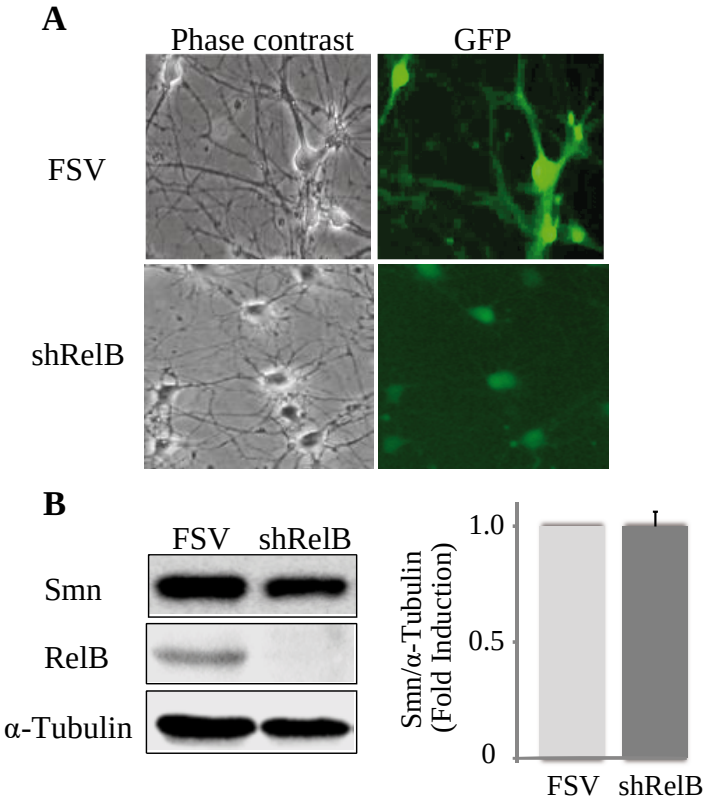

Figure 35. RelB inhibition has no effect on Smn protein: A) Representative microscopy images of 5 days FSV- (control) or shRelB-transduced MNs: phase contrast (left) and GFP (right) in the same microscopic field. **B) Immunoblot analysis of Smn protein level in shRelB transduction -** After seven days of interference with FSV (control) or shRelB lentiviral constructs, cell extracts were collected and were probed with antibodies against Smn or RelB proteins. All the membranes were reprobed with an antibody against α-Tubulin as a loading control. The graph represents the Smn protein level after FSV or shRelB transduction. Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars).

3.1.4. IKKα inhibition decreases Smn protein level in cultured MNs

In our previous study, we have demonstrated that in the developmental model of spinal cord MNs, the inhibition of IKKα, one of the two catalytic subunits in NF-κB pathway, even in the presence of NTFs affects the MN survival by inducing apoptotic cell death (Mincheva et al. 2011). In the present study, to assess the relevance of IKKα, in regulating Smn protein level in spinal cord MNs, we have performed lentiviral-based knockdown of IKKα.

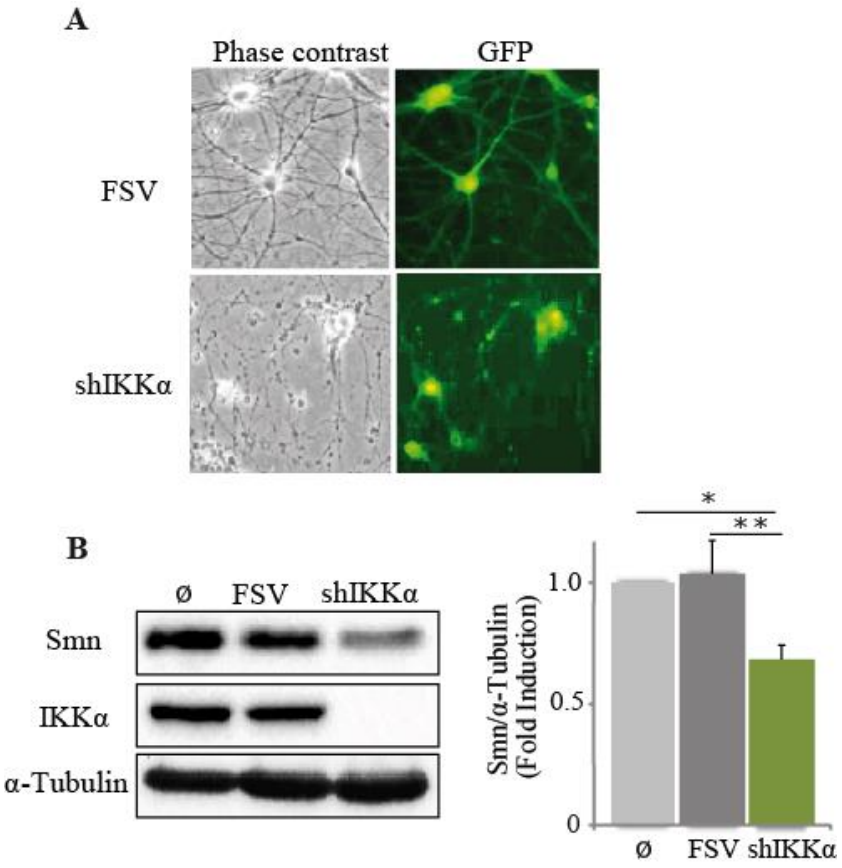

Figure 36. Knockdown of IKKα decreases Smn protein level: A) Representative microscopy images of 3 days of FSV (control) or shIKKα-transduced MNs: phase contrast (left) and GFP (right) of the same microscopic field. **B) Representative immunoblot for Smn protein level in shIKKα-transduced MNs**. After five days of shIKKα transduction, the cell lysates were collected and analyzed by immunoblot technique using specific antibodies against IKKα and Smn. α-Tubulin was used as a loading control. The graph represents the Smn protein level after FSV or shIKKα transduction or the non-transduced cells. Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using one-way ANOVA test and Bonferroni post-hoc multiple comparisons (*p<0.05; **p<0.005).

Cultured MNs (80000 cells/well) were transduced with the lentiviral particles of shIKKα or FSV. As shown in **Figure 36A**, the frequency of transduction is 99% of the cells present in the culture dish (GFP^+ cells monitored by fluorescence microscope). The level of IKKα protein in shIKKα-transduced cultures was clearly reduced compared to the FSV-

transduced cells or to the non-transduced cells (ø) **(Figure 36B)**. Knockdown of IKKα protein in the cultured embryonic spinal cord MNs significantly decreased the Smn protein level when compared to the controls; non-transduced cells (ø) or to the FSV-transduced cells **(Figure 36B)**.

3.1.5. IKKβ inhibition on the Smn protein level in NTF-maintained MNs

IKKβ knockout mice studies suggest that IKKβ is the predominant kinase responsible for IκBα, and p105 phosphorylation (Li et al. 1999), and also, the knockdown of IKKβ in NTF-supplemented MNs induces apoptotic cell death (Mincheva et al. 2011). To assess the relevance of IKKβ in regulating the Smn protein level, we performed lentiviral-based knockdown of IKKβ in MNs.

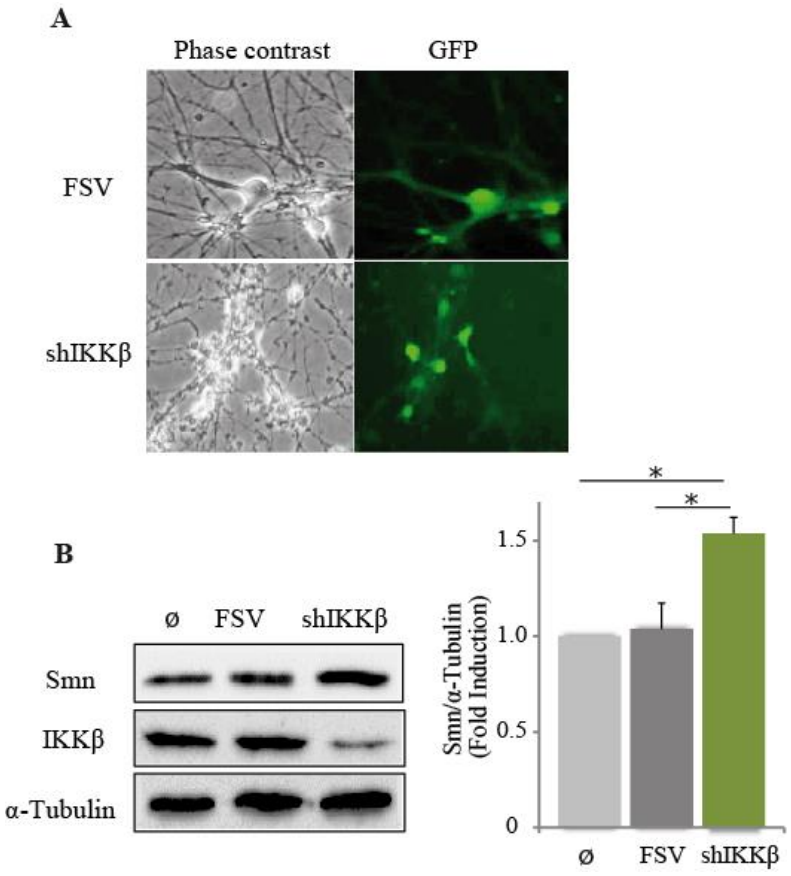

Figure 37. IKKβ inhibition increases Smn protein: A) Representative microscopy images of 3 days of FSV (control) or shIKKβ-transduced MNs: phase contrast (left) and GFP (right) of the same microscopic field. **B) Representative immunoblot for Smn protein level in IKKβ inhibited MNs.** After five days of shIKKβ transduction, cells were collected, and the protein extracts were analyzed by immunoblot. The membranes were probed with specific antibodies against IKKβ and Smn**.** For the loading control, all the membranes were reprobed with an antibody against α-Tubulin. The graph represents the Smn protein level after FSV or shIKKβ or non-transduced cells. Graph values are the mean for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using one-way ANOVA test and Bonferroni *post-hoc* multiple comparisons (*p<0.001).

Cultured MNs (80000 cells/well) from mouse embryos spinal cords were maintained in the presence of NTFs. Lentiviral particles of shIKKβ or FSV were added to the culture. Twenty hours later, the medium was washed and replaced with fresh medium containing APH. The frequency of transduction was 99% of the cells present in the culture dish (GFP positive cells monitored by fluorescence microscope) **(Figure 37A)**. IKKβ protein level in shIKKβ-transduced culture was reduced compared to the FSV-transduced cells or to the non-transduced cells (ø) **(Figure 37B)**. The Smn protein level in the shIKKβ-transduced MN cultures was significantly increased compared to the MN culture transduced with the FSV-transduced cells or to the non-transduced MNs **(Figure 37B)**.

3.1.6. Effect of both IKKα and IKKβ reduction in Smn protein level in MNs

IKKα and IKKβ kinases have different functions based on their cellular distribution. In the present work, we have demonstrated that IKKα knockdown significantly decreases Smn protein level, whereas, the inhibition of IKKβ increases Smn protein significantly. To further analyze the effect of IKKα, and IKKβ reduction on Smn, we performed lentiviral-based double knockdown of IKKα and IKKβ in the developmental model of spinal cord MNs.

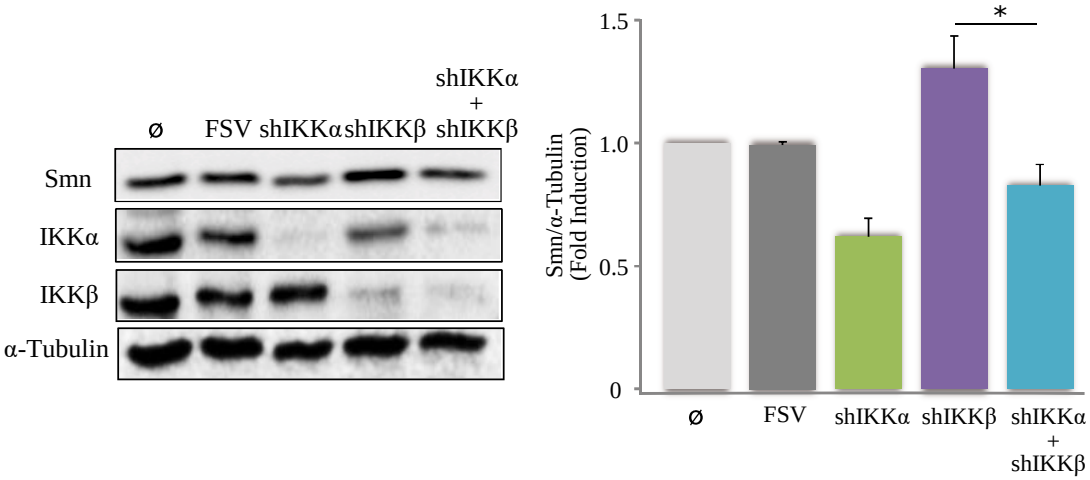

Figure 38. Immunoblot analysis of Smn protein levels in shIKKα and shIKKβ transduction: After four days, the cell lysates from MNs transduced with shIKKα and/or shIKKβ were analyzed by immunoblot for Smn protein. The membranes were probed with anti-Smn, anti-IKKα or anti-IKKβ antibodies. For the loading control, all the membranes were reprobed with an antibody against α-Tubulin. The graph represents the Smn protein level after FSV or shIKKα and/or shIKKβ transduction or the non-transduced cells. Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using one-way ANOVA test and Bonferroni post-hoc multiple comparisons (*p<0.05).

MNs were transduced with FSV control or shIKKα or shIKKβ or shIKKα plus shIKKβ. The level of IKKα and IKKβ protein in shIKKα plus shIKKβ-transduced cultures was clearly reduced compared to the control cells **(Figure 38)**. As we demonstrated before,

IKKβ protein reduction significantly increased Smn protein level. However, the inhibition of both IKKα and IKKβ proteins significantly decreased the Smn protein level compared to the shIKKβ-transduced cells **(Figure 38)**. This result shows that in the absence of the IKK complex Smn protein level is not significantly different to control, suggesting that Smn increase observed in IKKβ-reduced cells can be prevented by IKKα reduction.

3.1.7. Knockdown of IKKα and/or IKKβ decreases RelA phosphorylation

Our previous, and current results demonstrated that the canonical NF-κB pathway is essential for the regulation of Smn protein level in MNs. In this pathway, the activated IKK complex phosphorylates IκBα which is degraded via the 26S proteasome, thereby exposing the NLS on RelA, and inducing nuclear translocation of p50/RelA dimers (Perkins 2007). NTFs can induce IKK phosphorylation and nuclear translocation of RelA. However, in the absence of NTFs, RelA is maintained primarily in the cytoplasm (Mincheva et al. 2011). This study clearly indicates that the nuclear translocation of RelA depends on the activation of the IKK complex. To investigate the effect of IKKs knockdown on RelA protein level, and activation, we assessed the level of RelA phosphorylation (p-RelA) at Ser536 residue and total RelA protein in shIKKα or shIKKβ MNs. Inhibition of IKKα, and IKKβ protein significantly decreased the p-RelA level **(Figure 39A)**, whereas, the total RelA protein level **(Figure 39B)** was not altered. This result demonstrates that IKK complex is essential for the NF-κB pathway activation.

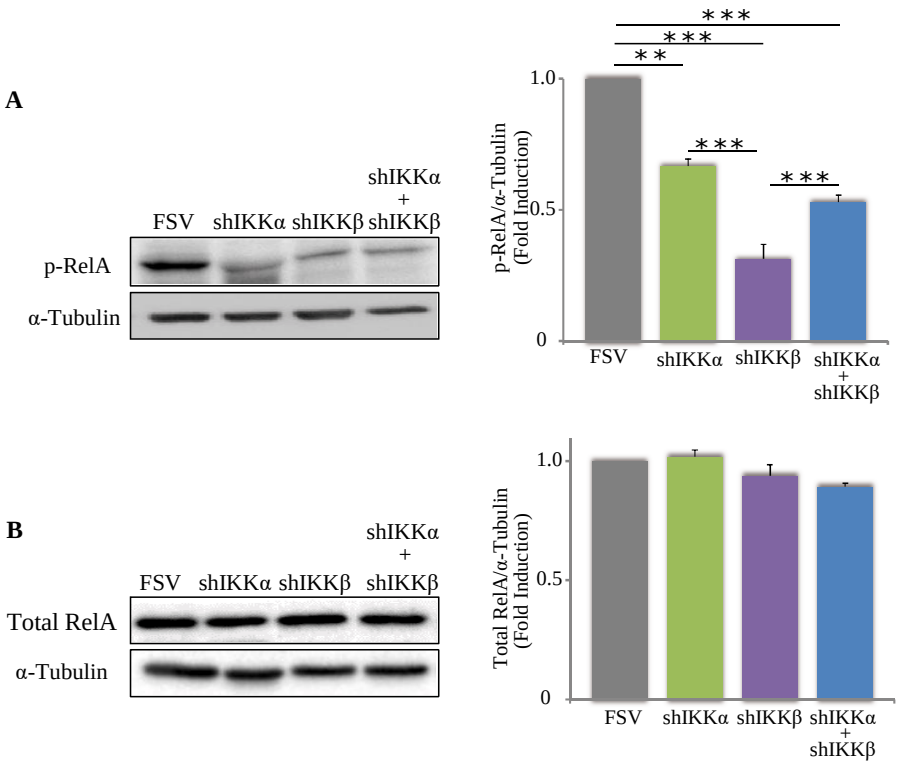

Figure 39. Immunoblot analysis of p-RelA (Ser536) in shIKKα and/or shIKKβ transduced cells. NTF-maintained MNs were transduced with lentivirus of shIKKα or shIKKβ or FSV. After four days, MNs were collected and analyzed by immunoblot technique.The protein extracts were probed with the p-RelA (Ser536) antibody. All the membranes were reprobed with an antibody against α-Tubulin, used as a loading control. The graphs represent the p-RelA (Ser536) level after FSV or shIKKα and/or shIKKβ transduction. Graph values are the mean of the expression level of p-RelA for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using one-way ANOVA test and Bonferroni post-hoc multiple comparisons (**p<0.005; ***p<0.0005). **B) Immunoblot analysis of total RelA in shIKKα and/or shIKKβ transduction.** Cells were collected, and the lysates of MNs were probed with RelA antibody. All the membranes were reprobed with an antibody against α-Tubulin, used as a loading control. The graph represents the total RelA level after FSV or shIKKα and/or shIKKβ transduction. Graph values are the mean of the expression level of Smn protein for each condition of three independent experiments ± SEM (error bars).

3.1.8. Effect of IKKα or IKKβ or RelA inhibition on Smn mRNA in embryonic spinal MNs

In the present study, we have demonstrated that canonical NF-κB pathway activation regulates the Smn protein expression. Inhibition of RelA protein or the IKKα protein induces a significant reduction in Smn protein level, whereas the knockdown of IKKβ significantly increases the Smn protein expression level. To assess the role of NF-κB pathway in the regulation of *Smn* mRNA level, we performed lentiviral-based knockdown of IKKα or IKKβ or RelA in MNs.

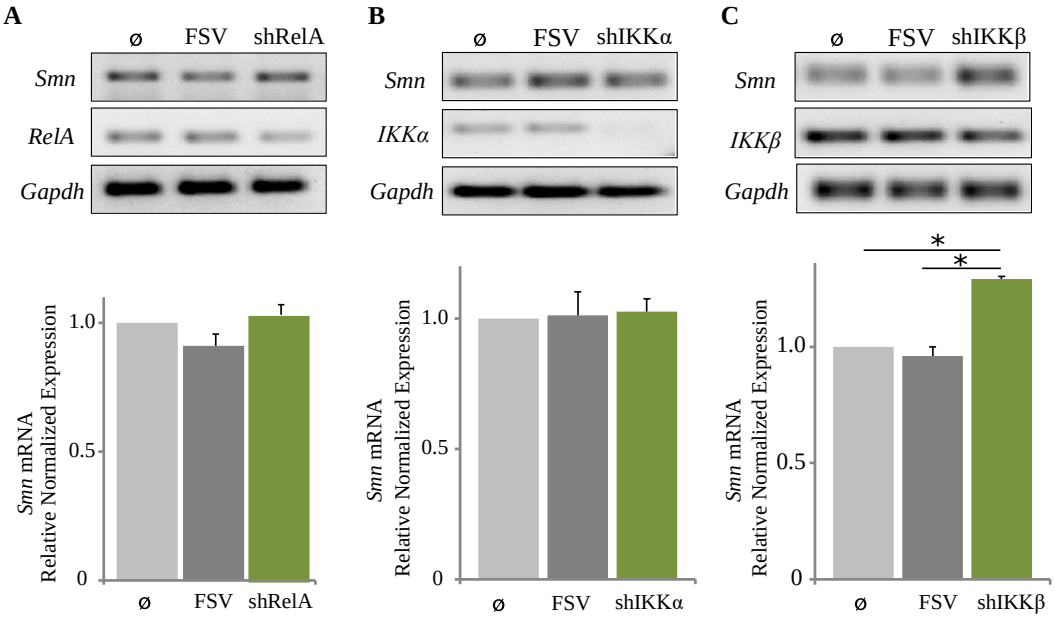

Figure 40. Semi-quantitative RT-PCR analysis of *Smn* mRNA level in RelA-, shIKKα-, and shIKKβ-transduced MNs: After four days, total RNA from MN cell extracts of shRelA or -shIKKα or -shIKKβ or FSV-transduced cells were collected, and analyzed by semi-quantitative RT-PCR using specific primers for *Smn* gene. The house-keeping gene, *Gapdh* was used as a control**. A)** The graph represents the expression level of *Smn* mRNA after FSV or shRelA transduction. **B)** The graph represents *Smn* mRNA level after FSV or shIKKα transduction. **C)** The graph represents the expression level of *Smn* mRNA after FSV or shIKKβ transduction. Graph values are the mean of the expression level of *Smn* mRNA for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using one-way ANOVA test and Bonferroni post-hoc multiple comparisons (*p<0.05).

MNs from E12.5 mouse embryos were plated and maintained in the presence of the NTFs (300000 cells/condition). Culture medium containing NTFs, and lentivirus of shRelA or -shIKKα or -shIKKβ or FSV was added to replace the old medium after two hours of plating. Total RNA from MN cell extracts was obtained, and one µg of RNA was first to reverse transcribed into cDNA using a reverse transcriptase. The resulting cDNA was used as templates for subsequent PCR amplification using primers specific for *Smn* gene. There were no changes in *Smn* mRNA levels in shRelA **(Figure 40A)** or shIKKα-transduced MNs **(Figure 40B)**, whereas inhibition of IKKβ protein significantly increases *Smn* mRNA level **(Figure 40C)**.

3.1.9. Quantitative RT-PCR analysis of Smn mRNA in shIKKβ-transduced MNs

To determine whether the changes of Smn protein level after IKKβ knockdown resulted from the activation or the inhibition of *Smn* gene expression, we quantified by real-time RT-PCR (qRT-PCR) the *Smn* mRNA. *Gapdh* gene was used as a control. Cultured MNs (300000 cells/condition) were transduced with shIKKβ or FSV lentiviral constructs. After four days of transduction, total RNA was extracted, and reverse transcribed to cDNA, used as a template to quantify *Smn* transcript level. Results shown in **Figure 41** reveal that IKKβ reduction causes an increase in *Smn* mRNA expression. These results demonstrate that IKKβ reduction regulates Smn at the transcriptional level.

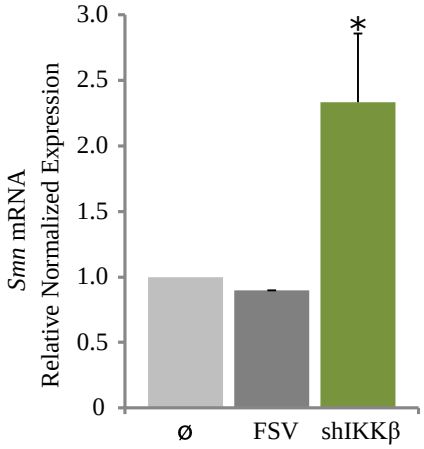

Figure 41. qRT-PCR analysis of *Smn* mRNA level in shIKKβ-transduced MNs: Graph values are the mean of *Smn* gene expression for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using one-way ANOVA test and Bonferroni *post-hoc* multiple comparisons *(*p<0.05)*.

3.1.10. Smn protein is degraded via ubiquitin/proteasome pathway (UPP)

Intracellular proteins can be degraded or cleaved by several different proteolytic systems. The proteasome process is an important mechanism of intracellular protein

degradation (Lecker et al. 2006). Previous studies have demonstrated that the SMN degradation is mediated via UPP in SMA patient-derived fibroblast (Burnett et al. 2009; Chang et al. 2004). To investigate whether MNs of our culture system Smn protein is degraded by UPP, cells were treated with the proteasome inhibitor MG-132.

After six days of plating, MNs were treated with MG-132. It has been observed that the presence of the proteasome inhibitor, MG-132, in the culture medium significantly increased Smn protein level in the MNs as compared to the non-treated cells **(Figure 42)**. This result gives clear evidence that the degradation of Smn protein is mediated via the UPP and demonstrate that pharmacological proteasome inhibition up-regulates Smn protein level.

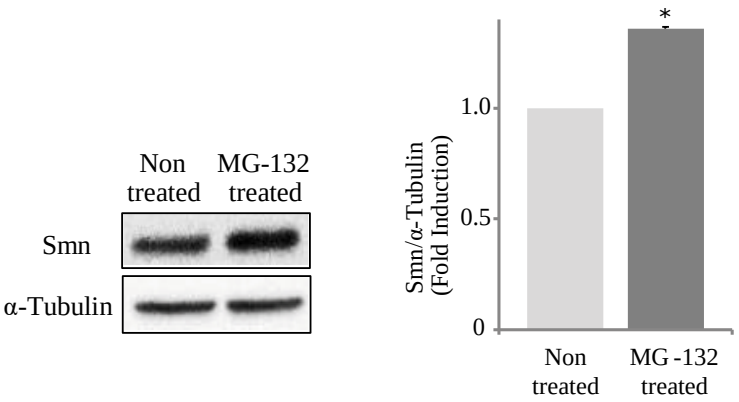

Figure 42. Smn is degraded by the UPP: After six days of plating, MNs were treated with MG-132 (5 µM) for 18 h. The cells were lysed, and the protein extracts were submitted to immunoblot analysis. All the membranes were reprobed with an antibody against α-Tubulin as a loading control. The graph represents the Smn protein level after MG-132 treatment. Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using student´s t-test (*p<0.05).

3.1.11. Effect of IKKα and/or IKKβ reduction on CREB protein of NTF-maintained MNs

It has been previously described that the transcription factor CREB regulates Smn protein expression (Majumder et al. 2004). CREB is a transcription factor which interacts with the cAMP response elements (CRE) sequences in DNA, and thereby increasing or decreasing the transcription of the downstream genes. In our previous study, we have demonstrated that NF-κB regulates CREB protein (Mincheva et al. 2011). Therefore, we decided to evaluate whether IKKs regulate Smn through CREB. In this context, we analyzed whether the differential regulation of Smn protein expression by IKKα and IKKβ can be mediated by CREB. With this regard, we assessed the level of CREB in the IKKα or IKKβ-reduced spinal cord MNs. IKKα or IKKβ in MNs is inhibited as described in above experiments. Analysing CREB level in double knockdown (IKKα, and IKKβ) was also

carried out. In our result, the CREB level was significantly reduced in shIKKα, and in double knockdown MN culture when compared to control **(Figure 43)**. In IKKβ inhibited MNs, the CREB level was significantly increased in comparison with control **(Figure 43)**. These results thus suggest that the regulation of Smn by IKKα, IKKβ or RelA could be mediated through CREB.

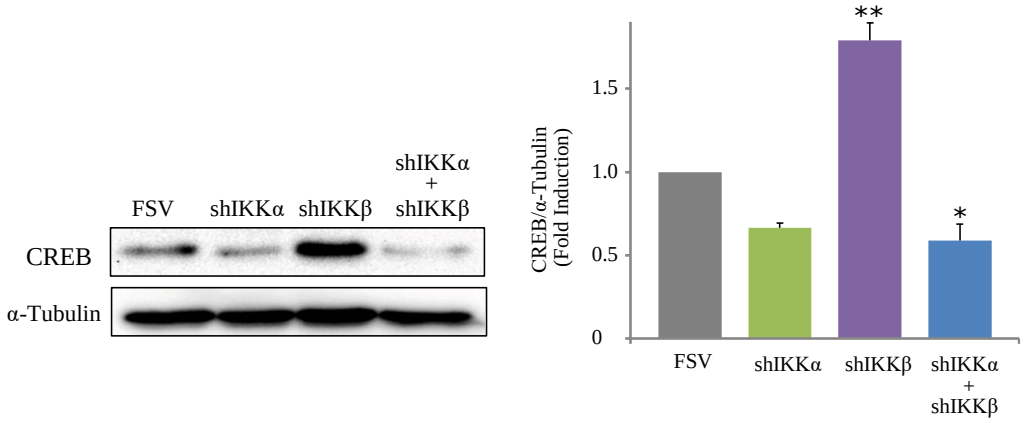

Figure 43. Immunoblot analysis of CREB protein level in shIKKα or/and shIKKβ transduction: The MNs were transduced with shIKKα or/and shIKKβ lentiviral particles. After four days, the protein extracts were collected, and submitted to immunoblot analysis. The membranes were probed with an anti-CREB antibody. All the membranes were reprobed with an antibody against α-Tubulin as a loading control. The graph represents the Smn protein level after empty vector (FSV) or shRNAs transduction. Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using one-way ANOVA test and Bonferroni *post-hoc* multiple comparisons *(**P<0.0001; *P<0.05).*

3.1.2. Effect of CREB inhibition on Smn protein on NTF-maintained MNs

Our previous result suggested that the members of NF-kB canonical pathway regulate Smn protein through CREB. To further confirm the role of CREB in regulating Smn, we have evaluated Smn level in CREB downregulated MNs.

We have downregulated CREB by the lentiviral method. Isolated MNs were maintained in the presence of the NTFs, and transduced with shCREB or FSV. The frequency of transduction was 99% of the cells present in the culture dish assessed by GFP^+ cells monitored by fluorescence microscopy **(Figure 45A).** The level of CREB protein in shCREB-transduced cultures was clearly reduced in comparison to the FSV-transduced cells. Inhibition of CREB protein significantly decreased the Smn protein level **(Figure 45B),** indicating that the transcription factor CREB regulates Smn protein.

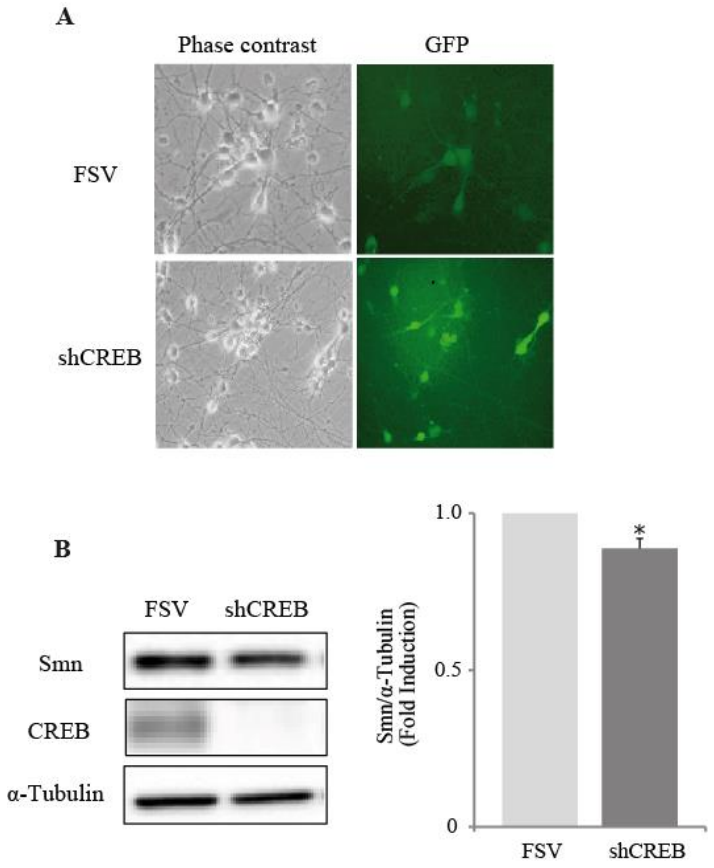

Figure 44. shCREB transduction reduces Smn protein: A) Representative microscopy images of 3 days FSV (control) or shCREB-transduced MNs: phase contrast (left) and GFP (right) of the same microscopic field. **B) Immunoblot analysis of Smn protein levels in shCREB transduction-** After seven days of transduction with shCREB lentiviral particles, MN cell extracts were collected, and analyzed by immunoblot using specific antibodies against CREB, and Smn. All the membranes were reprobed with an antibody against α-Tubulin as a loading control. The graph represents the Smn protein level after FSV or shCREB transduction. Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using student´s t-test (*p<0.05).

3.1.13. Effect of IKKα overexpression on Smn protein on NTF-maintained MNs

In the present study, we have demonstrated that the inhibition of IKKα protein decreases the Smn protein level. To elucidate whether IKKα overexpression regulates the Smn protein level in MNs, we overexpressed IKKα protein by the lentiviral method. MNs were plated, and maintained in the presence of the NTFs. Results demonstrate that the IKKα overexpression did not alter the Smn protein expression in our developmental model of cultured MNs **(Figure 45)**.

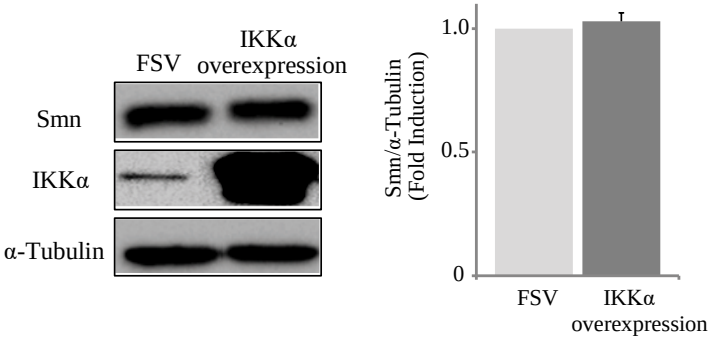

Figure 45. Immunoblot analysis of Smn protein level in IKKα overexpressed MNs: MNs maintained with lentiviral constructs of FSV (control), or IKKα overexpressed lentiviral constructs were collected after five days of transduction. The protein extracts were analyzed by immunoblot using specific antibodies against Smn and IKKα antibodies. All the membranes were reprobed with an antibody against α-Tubulin as a loading control. The graph represents the Smn protein level after FSV or shRelA transduction. Graph values are the mean of the Smn protein level for each condition of three independent experiments ± SEM (error bars).

Part 2: NF-κB pathway is altered in SMA

In the present study, we demonstrated that the different members of NF-κB signaling pathway regulate Smn protein. To determine whether the NF-κB signaling pathway is altered in SMA, we have measured the protein level of several members of NF-kB pathway in Smn-deficient MNs.

3.2.1. Expression level of p-RelA, Total RelA, IKKα and IKKβ proteins in lentivirus Smn-reduced MNs

RNAi is an appropriate tool to generate an *in vitro* model of Smn loss-of-function (Dykxhoorn et al. 2003). We have previously developed an *in vitro* model of SMA using lentiviral RNAi methods to downregulate the Smn protein level of isolated mouse spinal cord MNs. In brief, we have generated shRNA sequence targeting to specific sites of the mouse (shSmn). MNs were isolated from mouse embryos (E12.5) and maintained in the presence of NTFs. Three hours after plating, the culture medium was changed, and medium containing NTFs and lentivirus of shSmn or shRNA empty vector (EV) was added. Twenty hours later the medium was washed, and substituted by fresh medium supplemented with NTFs. After 2 or 3 days, most of the cells present in the culture dishes were GFP^+ (as monitored by fluorescence microscopy), showing a nearly 99% frequency of transduction of the cells. The efficiency of shRNA was monitored; Smn protein level was checked by immunoblot analysis. Smn knockdown conditions show the gradual decrease of Smn protein level after several days in culture (3, 6, 9, and 12). In these cells, it was also observed neurodegeneration with disturbed soma, and neurite degeneration on day 6, and at day 12 cell death occurred. To confirm that the cellular effect of neurite degeneration was due to an 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 (Garcera et al. 2011).

We have now used this model to analyze the alteration in the NF-κB pathway in Smn-reduced MNs. To this end, we carried out a lentiviral-based knockdown of Smn in cultured MNs. Even though the Smn protein level dropped in comparison to the EV or the control cultures from 3 days of interference, the reduced levels were maintained after 9 days, and 12 days (Garcera et al. 2011). Hence, we collected the cell lysates after 12 days of plating.

3.2.2. RelA phosphorylation is decreased in Smn-reduced MNs

To determine whether the NF-κB signaling pathway is altered in SMA, we initially studied the level of phospho-RelA (p-RelA), and total RelA in lentiviral Smn-reduced MNs. There was a significant reduction in p-RelA in Smn-reduced condition compared with the control (EV) **(Figure 46A)**. The total RelA protein level in Smn-reduced MN culture remained unchanged **(Figure 46B)**.

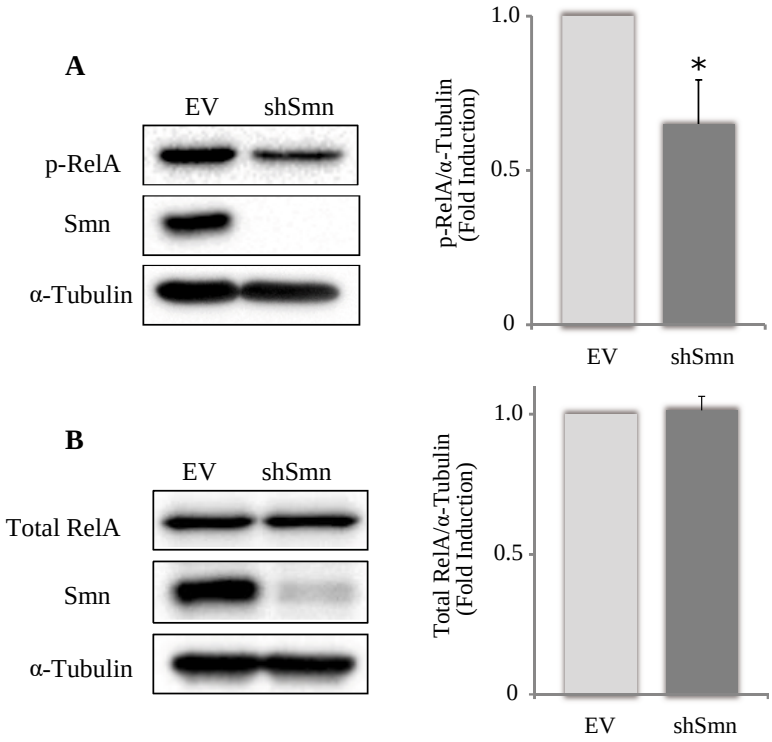

Figure 46. A) Immunoblot analysis of p-RelA in shSmn-transduced MNs- MNs were cultured in the presence of NTFs, and transduced with shSmn lentiviral constructs. After 12 days of interference, cells were collected, and were submitted to immunoblot. The protein extracts were probed with anti-p-RelA (Ser536) antibody. All the membranes were reprobed with an antibody against α-Tubulin, used as a loading control. The graph represents the p-RelA (Ser536) level after EV or shSmn transduction. Graph values are the mean of the expression level of p-RelA for each condition of three independent experiments ± SEM (error bars). Asterisk indicates significant differences between data from the experiments using Student's t-test (*p<0.005)**. B) Immunoblot analysis of total RelA in shSmn-transduced MNs -** After 12 days of interference, the shSmn-transduced MNs were collected, and analysed using immunoblot technique. The membranes were probed with an anti-RelA antibody. All the membranes were reprobed with an antibody against α-Tubulin, used as a loading control. The graph represents the total RelA level after EV or shSMN transduction. Graph values are the mean of the expression level of Smn protein for each condition of three independent experiments ± SEM (error bars).

3.2.3. Effect of Smn reduction on IKKα protein

To determine IKKα level in Smn-deficient cells, isolated spinal cord MNs from E12.5 mouse were maintained in the presence of NTFs, and transduced with the lentivirus of shSmn or EV. Twenty hours later the medium was washed, and substituted by fresh medium supplemented with NTFs. After 2 or 3 days most of the cells present in the culture dishes

were GFP$^+$ (as monitored by fluorescence microscopy), showing a nearly 99% frequency of transduction of the cells. The cell lysates were collected after 12 days of plating, and were submitted to immunoblot analysis. Results show a significant reduction of IKKα in Smn-reduced MNs **(Figure 47)**.

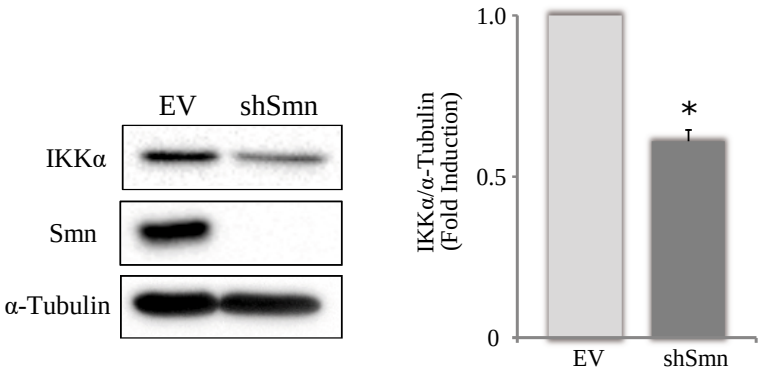

Figure 47. Immunoblot analysis of IKKα protein level in shSmn-transduced MNs. The protein extracts of shSmn-transduced MNs were analysed by immunoblot technique. The membranes were probed with anti-IKKα, and anti-Smn antibodies. All the membranes were reprobed with an antibody against α-Tubulin, used as a loading control. The graph represents the expression level of IKKα protein after EV or shSmn-transduced cells. Graph values are the mean of the expression level of IKKα protein for each condition of three independent experiments ± SEM (error bars). The Asterisk indicates significant differences between data from the experiments using Student's t-test *(*p<0.005)*.

3.2.4. Smn reduction decreases IKKβ protein

To study the level of IKKβ protein in Smn-reduced MNs, cultured MNs were transduced using lentivirus of shSmn or EV. The IKKβ protein expression level in the shSmn-transduced MNs were significantly decreased compared to the MN culture transduced with the EV control **(Figure 48)**.

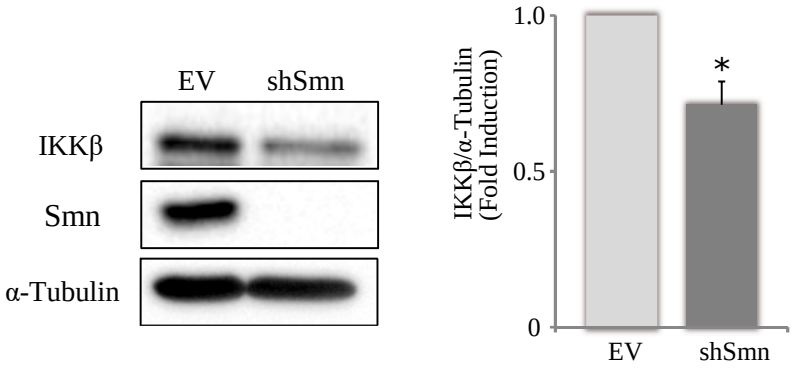

Figure 48. Immunoblot analysis of IKKβ protein levels in shSmn-transduced MNs. MNs were cultured in the presence of NTFs. After 12 days of interference, cells were collected, and the protein extracts of MNs transduced with shSmn were probed with anti-IKKβ, and anti-Smn antibodies. For the loading control, all the membranes were reprobed with an antibody against α-Tubulin. The graph represent the IKKβ protein level after EV or shSmn transduction. Graph values are the mean of the IKKβ protein level for each condition of three independent experiments ± SEM (error bars). The asterisk indicates significant differences between data from the experiments using Student's t-test *(*p<0.05)*.

3.2.5. Analysis of NF-κB pathway members in cultured MNs from SMA mouse model

To determine whether the reduction in the level of p-RelA, IKKα, and IKKβ can also be detected in MNs from an *in vivo* model of SMA, we have used the Smn null mouse with a human *SMN2* transgene (*Smn-/-;SMN2+/+)*, that resembles the human SMA type I **(Figure 49)**. We dissected, and genotyped the E13 embryos obtained from the cross of two (*Smn+/-;SMN2+/+)* mutants.

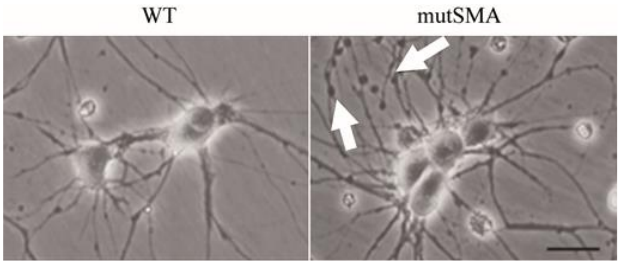

Figure 49. Representative images showing 8 days cultured isolated MNs from WT and mutSMA. Arrows indicate neurite degeneration (Garcera et al. 2013).

3.2.6. p-RelA level is decreased in SMA mouse model

After genotyping, MNs from WT and mutSMA E12.5 mice spinal cords were purified, and cultured separately. Twelve days after plating protein extracts were obtained, and submitted to immunoblot analysis using anti-p-RelA (Ser536), and anti-RelA antibody. Results show that p-RelA level was significantly reduced in MNs from mutSMA **(Figure 50A),** and there was no change in total RelA protein level **(Figure 50B)**. These results suggest that Smn-reduced MNs obtained from an *in vivo* model of SMA show decreased phosphorylation of RelA compared with MNs cultured from their WT littermate embryos.

3.2.7. IKKα is decreased in MNs from a mouse model of SMA

To investigate the level of IKKα in MNs from SMA mouse model, MNs were genotyped, isolated, and the MNs from WT and the mutSMA spinal cord were cultured separately. The immunoblot analysis shows a significant decrease in IKKα protein in MNs from mutSMA compared with the MNs from WT littermates at day 12 in culture **(Figure 51)**.

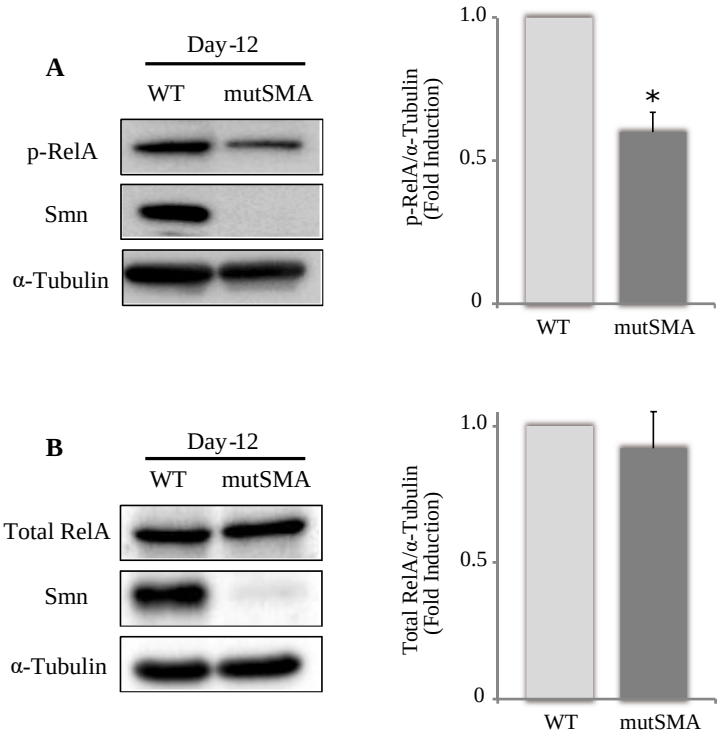

Figure 50. A) Immunoblot analysis of p-RelA in SMA MNs- Protein extracts of WT and mutSMA of 12 days cultures were probed with an anti-p-RelA, and anti-Smn antibodies by immunoblot analysis. Membranes were reprobed with an antibody against α-Tubulin as a loading control. Graph values represent the expression of p-RelA versus α-Tubulin and corresponds to the quantification of three independent experiments ± SEM. Asterisks indicate significant differences using Student's t-test *(*p<0.05)*. **B) Immunoblot analysis of total RelA in SMA MNs-** Cell extracts of WT and mutSMA of 12 days cultures were probed with an anti-RelA, and anti-Smn antibodies by immunoblot analysis. Membranes were reprobed with an antibody against α-Tubulin as a loading control. Graph values represent the expression of RelA versus α-Tubulin and corresponds to the quantification of three independent experiments ± SEM.

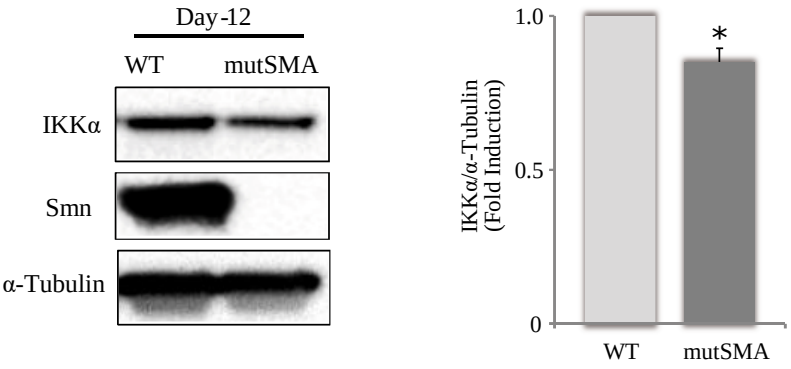

Figure 51. Immunoblot analysis of IKKα in WT and mutSMA MN culture: Protein extracts were collected from day 12 MN culture, and were analysed by immunoblot analysis. The membranes were probed with anti-IKKα, and anti-Smn antibody. Membranes were reprobed with anti-α-tubulin antibody, as a loading control. Graph values represent the expression of IKKα versus α-Tubulin and corresponds to the quantification of three independent experiments ± SEM. Asterisks indicate significant differences using Student's t-test *(*p<0.005)*.

3.2.8. MNs from a mouse model of SMA shows reduction in IKKβ protein

To examine the level of IKKβ protein in SMA mouse cells, MNs from genotyped WT and mutSMA spinal cords were purified, and cultured separately. Immunoblot analysis reveals that there was a significant reduction of IKKβ protein in MNs from mutSMA compared to WT controls **(Figure 52)**.

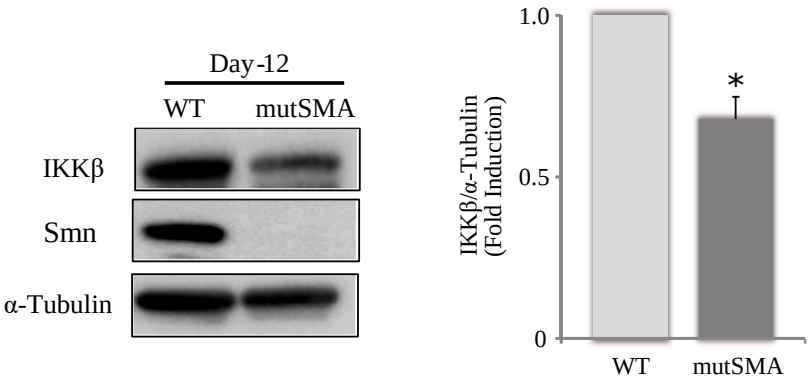

Figure 52. IKKβ protein level in WT and mutSMA MN cultures: After 12 days, cultured MNs were collected, and analysed by immunoblot technique. The membranes were probed with anti-IKKβ, and anti-Smn antibodies. Membranes were reprobed with an antibody against α-Tubulin as a loading control. Graph values represent the expression of IKKβ versus α-Tubulin and corresponds to the quantification of three independent experiments ± SEM. Asterisks indicate significant differences using Student's t-test *(*p<0.005)*

3.2.9. Time-course study on IKKα and IKKβ protein level in in vivo mouse model of SMA

To investigate whether the changes of IKKα, and IKKβ proteins level were time-dependent, cultured spinal cord MNs from WT and mutSMA were collected at different days of culture (2, 4, 6 and 8), and submitted to immunoblot analysis. Results show that there was a reduction of IKKα **(Figure 53),** and IKKβ **(Figure 54)** proteins in mutSMA MN cultures at Day-2, Day-4, Day-6, and Day-8 after plating compared to WT controls. These results indicate that the reduction of the two catalytic subunits of the IKK complex was initiated early, and was maintained throughout the experiment.

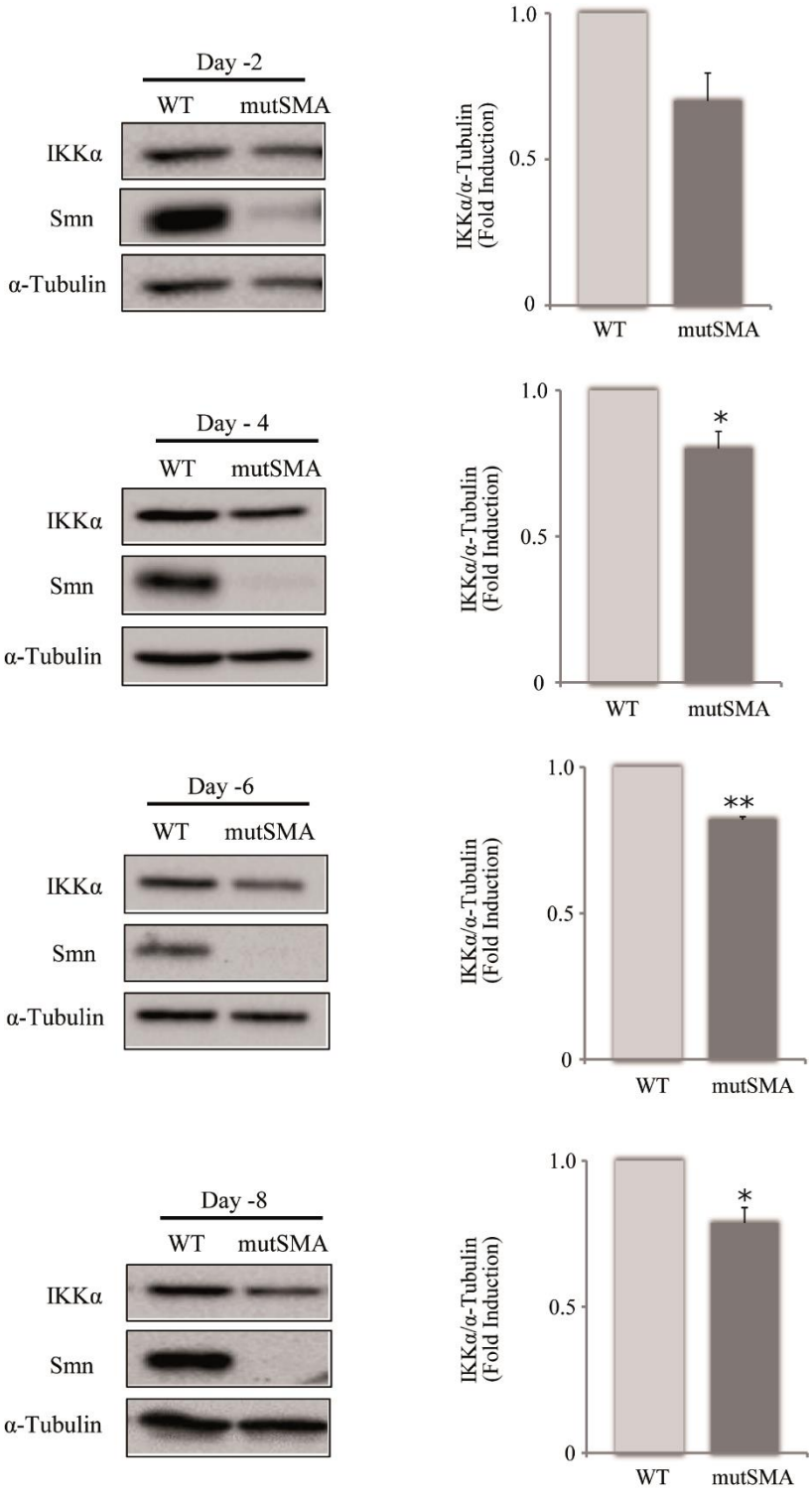

Figure 53. Time-course study on the expression level of IKKα in WT and mutSMA: Protein extracts of Day-2, Day-4, Day-6, Day-8 of cultured MNs were probed with anti-IKKα and anti-Smn antibodies. Membranes were re-probed with an anti-tubulin antibody as a loading control. Graph values represent the expression of IKKα versus α-Tubulin and correspond to the quantification of three independent experiments ± SEM. Asterisks indicate significant differences using Student's t-test *(*p<0.05; **p<0.005)*.

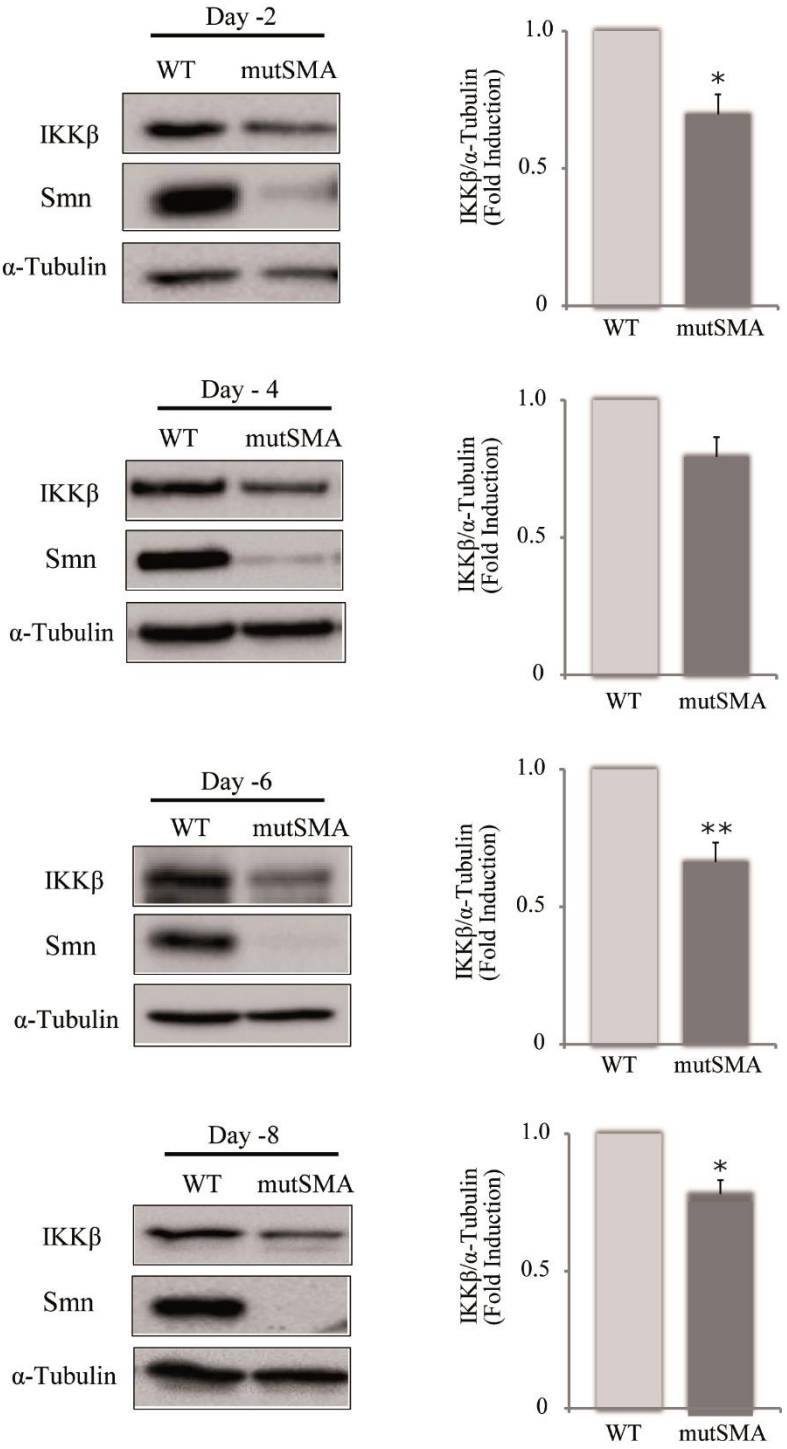

Figure 54. Time-course study on the expression level of IKKβ in WT and mutSMA: Protein extracts of Day-2, Day-4, Day-6, Day-8 of cultured MNs were probed with anti-IKKβ and anti-Smn antibodies. Membranes were re-probed with an anti-tubulin antibody as a loading control. Graph values represent the expression of IKKβ versus α-Tubulin and correspond to the quantification of three independent experiments ± SEM. Asterisks indicate significant differences using Student's t-test *(*p<0.05; **p<0.005).*

Discussion

Chapter IV

Discussion

SMA is a genetic disease caused by recessive mutations in *SMN1* gene (Lefebvre 1995) resulting in low levels of SMN protein. Reduction in SMN protein leads to degeneration of spinal MNs, and cause muscle weakness that is followed by symmetric limb paralysis, respiratory failure, and death (Crawford & Pardo 1996; Dubowitz 1978). Currently, there is no valid treatment for SMA. The development of therapeutic treatments requires a better understanding of the molecular mechanisms involved in the regulation of gene expression and neurodegeneration. The mechanisms of MN degeneration and neuronal death caused by low levels of SMN in SMA are unclear. Understanding the signaling pathways regulated by low levels of SMN that mediate neurodegeneration in SMA can contribute in identifying non-SMN targets as potential therapeutic targets to prevent neurodegeneration and neuronal death. NF-κB is an ubiquitously expressed transcription factor system involved in several physiological processes (Karin & Lin 2002). NF-κB pathway plays an essential role in NTF-induced MN survival of mouse embryonic spinal cord (Mincheva et al. 2011). This project had two broad aims: i) to investigate the involvement of NF-κB signaling pathway in the physiopathology of SMA and ii) to study the role of NF-κB pathway members in the regulation of Smn in the embryonic spinal cord MNs. To this end, we downregulated several members of NF-κB signaling pathway and analyzed the level of Smn protein in MNs from CD1 mouse, and we also analyzed the expression level of different members of NF-κB pathway in MNs obtained from SMA mouse model.

4.1. Smn-deficient MNs Exhibit Alteration in NF-κB Signaling Pathway

One of the most interesting findings in this study is the reduction of p-RelA in the Smn-deficient MNs. The NF-kB activation was evaluated by detecting phosphorylated p65 (Corbetta et al. 2005). At day 12 in culture, the lentiviral reduction in MNs primary culture from CD1 mouse resulted in the p-RelA reduction **(Figure 46)**. Immunoblot analysis revealed that the spinal cord MN culture from the severe SMA mouse model also shows a reduced level of p-RelA **(Figure 50)**. The total RelA level was not changed in both the cases **(Figure 46 & Figure 50)**. The reduction in p-RelA is not due to the availability of the total amount of RelA, but only due to the defect in its phosphorylation. This phosphorylation deficiency shows that the NF-κB signaling pathway activation is affected in Smn-deficient MNs. During development, the activation of NF-κB pathway has been associated with neurite growth (O'Neill & Kaltschmidt 1997; Gutierrez et al. 2008; Mémet 2006). In the adult nervous

system, NF-κB signaling pathway plays a vital role in synaptic signaling, and learning (Kaltschmidt & Kaltschmidt 2009), formation of long-term memory (Meffert et al. 2003; Mattson 2005), pre-, and postsynaptic space (Meffert et al. 2003), and in synaptic plasticity (Unlap & Jope 1995; Mattson et al. 2000; Kaltschmidt et al. 2006). The activity of this pathway is also associated with long-term potentiation of synaptic transmission (Meberg et al. 1996). Thus, a defect in this pathway could lead to severe impairment in the nervous system.

In agreement with it, NF-κB pathway plays a vital role in the pathogenesis of many chronic neurodegenerative diseases. The brain samples of AD patients show an increased NF-κB activity both in neurons, and glial cells (Kaltschmidt et al. 1999; Kaltschmidt B 1997). An increase of NF-κB activity has been linked to neuronal apoptosis, and the onset, and development of the disease in mice models of AD (Tg2576) (Niu et al. 2010). High NF-κB activity was observed in the dopaminergic neurons in the brains of PD patients (Hunot et al. 1997). Several studies suggest the involvement of NF-κB pathway in HD (Yu et al. 2000; Takano & Gusella 2002; Khoshnan et al. 2009; Khoshnan et al. 2004). Increase in NF-κB activity in glial cells, but not in MNs have been reported in the spinal cords of ALS patients (Migheli et al. 1997; Pyo et al. 2010). In response to the brain, and spinal cord ischemia and trauma, NF-κB activation has been observed (Xu et al. 2005). Also, it has been reported that the activation of NF-κB exerts a neuroprotective role in neurodegenerative diseases (Barkett & Gilmore 1999; Cardoso & Oliveira 2003; Fridmacher et al. 2003; Smith Darrell 2009).

4.2. IKKα and IKKβ are reduced in SMA

Another striking finding in this study is the reduction of IKKα, and IKKβ in the Smn-deficient MNs. At day-12 in culture, the immunoblot analysis of MNs, in which Smn is down-regulated showed a significant reduction of IKKα, and IKKβ **(Figure 47 & Figure 48)**. Authenticating this result, MNs from the severe SMA mouse model also showed a significant decrease in IKKα and IKKβ **(Figure 521 & Figure 52)**. The activation of NF-κB pathway depends on the phosphorylation of the two kinase subunits, IKKα and IKKβ (Israel 2010). Our results suggest that the defect in the phosphorylation of RelA could be due to the reduction in IKKα, and IKKβ protein level.

Which could be the cause of this defect in IKKα and IKKβ of the NF-κB signaling pathway? Besides the fact that the NF-κB signaling pathway activity depends on its upstream signaling pathways, and the hypothesis stating that the defects in NF-κB pathway activity could only be due to the defects in its upstream signaling pathways, our data propose that the

reduction of IKKα and IKKβ is the main reason behind the alteration in NF-κB pathway activity in SMA. The reduction/degradation of IKKα and IKKβ could be due to i) high level of Ca^{2+} influx, ii) increased the autophagic level or iii) the combination of both. In SMA, the abnormal increase of Ca^{2+}-dependent asynchronous release during prolonged stimulations suggested an altered intraterminal bulk Ca^{2+} concentration in the synapses (Ruiz et al. 2010). Moreover, the intracellular Ca^{2+}/calmodulin-dependent kinase II (CaMKII)/ phosphatidylinositol-3 kinase (PI3-K)/AKT/CREB cascade was found to be activated in the spinal cord explant cultures from the severe SMA mouse model (Branchu et al. 2013). In response to the intracellular free Ca^{2+} increase, caspases are activated. It has been demonstrated that the activated caspase-3 and caspase-7 can specifically proteolyze IKKβ both *in vivo* and *in-vitro*. The proteolysis was marginal for IKKα and undetectable for IKKγ**.** The amount of reduction in IKKβ and IKKα are well correlated with the proteolytic effect of caspase-3 and caspase-7 (Guilin Tang et al. 2001). Calcium regulates several physiological processes acting as an intracellular messenger. However, disruption of its homeostasis, due to an increase of free cytoplasmic Ca^{2+}, can induce cell death by apoptosis (Orrenius et al. 2003).

In Smn knock-down MN cultures and MNs from severe SMA mouse model, LC3-II protein level (an overall indicator of autophagy impairment) is increased, suggesting that the autophagy level is higher in SMA (Garcera et al. 2013; Periyakaruppiah et al. 2016). IKKα, IKKβ, and IKKγ are the targets for degradation by autophagy (Qing et al. 2006; Yan et al. 2007; Kim et al. 2010; Niida et al. 2010). The treatment of cells with geldanamycin, an inhibitor of Hsp90 (a major stability factor for the activation of IKK signalosome (Salminen et al. 2008; Hinz et al. 2007; Broemer et al. 2004)), induced the degradation of IKKα and IKKβ proteins via autophagy, and neither ubiquitination nor proteasomes were involved in the degradation of the IKK complex (Qing et al. 2006). Acute cellular stress can trigger the dissociation of Hsp90 from the IKK complex which inhibits NF-κB signaling (Salminen et al. 2008; Pittet et al. 2004). Moreover, geldanamycin stimulated the autophagic degradation of NIK, a critical kinase activating IKKα in the noncanonical NF-κB pathway (Qing et al. 2007). Moreover, IκBα is an autophagic substrate. At the first phase following stimulation with TNF treatment, IκBα is degraded through proteasome–dependent mechanism while at later stage it is cleared through the autophagic pathway (Colleran et al. 2011). In addition to the direct effects of autophagy on NF-κB signaling components, NF-κB pathway members can regulate autophagy. The TNF-dependent activation of NF-κB represses autophagy, whereas, loss of NF-κB activation in these cells triggers the reactivation of autophagy

(Djavaheri-Mergny et al. 2007; Djavaheri-Mergny et al. 2006). Inhibition of NF-κB activity results in an enhancement of starvation-induced autophagy (Fabre et al. 2007), and suppression of prolonged NF-κB activity promotes autophagy (Schlottmann et al. 2008). Our preliminary data shows reduced level of Hsp90 in Smn-deficient cells **(Figure 55)**. The possible interplay between NF-κB pathway members and autophagy in SMA has to be explored. Additionally, the combinatory effect of increased Ca^{2+} influx and autophagy on NF-κB pathway in SMA cannot be ruled out and needs detailed investigation.

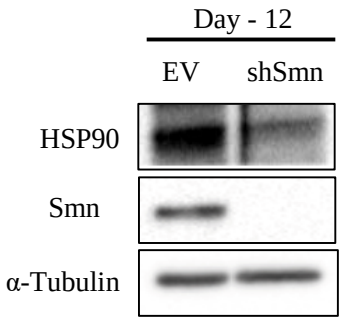

Figure 55. Immunoblot analysis of HSP90 in EV and shSmn MN culture: Protein extracts were collected from day 12 MN culture, and were analyzed by immunoblot analysis. The membranes were probed with the anti-HSP90, and anti-Smn antibody. Membranes were reprobed with an anti-α-tubulin antibody, as a loading control.

The time course study in the MNs from the severe SMA mouse model clearly showed the reduction in the two kinases, IKKα and IKKβ followed the same trend from Day-2 in culture to Day-12 MN culture **(Figure 521, Figure 52, Figure 53, & Figure 54)**. This clearly reveals that the NF-κB signaling pathway activity is reduced in MNs even in the earlier stage of the disease. NF-κB transcriptional activity in the spinal cord is first observed at the beginning of motoneuron neurotrophic factor dependence and the programmed cell death period (Schmidt-Ullrich et al. 1996; Yeo & Gautier 2004), The double knockout of IKKα and IKKβ causes defects in neural tube closure due to massive apoptosis of the neuroepithelium (Li et al. 2000). During embryogenesis, the blockade of NF-κB reduces neurite length and branching (Gavaldà et al. 2009; Gutierrez et al. 2008). These data determines the vital role of NF-κB pathway during development. Our data shows that the early defect in NF-κB signaling pathway may contribute heavily to the neurodegeneration in SMA, and also supports the belief of describing SMA not only as a neurodegenerative disease but also as a neurodevelopmental disease.

4.3. NF-κB Signaling Pathway, NTFs, and MN survival

In the present work, we also analyzed the role of NF-κB pathway in the regulation of Smn protein, a protein which is essential during MN embryonic development (Schrank et al. 1997) and whose reduction causes the lower MN neurodegenerative disorder, Spinal Muscular Atrophy (Sumner 2006). The role of NF-κB in regulating the genes related to survival has been well documented (Sarnico et al. 2009). Our group, for the first time, validated the essential role of NF-κB pathway in NTF-induced MN survival from mouse embryonic spinal cord (Mincheva et al. 2011). In the NS, NF-κB is widely expressed (O'Neill & Kaltschmidt 1997; Bhakar et al. 2002; Yalcin et al. 2003) and is activated by several NTFs. A wide range of NTFs are involved in MN survival during development (Henderson 1988). Different families of NTFs promote survival of distinct sub-population of MNs (Gould & Enomoto 2015). The physiological effect of these factors is due to their binding to specific membrane receptors on neurons and promoting the activation of different signaling pathways and transcription factors, including NF-kB pathway. We used a cocktail of NTFs containing CNTF/CT-1, BDNF, GDNF and HGF to achieve the maximal MN survival *in vitro* (Gou-Fabregas et al. 2009; Arce et al. 1999; Mincheva et al. 2011). These NTFs individually, CNTF, CT-,1 and BDNF in sensory neurons (Middleton 2000; Gallagher et al. 2007; Gutierrez et al. 2005), GDNF in astrocytes (Chu et al. 2008; Kaltschmidt et al. 2005), and can stimulate the NF-κB signaling pathway. Several pieces of evidence indicate that NF-κB pathway regulates cell survival both in CNS, and PNS (Mémet 2006). Studies on embryonic hippocampal cells under stressing conditions (Cheng et al. 1994; Barger et al. 1995; Mattson et al. 1997) determined that the activation of NF-κB pathway promotes neuronal survival. In cerebellar granule neurons, trophic factor deprivation induces NF-κB inactivation, leading to cell death (Kovács et al. 2004), and the NF-κB activity is required for the survival of NGF-dependent sympathetic neurons (Maggirwar et al. 1998).

4.4. Role of Canonical and Non-canonical Pathway on Smn

In the CNS, the NF-κB pathway can be activated by three ways namely canonical, non-canonical, and atypical activation pathways. In spinal cord MN culture, NTFs activate both the canonical and non-canonical NF-κB signaling activation pathways. NTFs stimulation activates the canonical pathway by phosphorylating both the IKK kinases, inducing IκB endogenous protein reduction which is followed by a characteristic resynthesis of IκBα and nuclear translocation of RelA. The degradation of the p100 precursor to the p52 was observed in NTFs stimulated MNs whereas such degradation was absent in non-NTF-stimulated MNs. (Mincheva et al. 2011). Although there are evidences illustrating that in

nervous system atypical NF-κB activation pathway is stimulated in response to NGF, and CNTF as NTFs (Bui et al. 2001; Gallagher et al. 2007), our previous unpublished data demonstrates that both NTFs-stimulated and non-NTFs-stimulated MN cultures show no detectable levels of phosphorylation in IκBα at the Tyr42 residue indicating that atypical activation pathway is not stimulated by NTFs in our MN culture system.

Even both pathways are activated by NTFs, canonical, and non-canonical pathways have different effects on MN survival. The pharmacological inhibition and the lentiviral knockdown of RelA protein decreased the NTF-induced MN survival, whereas the inhibition of RelB exhibited no change in survival of MN cells (Mincheva et al. 2011). Likewise, the role of canonical and non-canonical pathways in regulating Smn protein is different. The pharmacological inhibitor peptide, SN50, decreased Smn protein level in the MNs **(Figure 33)**. The lentiviral inhibition of the DNA-binding subunit, RelA, caused a reduction of Smn, whereas, the lentiviral-based knockdown of RelB protein did not affect Smn **(Figure 34 & Figure 35).** Our data conclude that only the canonical pathway regulates Smn protein whereas the non-canonical pathway have no effect on Smn level. Previous studies suggest that p52/RelB have an affinity for κB elements distinct from p50/RelA, and might, therefore, regulate a different subset of NF-κB target genes (Wang et al. 2012; Bonizzi et al. 2004). Thus, our results support this hypothesis in Smn regulation.

4.5. Role of IKKα and IKKβ on Smn

The reduction of IKKα shows a substantial decrease in Smn protein but the IKKβ reduction increased Smn protein **(Figure 36 & Figure 37)**. Likewise, IKKα and IKKβ had inverse functions on β-catenin (a protein having a dual role of regulating the coordination of cell–cell adhesion, and gene transcription), IKKβ negatively regulates its activity, whereas, IKKα increases its protein levels (Carayol & Wang 2006; Lamberti et al. 2001). Aside from their similarities in localization, structural (Häcker & Karin 2006; Woronicz 1997; Zandi et al. 1997), and kinase activation (Kwak et al. 2000), IKKα and IKKβ have several substrates, and are targeted through NF-κB-independent mechanisms, which considerably widens the roles of these kinases (Chariot 2009). Our results demonstrating that IKKα, and IKKβ exhibit opposite effect on Smn supports the hypothesis that both the kinases have distinct substrates and enhances its support to discard the suggestion that IKKα and IKKβ would probably have overlapping functions. A representative scheme for the role of IKKα and IKKβ in regulating Smn protein is shown in **Figure 56.**

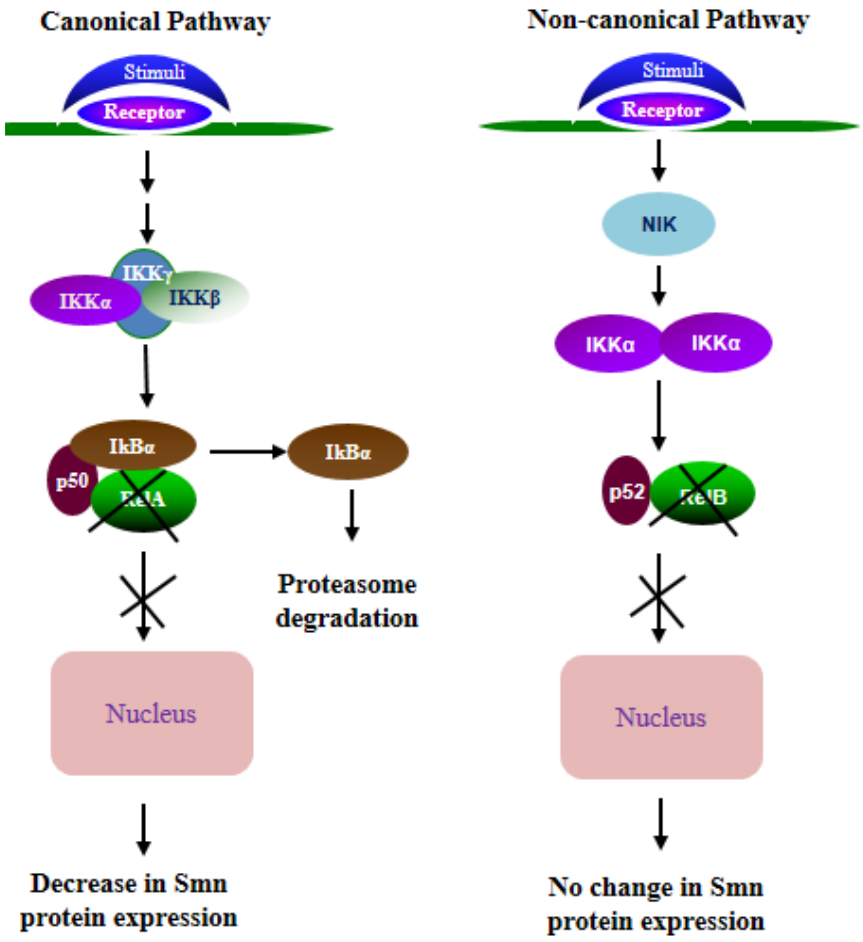

Figure 56. A representative Scheme for the role of IKKα and IKKβ on Smn.

4.6. IKKα and IKKβ are Essential for the RelA phosphorylation in MNs

The lentiviral-based knockdown of IKKα or IKKβ and the double knockdown of IKKα plus IKKβ in our MN culture system showed a significant decrease in of RelA phosphorylation at Ser536 residue **(Figure 39)**. NTF-induced NF-κB pathway activation depends on IKKα and IKKβ. The two kinases are phosphorylated and activated in response to the stimuli that induce NF-κB pathway activation (Delhase 1999), and the stimulation with NTFs increases IKKs phosphorylation level (Mincheva et al. 2011). Our results confirm that the two catalytic subunits IKKα and IKKβ are essential for NF- κB canonical pathway activation and the inhibition of both or either one of the subunits blocks the NF-κB canonical pathway activation.

Although both kinases are necessary for the pathway activation, they have different levels of effect. Our previous unpublished data show that in MNs, IKKα knockdown showed higher levels of RelA in the nucleus (65%) compared to that of IKKβ knockdown (25%).

The similar result has also been demonstrated in mouse embryonic fibroblast (MEF) cell line where inhibition of IKKβ completely blocks the nuclear localization of RelA in response to TNF stimulation whereas blocking the expression of IKKα partially affects its localization (Huang et al. 2007). In the present study, the p-RelA level in IKKα or IKKβ downregulated MN also reflects the similar pattern NF-κB pathway activation **(Figure 39)**. Thus, IKKβ is dominant than IKKα in activating NF-kB pathway, based on its higher level of activity for IkBα compared with IKKα (Li & Karin 1998; Mercurio 1997; Nakano et al. 1998; Woronicz 1997; Yin, Christerson, et al. 1998; Yin, Yamamoto, et al. 1998), and the failure of NF-kB pathway activation when *IKKβ* gene is disrupted in mice (Q Li et al. 1999). Thus, our results show that both kinases are required for the activation of NF-κB pathway, and the increase of Smn in IKKβ knockdown cells can be NF-κB independent.

4.7. Role of IKKα in the Absence of IKKβ on Smn

The double knockdown of IKKα plus IKKβ in our MN culture illustrate that the substantial increase of Smn protein expression in IKKβ knockdown MNs is significantly reduced in IKKα plus IKKβ double knockdown condition **(Figure 38).** This result explains that IKKα plays a significant role in the absence of IKKβ in regulating Smn. Several studies confirm the role of IKKα independent of its function in NF-κB pathway. *IKKα* KO mice die few days after birth and show defects affecting multiple morphogenetic events, including limb and skeletal patterning, and show keratinocyte hyperproliferation without differentiation (Hu et al. 1999; Qiutang Li et al. 1999; Takeda et al. 1999). IKKα controls keratinocyte differentiation, but this effect does not rely on NF-κB or the kinase activity of IKKα (Hu et al. 2001). Compared to IKKβ, the IKKα subunit shows much more diverse functions. IKKα also functions in apoptosis, cell cycle, and tumor progression in different types of cancer (Fernández-Majada et al. 2007; Hoberg et al. 2004; Park et al. 2005; Tu et al. 2006; Shiah et al. 2006; Hirata et al. 2006; Furuya et al. 2007; Luo et al. 2007). Thus, our double knock-down study suggests that IKKα may regulate Smn in the absence of IKKβ, which is independent of its kinase role in the activation of NF-κB pathway.

4.8. Role of NF-κB Pathway on Smn mRNA

The increase in *Smn* mRNA level in shIKKβ-transduced embryonic MN culture is substantiated the transcriptional regulation of Smn in the absence of IKKβ **(Figure 40C & Figure 41)**. Additionally, our analysis exhibits no change in *Smn* mRNA level in shRelA- or shIKKα-transduced MN culture **(Figure 40A & Figure 40B)**. This observation indicates that the inhibition of IKKα and RelA could affect *Smn* at the post-translational level. SMN can be ubiquitinated by Mib1 (Kwon et al. 2013) and degraded by the proteasome (Chang et al.

2004). Ubiquitination is a post-translational modification where ubiquitin is attached to a substrate protein. The addition of ubiquitin can affect proteins in many ways: It can signal for their degradation via the proteasome, modify their cellular location, change their activity, and promote or prevent protein interactions (Glickman & Ciechanover 2002; Mukhopadhyay & Riezman 2007; Schnell & Hicke 2003). To make out that Smn undergoes ubiquitination and proteasomal degradation, we pharmacologically inhibited MNs with MG-132, a proteasome inhibitor. Our results show that the pharmacological inhibition of proteasome by MG-132 increases Smn protein level in MNs **(Figure 42)**, reinforcing the previously reported role of the proteasome in SMN protein stability. The proteasomal degradation of Smn in shIKKα- or shRelA-tranduced MNs needs further explorations.

The reduction of Smn protein when there is no alteration in *Smn* mRNA could also be due to calpain cleavage. The inhibition of NF-κB pathway members leads to apoptotic cell death even in the presence of NTFs. The shIKKβ-, shIKKα-, and shRelA-transduced MN cultures showed an increase of the activation-specific Caspase-3 fragment and the knockdown of RelA also causes an increase of the pro-apoptotic protein, Bim (Mincheva et al. 2011). Caspases, members of the cysteine protease family, are known to be critical effectors of cellular apoptosis in central nervous system. More recently, the calcium-dependent proteases, calpains, have been implicated in cellular apoptotic processes (Raynaud & Marcilhac 2006). Cross-talk between calpains and caspases has been reported during apoptosis of neuronal cells induced by a prion protein fragment (O'Donovan et al. 2001). Moreover, calpains can be activated via caspase-mediated cleavage of calpastatin (MI Pörn-Ares et al. 1998.). It has been demonstrated that SMN is cleaved by cytosolic, but not nuclear calpains (Fuentes et al. 2010), and our recent study shows that calpain can regulate Smn protein level (Periyakaruppiah et al. 2016).

4.9. Role of CREB in Regulating Smn

In shIKKα-, and in shIKKα plus shIKKβ-transduced MN cultures, we observed a decrease in CREB protein level whereas we found a significant increase in CREB protein level in shIKKβ-transduced cells **(Figure 43)**. Our previous study indicates that the blockade of NF-κB pathway by the lentiviral transduction of RelA shows a decrease in CREB protein (Mincheva et al. 2011). The shCREB-transduced cells show a significant reduction in Smn protein **(Figure 44)**. It has been illustrated that CREB can regulate Smn, and acts as the primary transactivating factor for SMN gene transcription. The *SMN* promoter has CRE sequence due to which it can interact with the CREB family of proteins. CREB specifically interacts with CRE-II sequence in the *SMN* promotor and positively regulates the SMN gene.

Also, the treatment with cAMP-elevating agents increases expression the SMN transcript in human fibroblasts from type 1 SMA patients (SMN1-/-: SMN2), mouse fibroblasts derived from SMA mice (Smn-/-: SMN2), and HeLa cells (Majumder et al. 2004). In type 1, and type 2 SMA-like mice, and in the co-culture SMA spinal cord explants, NMDA treatment stimulates CREB phosphorylation and thereby inducing the expression of SMN (Biondi et al. 2010). Also, the inhibition of the MEK/ERK/Elk-1 pathway stimulates the AKT/CREB pathway activation, resulting in an enhanced SMN expression in the spinal cord of SMA-like mice and human SMA myotubes (Branchu et al. 2013). Collectively, our results also show that Smn regulation can be mediated through CREB.

Though shCREB transduction shows significant reduction of Smn protein, our result indicates that the reduction is only around 20%. This result indicates that Smn may have different transcription factors. SMN is a target gene also for Elk-1 (Demir et al. 2011), Stat5 (Ting et al. 2007; Farooq et al. 2011), IRF-1 (Baron-Delage et al. 2000). Apart from these transcription factors, SMN gene also has putative binding sites for transcription factors such as AP-2, GATA-2, HNF-3, N-Oct-3, and YY1 (Echaniz-Laguna et al. 1999).

4.10. Possible Nuclear Role of IKKα in the Absence of IKKβ in MNs

The role of IKKα in regulating Smn in the absence of IKKβ can be explained by its documented nuclear role. The diverse functions of IKKα and IKKβ are due to their distinct distribution in the cell. IKKβ is distributed mainly in the cytoplasm, whereas IKKα shows both cytoplasmic and nuclear localization due to the NLS sequence in the kinase domain (Anest et al. 2003; Birbach et al. 2002; Huang et al. 2012; Huang et al. 2007; Yamamoto et al. 2003). Studies demonstrated the crucial nuclear role of IKKα through the identification of a functional NLS in the IKKα kinase domain. Inactivation of this NLS represses keratinocyte differentiation, indicating that IKKα exerts its function within the nucleus of basal keratinocytes in the epidermis which is independent of the kinase activity of IKKα (Sil et al. 2004). Several IKK knockout MEF cells showed that the histone acetyltransferase, CBP phosphorylation was abolished in IKKα-/- MEF cells, but was still activated in IKKβ-/-and IKKγ-/-MEF cells, in which nuclear IKKα remains activated (Huang et al. 2007).

IKKα translocates into the nucleus to mediate both NF-kB-dependent, and NF-κB-independent gene expression (Birbach et al. 2002; Ear et al. 2005; Massa et al. 2005). IKKα can enter the nucleus in response to diverse stimuli involved in inflammation, apoptosis, immune response and cancer (Huang & Hung 2013). Akt, a serine/threonine kinase, has been shown to increase the activity of IKKα (Ozes et al. 1999), and the cocktail of NTFs stimulate

the activity of Akt in embryonic spinal cord MNs (Mincheva et al. 2011). The binding of transcription factors to promoter regions within DNA can potentially trigger chromatin remodeling, thereby inducing a mechanism for regulating the transcription of the gene (Natoli et al. 2005). In NF-κB dependent gene transcription the nuclear IKKα enhances transactivation (Jiang et al. 2003), and DNA binding of p65 (Gloire et al. 2007). Also, IKKα also takes part in chromatin regulation through its interaction with CBP, (Huang et al. 2007; Yamamoto et al. 2003). In the nucleus, the association between IKKα and CBP induces IKKα-mediated phosphorylation of histone H3 at Ser-10 *in vitro* (Anest et al. 2003; Yamamoto et al. 2003). Moreover, IKKα interacts with the transactivation domain of CBP and mediates the acetylation of specific residues in histone H3 (Yamamoto et al. 2003). The nuclear IKKα binds directly to CBP and phosphorylates its HAT domain at Ser1382 and Ser1386 to enhance the enzymatic activity of CBP on histone acetylation (Huang et al. 2007). Thus, nuclear IKKα can mediate both histone H3 phosphorylation and acetylation implying that nuclear IKKα may function as a common epigenetic regulator for gene transcription. The chromatin regulation by nuclear IKKα is not specific for NF-κB-targeted genes. It also affects gene transcription regulated through targeting various transcription factors or cofactors. For example, in response to EGF (Anest et al. 2004), and UV (Dong et al. 2011), the nuclear IKKα mediates histone H3 phosphorylation in an NF-κB-independent manner which leads to c-fos upregulation. In short, IKKα may regulate Smn in the absence of IKKβ in an NF-κB independent manner in the embryonic spinal cord MNs which is supported by the well-documented nuclear role of IKKα. Here we proposed the schematic model for the possible nuclear role of IKKα in regulating Smn protein **(Figure 57)**.

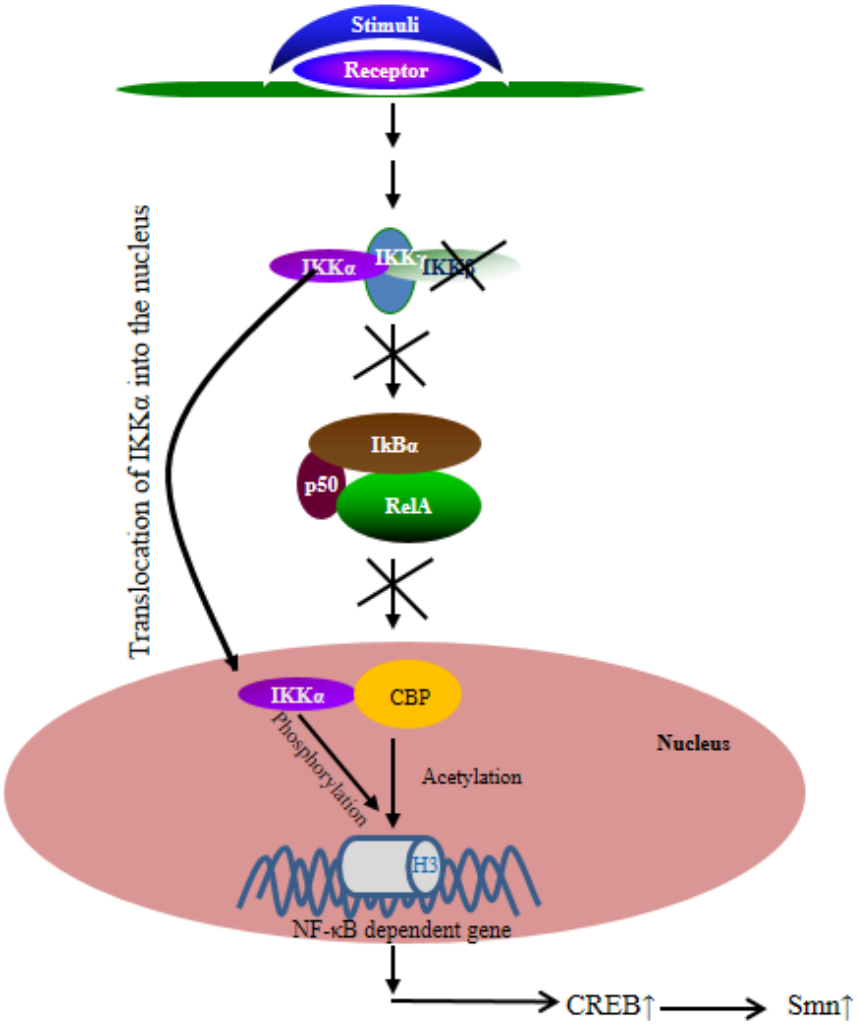

Figure 57. Proposed Scheme for the Possible Nuclear Role of IKKα

In our culture system, IKKα overexpression did not alter the Smn protein level **(Figure 45)**. Previous studies revealed that IKKα is upstream of IKKβ in mediating the regulation of NF-kB directed gene expression (Yamamoto et al. 2000). In a study to determine the ability of IKKα and IKKβ to activate an NF-κB-dependent reporter gene in transiently transfected 293 cells, the overexpression of IKKβ gave greater activation of the NF-κB reporter gene than IKKα overexpression did. Moreover, the authors demonstrated that the increased expression of NF-κB reporter decreases in higher fold overexpression of IKKα (Woronicz 1997).

4.11. Possible Molecular Mechanisms for Neurodegeneration in SMA

What could be the possible molecular mechanisms through which NF-κB mediate neurodegeneration in SMA? SMN deficiency may result in intracellular stress that might activate intracellular signaling cascades that lead to neurodegeneration in SMA. The ROCK and the JNK signaling pathways have been shown to be activated by the low levels of SMN in *in-vitro* and *in vivo* SMA models (Genabai et al. 2015; Bowerman et al. 2007; Nolle et al. 2011). The downregulation of phospho-AKT is shown in the spinal cords of SMAΔ7 mice and human SMA patients (Genabai et al. 2015). ROCK activation in SMA might be involved in the activation of PTEN that leads to inactivation of PI3-K/AKT cascade. Activated ROCK pathway mediates neurodegeneration by disruption of cytoskeleton stability (Yamaguchi et al. 2001). The JNK signaling pathway was activated in the spinal cords of SMA patient and SMAΔ7 mice, in cultured spinal cord motor neurons from SMA mice and also, the knockdown of SMN using RNAi also resulted in JNK activation in cultured neurons (Genabai et al. 2015). Reduced AKT phosphorylation in the SMA spinal cords is consistent with suppression of AKT pathway during JNK activation (Sunayama et al. 2005). Two JNK signaling modules, ASK1/MKK4/JNK3 and MEKK1/MKK7/JNK3, mediate *in vivo* phosphorylation of c-Jun that causes the degeneration and apoptosis of neurons (Genabai et al. 2015). The cross talk between these activated and inactivated signaling cascades with NF-κB signaling pathway in SMA needs an elaborate investigation.

It has been demonstrated that the activation of RhoA/ROCK pathway regulates NF-κB signaling pathway (Xie et al. 2013). But, our data show reduced NF-κB signaling pathway activity in SMA. This could be due to the reduction of IKKα, and IKKβ, the two kinases important for the activity of this pathway. The anti-apoptotic function of NF-kB is mediated in part through its ability to downregulate JNK activation (Papa et al. 2004; Nakano 2004). The JNK cascade is also important in regulating cell death decisions, and the prolonged JNK activation promotes apoptosis (G Tang et al. 2001). Activation of NF-kB by TNF-α induces the expression of GADD45b, XIAP, and other molecules that downregulate JNK activation. Because, the NF-κB pathway activity is reduced in SMA, the inhibition of the activated JNK pathway which leads to neurodegeneration, and neuronal death cannot be prevented. The Akt pathway is considered as the upstream pathway of NF-κB pathway (Mincheva et al. 2011). But, evidence illustrate the possibility of NF-κB pathway being the upstream signaling pathway of AKT pathway (Meng et al. 2002), and we propose that the inactivation of PI3-K/AKT in SMA is due to the defect in NF-κB pathway.

In summary, our findings shed light on the molecular mechanisms implicated in the neurodegeneration and neuronal death in SMA. The defect in IKKα and IKKβ could be a key to activate the intracellular signaling cascades that are responsible for the neurodegeneration in SMA. The ability of NF-κB pathway members to regulate Smn level suggests that this pathway member could be a possible therapeutic target, which could also be explored to complement current therapies approaches to enhance the survival.

Conclusions

Conclusions

1. NF-kB inhibitor, SN50 decreases the Smn protein level in NTFs-stimulated spinal cord MNs.
2. Inhibition of RelA reduces Smn level, whereas RelB, inhibition has no effect on Smn in spinal cord MNs.
3. Inhibition of IKKα decreases Smn protein level in spinal cord MNs, but the inhibition of IKKβ increases Smn protein level.
4. The double knockdown of IKKα and IKKβ decreases Smn protein level compared to the shIKKβ-transduced cells.
5. Inhibition of IKKα, and/or IKKβ protein reduces RelA phosphorylation. In NTFs-maintained spinal cord MNs.
6. Inhibition of RelA and IKKα shows no alteration in Smn mRNA level, whereas shIKKβ-transduced cells increase the Smn mRNA level in the embryonic spinal cord MNs.
7. The presence of the proteasome inhibitor, MG-132, in the culture medium increases Smn protein level in the MNs.
8. Inhibition of IKKα or IKKα and IKKβ decreases CREB level, whereas the inhibition of IKKβ increases CREB level in cultured MNs.
9. Inhibition of CREB reduces Smn level in the NTFs-maintained MNs.
10. Overexpression of IKKα in spinal cord MNs shows no effect on Smn protein level.
11. Smn-deficient MNs exhibit reduced RelA phosphorylation with no change in total RelA protein.
12. IKKα protein level decreases in Smn-reduced MNs.
13. Inhibition of Smn protein reduces IKKβ protein level in cultured MNs.
14. Cultured MNs from severe SMA mouse model show reduction in RelA phosphorylation.
15. SMA mutant spinal cord MNs exhibit decreased the level of IKKα.
16. Isolated MNs from the SMA mouse spinal cord has reduced the level of IKKβ.
17. SMA mutant MNs show decreased the level of RelA phosphorylation, IKKα, and IKKβ from Day-2 in culture to Day-12 MN culture.

APPENDIX

Smn-deficient Motoneuron-like, NSC-34 cells exhibit hyperexcitability

Short stay: (Oct-2014 to Dec 2014)

Department of Medical Physiology and Biophysics

School of Medicine, University of Seville

Seville - Spain

Guidance: Prof. Lucia Tabares

Chapter I Introduction

Low levels of SMN protein result in SMA, the most frequent genetic cause of early childhood lethality (Crawford & Pardo 1996). SMA is an autosomal recessive degenerative disease characterized by muscular weakness, and atrophy of limb, and trunk muscles. Currently, there is no effective treatment for SMA. SMN is encoded by two genes in chromosome 5q, *SMN1* which produces full-length SMN (SMN-FL), and *SMN2* which majorly produces a unstable truncated form of SMN (SMNΔ7) and a small amount of SMN-FL (Gennarelli et al. 1995; Lorson et al. 1999; Monani et al. 1999). In the absence of a functional *SMN1* gene, the severity of the disease depends on the amount of SMN-FL produced by *SMN2* (*SMN2* copy number varies in the population).

The best-characterized SMN function is its participation in the assembly of small nuclear ribonucleoproteins (snRNP) as part of multiprotein complexes in the spliceosomes (Fischer et al. 1997; Liu et al. 1997; Meister et al. 2001; Pellizzoni et al. 2002). It is still not understood, however, why reduced levels of the ubiquitously expressed SMN protein specifically causes MN death. Although disturbed RNA processing could affect some specific functions of MNs, interestingly, SMN is also found in axons *in vivo* (Pagliardini et al. 2000), and at branch points and growth cones in cultured MNs (Jablonka et al. 2001), what suggest that SMN has functions in axons and synapses which are independent of its role in snRNP biogenesis (Carrel et al. 2006). In axons, SMN has been shown to form a complex with other proteins and transport mRNA for beta-actin (Rossoll et al. 2003). In animal models of SMA SMN axonal transport is reduced and the truncated form of the protein (SMNΔ7) is not transported.

Of special interest is the particular early onset vulnerability of MNs with low SMN levels. Typically, as MNs develop, their soma size increase and they become morphologically more complex by the development of dendritic arborizations, and axonal elongation (Carrascal et al. 2005). These morphological changes are accompanied by shifts in the membrane electrical properties, namely a decrease in input resistance, more hyperpolarized resting membrane potentials, and the ability to fire repetitively during sustained depolarization stimuli (Carrascal et al. 2005; Gao & Ziskind-Conhaim 1998). However, in isolated intact spinal cord preparation from SMNΔ7 mice it has been found that SMA MNs have a high input resistance and a low threshold for eliciting APs, what make them *hyperexcitable* (Mentis et al. 2011); in addition, SMA MNs show APs of greater amplitude, and of larger rate of rising than controls (Mentis et al. 2011). MN *hyperexcitability*

and altered firing of APs also has been described in SOD G93A transgenic mice, a model of ALS, however, with no alteration of the passive membrane properties (Kuo et al. 2004; Pieri et al. 2003). The hyperexcitability of MNs in the G93A mouse model has been attributed to an increase in Na^+ current (Kuo et al. 2004; Pieri et al. 2003; Kuo et al. 2005).

In the present study, our goal is to understand the anomalous membrane electrical properties of Smn-deficient MN-like cell line, NSC-34.

Hypothesis & Objectives

Hypothesis &Objectives

Precedent

Lumbar spinal MNs are hyperexcitable in SMA (Mentis et al. 2011).

Hypothesis

- MNs electrical phenotype is determined by the expression of different types of membrane ion conductances. SMN regulates the expression of one/several membrane ion channel type(s) necessary for MN fate and/or maintenance. When SMN is decreased electrical excitability is altered.

Objectives

- To characterize the ion currents in Smn-reduced MN-like cell line, NSC-34.

Appendix

Materials & Methods

2.1. The culture of NSC-34 cell line.

The maintenance procedure for the NSC-34 cells has been given in detail in the section **2.1.2**. The cells grow right on the surface of the conventional tissue culture plastic; no special coating is required. For our experiment, 50000 cells were plated in M12 plates over laminin coated coverslips, and after 2 h the medium is changed to differentiation medium (1% FBS), and shSMN or EV lentiviral constructs were added. After 18 h the médium was replaced by fresh médium (DMEM +1% FBS). Electrophysiological recording was done after 72 h.

2.2. Patch Clamp Technique:

Patch clamp is the technique to study the transient change in electrical current across the cell membrane. The technique uses an electrode inside a glass pipette, containing a salt solution resembling the fluid usually found within the cell, which is lowered to the cell membrane where a tight seal is formed. When a little suction is applied to the pipette, the "patch" of the membrane within the pipette ruptures, permitting access to the whole cell. The electrode, which is connected to specialized circuitry, can then be used to measure the currents passing through the ion channels of the cell.

Patch clamp technique can be operated in voltage clamp and current clamp mode. Voltage clamp allows an experimenter to "clamp" the cell potential at a chosen value. This makes it possible to measure voltage specific activity of ionic channels. Current clamp records the membrane potential by injecting current pulses into a cell and measuring potential changes in response to it. This technique is used to study how a cell responds when electrical current enters a cell. This type of recording is performed to study action potential firing of the neuron.

Patch clamp studies are conducted in five basic configurations-cell-attached mode, whole-cell mode, inside-out mode, outside-out mode and perforated patch mode. We performed Whole-cell recording, where a negative pressure is applied through the pipette which ruptures the patch of the membrane exposing the whole cell to the pipette solution. The resistance is lower and better electrical access to the cell interiors.

Patch clamp setup primarily comprises of components that facilitate seal formation by the pipette and recording the amplified current/voltage signals from the cell. Pipette Pullers are used to pull patch pipettes from glass capillary tubes. Microscopic movements and vibrations are undesirable for patching the cell as they can interrupt the recording, so a vibration isolation table is used to dampen these vibrations. The air suspension table reduces

vibrations to a few Hertz, at which patch recording is possible. Patch clamp has a Faraday cage surrounding so as to shield the sensitive patch clamp preamplifier from electrical noise. Inside wall of it may have shelves or brackets. An inverted microscope offering micrometer resolution is used for viewing access to the cell by a patch pipette. Focusing is accomplished by moving the objective rather than the microscope stage. To place the pipette in cells as small as a few micrometers, it is essential to precisely control the movement of the patch pipette in the sub-micrometer range. Also, the position of the pipette should be drift-free after seal formation to maintain stable recording for several minutes. This is achieved by micromanipulators. Amplifier (HEKA EPC 10) is used for recording whole-cell current or voltages. Patch clamp recordings are analyzed on a personal computer using Patch master software.

To characterize the active electrical property of Smn-deficient NSC-34 cells, the Whole-cell voltage-clamp recordings were carried out at RT. The electrodes with the resistance of 2-3 MΩ were prepared using a puller (Sutter Instrument Co., ModelP-97). A chlorinated silver wire was used as a reference electrode. For our experiment we used the pipette solution consisted of (in mM) 135 K^+-gluconate, 10 KCl, 9 NaCl, 1 $MgCl_2$, 1 EGTA, 3 Mg-ATP, 0.3 Na-GTP, and 10 HEPES, pH 7.35 (295 mOsm/l). The bath solution consisted of (mM) 140 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 Glucose, pH 7.4 (310 mOsm/l).

Appendix-Chapter III

Results & Discussions

Smn-deficient-NSC-34 cells exhibit increased Na^+ current density

We have measured Na^+- and K^+- currents using the whole-cell configuration of the patch clamp technique. The currents were recorded in response to 5 or 10 ms depolarizing voltage steps, from a holding potential of -70 mV. The amplitude of Na^+- and K^+- currents was normalized to the membrane capacitance of the cell. Smn-deficient NSC-34 cells exhibited larger inward current with no change in outward current when compared to EV-transduced cells. **(Figure 58).**

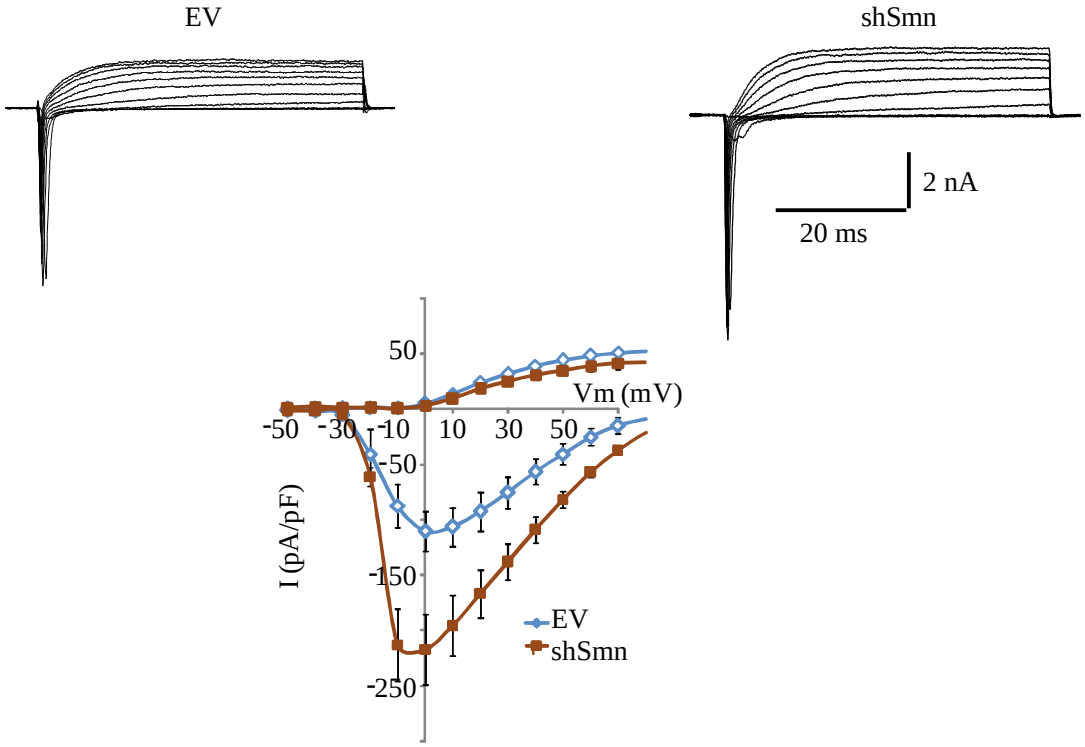

Figure 58. **The Na^+ current density is increased in Smn-deficient MNs**. Examples of families of total whole-cell currents in EV and shSmn-transduced NSC-34 cells. I/V curves (right panel) shows an increase in inward current in Smn-deficient NSC-34 cells (red symbols) compared to EV-transduced cells (blue symbols). However, the size of the outward currents at the end of the pulse was not significantly different. Current amplitude was normalized to cell size by dividing the absolute value of the peak current by the membrane capacitance.

Our preliminary study shows that the shSmn-transduced NSC-34 cells are hyperexcitable consisting of increased Na^+ current density as previously reported (Liu et al. 2015). Like in ALS, SMA MNs are hyperexcitable, i.e. they show high input resistance, low activation threshold, action potentials (APs) of large amplitude and fast-rising rate, and high AP firing frequency (Mentis et al. 2011; Liu et al. 2015). However, the mechanism through which the Na^+ current density increases remain unexplored. SMA MNs have reduced proprioceptive synaptic inputs (Mentis et al. 2011; Ling et al. 2012), and defective

neurotransmitter release (Kong et al. 2009; Ruiz & Tabares 2014; Tejero et al. 2016). Hence, hyperexcitability could be a homeostatic mechanism to compensate for these deficits. Also, very recently, it has been shown that MN hyperexcitability is caused by Smn-deficiency at excitatory premotor interneurons (INs), and that low Smn levels in MNs alone do not produce hyperexcitability (Simon et al. 2016). It is proposed that hyperexcitability results from the decrease in the number of excitatory inputs in MNs and, therefore, on the activity of the motor circuitry. However, the origin of hyperexcitability in Smn-deficient MNs and the role of hyperexcitability in the pathogenesis of SMA need to be further explored in future studies.

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