Study of local protein synthesis in growth cones of embryonic mouse motor neurons

Analyse lokalen Proteinsynthese in Wachstumskegeln von embryonalen Maus Motoneuronen

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Contents

1 SUMMARY

In cultured motoneurons of a mouse model for the motoneuron disease spinal muscular atrophy (SMA), reduced levels of the protein SMN (survival of motoneurons) cause defects in axonal growth. This correlates with reduced β-actin mRNA and protein in growth cones, indicating that anterograde transport and local translation of β-actin mRNA are crucial for motoneuron function. However, direct evidence that indeed local translation is a physiological phenomenon in growth cones of motoneurons was missing. Here, a lentiviral GFP-based reporter construct was established to monitor local protein synthesis of β-actin mRNA. Time-lapse imaging of fluorescence recovery after photobleaching (FRAP) in living motoneurons revealed that β-actin is locally translated in the growth cones of embryonic motoneurons. Interestingly, local translation of the β-actin reporter construct was differentially regulated by different laminin isoforms, indicating that laminins provide extracellular cues for the regulation of local translation in growth cones. Notably, local translation of β-actin mRNA was deregulated when motoneurons of a mouse model for type I SMA (*Smn*$^{-/-}$*; SMN2*) were analyzed. *In situ* hybridization revealed reduced levels of β-actin mRNA in the axons of *Smn*$^{-/-}$*; SMN2* motoneurons. The distribution of the β-actin mRNA was not modified by different laminin isoforms as revealed by *in situ* hybridization against the mRNA of the eGFP encoding element of the β-actin reporter. In case of the mRNA of α-actin and γ-actin isoforms, the endogenous mRNA did not localize to the axons and the localization pattern was not affected by the SMN levels expressed in the cell. Taken together our findings suggest that regulation of local translation of β-actin in growth cones of motoneurons critically depends on laminin signaling and the amount of SMN protein.

Embryonic stem cell (ESC)-derived motoneurons are an excellent *in vitro* system to sort out biochemical and cellular pathways which are defective in neurodegenerative diseases like SMA. Here, a protocol for the differentiation and antibody-mediated enrichment of ESC-derived motoneurons is presented, which was optimized during the course of this study. Notably, this study contributes the production and purification of highly active recombinant sonic hedgehog (Shh), which was needed for the efficient differentiation of mouse ESCs to motoneurons. ESC-derived motoneurons will now offer high amounts of cellular material to allow the biochemical identification of disease-relevant molecular components involved in regulated local protein synthesis in axons and growth cones of motoneurons.

2 Zusammenfassung

In kultivierten Motoneuronen eines Maus-Models für die Motoneuronen-Erkrankung Spinale Muskelatrophie (SMA) verursachen verminderte Mengen des Proteins SMN (*survival of motoneurons*) Schäden im axonalen Wachstum. Dies korreliert mit einer verminderten Menge an β-Aktin kodierender mRNA und β-Aktin Protein. Dies impliziert, dass anterograder Transport und lokale Translation von β-Aktin mRNA für die Motoneuronfunktion notwendig ist. Bislang gab es jedoch keinen direkten Nachweiß funktioneller lokaler Translation in Wachstumskegeln von Motoneuronen.

In dieser Arbeit wurde ein lentivirales GFP-basierendes Reporterkonstrukt etabliert, welches lokale Proteinsynthese von β-Aktin mRNA nachweißt. Zeitraffermikroskopie von GFP-vermittelter Fluoerszenzregeneration nach Fotobleichung (*fluorescence recovery after photobleaching;* FRAP) in lebenden Motoneuronen zeigte, dass β-Aktin in Wachstumskegeln embryonaler Motoneuronen lokal translatiert wird. Interessanterweise wurde die lokale Translation des β-Aktin Reporterkonstrukts differentiell durch verschiedene Laminin-Isoformen reguliert. Dies gibt einen Hinweis, dass Laminin als extrazelluläres Signalmolekül die Regulation der lokalen Translation in Wachstumskegeln reguliert. Die lokale Translation von β-Aktin mRNA war dereguliert wenn Motoneurone eines Mausmodels für die Typ I SMA (*Smn$^{-/-}$;SMN2*) analysiert wurden. *In situ* Hybridisierung bestätigte eine Reduktion von β-Aktin mRNA in den Axonen von *Smn$^{-/-}$;SMN2* Motoneuronen. Die Verteilung der β-Aktin mRNA wurde von verschiedenen Laminin-Isoformen nicht beeinflusst, wie durch *in situ* Hybridisierung gegen eGFP kodierende Elemente des β-Aktin Reporters bestätigt werden konnte. Im Fall der mRNA für α-Aktin und γ-Aktin Isoformen wurde keine axonale Lokalisierung der endogenen mRNAs festgestellt und das Lokalisierungsmuster dieser mRNAs war durch reduzierte zelluläre SMN Mengen nicht beeinflusst. Zusammenfassend deuten diese Befunde darauf hin, dass die lokale Translation von β-Aktin in Wachstumskegeln von Motoneuronen von Laminin-Signalgebung und von der Menge an SMN Protein abhängt.

Motoneurone aus embryonalen Stammzellen sind ein etabliertes *in vitro* System um biochemische und zelluläre Signalwege zu identifizieren, die in neurodegenerativen Erkrankungen wie SMA betroffen sind. Hier wird ein Protokoll zur Differenzierung und Antikörper-gestützten Anreicherung von Motoneuron aus embryonalen Stammzellen präsentiert, welches im Rahmen dieser Arbeit optimiert wurde. Im Besonderen wird die Herstellung und Reinigung von hochaktivem Sonic Hedgehog (Shh) vorgestellt, welches für die effiziente Differenzierung von embryonalen Stammzellen der Maus notwendig war.

Motoneurone aus embryonalen Stammzellen werden in zukünftigen Studien nun große Mengen an zellulärem Material liefern, und somit auf biochemischer Ebene die Identifizierung von krankheitsrelevanten molekularen Komponenten ermöglichen, die in der Regulation der lokalen Proteinsynthese in Axonen und Wachstumskegeln von Motoneuronen involviert sind.

3 Introduction

The neuromuscular system enables the regular movements of skeletal muscles via motoneurons that excite them. Neuronal activity in motoneurons causes the release of neurotransmitters that bind to the post-synaptic receptors at the neuromuscular junction thus mediating the electric activation of the skeletal muscle. During development, motoneurons have to find their path from the ventral spinal cord towards the skeletal muscles. Within this growth process, motoneuron pathfinding and survival depends on a series of fundamental differentiation mechanisms before motoneurons axons reach the skeletal muscle cells. (deLapeyriere and Henderson, 1997; Hamburger and Yip, 1984; Sanes and Lichtman, 1999). Motoneuron growth is a long-distance growth mechanism that requires continous local communication of the growth cone with extracellular cues that enable proper local growth cone physiology and pathfinding.

Several lines of evidence point to a major impact of local protein synthesis in growth cones of motoneurons thus ensuring the local availability of key substrates for regulated growth, differentiation and even presynaptic differentiation and axon maintenance.

3.1 mRNA transport and local protein synthesis

Local protein synthesis has been implemented by organisms in an evolutionarily conserved manner to spatially and temporally compartmentalize the availability of proteins (citation). Sorting of defined mRNA species to distinct subcellular regions is observed in many different cell types, eg.MBP (myeline basic protein mRNA in oligodendrocytes. Bicoid and nanos mRNA in drosophila oocytes, β-actin mRNA leading edge of a a migrating chick fibroblast and particularly in neurons (for review see Mohr and Richter, 2001). In neurons, this provides an efficient manner of compartmentalizing the protein synthesis which is an important means to respond to the external cue in a synapse specific manner in dendrites as well as growth cones of axons (citation). This increases the computational capacity several folds in case of dendrites and helps in path finding during axon extension.

Initial studies using radio labeled amino acid incorporation in isolated dendrites convincingly revealed the phenomenon of localized protein synthesis (Rao and Steward 1991). Consequently this pointed to a local availability of mRNA molecules and a protein synthesis machinery in far distance from neuronal somata (Rao and Steward 1991). The landmark discovery that the cytoskeletal protein β-actin is a prominent protein which is expressed, at least in part, by local protein translation from localized β-actin mRNA molecules implicated the existence of specific target sequences for regulated mRNA transport. The actin

cytoskeleton plays an important role in axon initiation, growth, guidance, and also in synaptogenesis and synapse maintainance (for review see Luo, 2002). β-actin protein is highly enriched in distal parts of axons and growth cones. Specific mRNA encoded motifs in the 3' UTR of β-actin are important for the transport of the β-actin encoding transcript (Kislauskis et al., 1993). A 54-bp domain called zipcode (Kislauskis et al., 1994) is bound by still barely defined protein complexes that are involved in this process (Ross et al., 1997; Zhang et al., 2001; Gu et al., 2002). Transport with help of small nuclear ribonucleoprotein particles (snRNPs) carrying β-actin mRNA to axons contributes to this specific distribution (Bassell et al., 1998; Zhang et al., 1999, 2001).
Various attempts have been made in order to visualize the transport of mRNA to the periphery and to understand the kinetics and distribution of mRNA within the cells. Labelled *in vitro* transcribed MAP2 and CamKIIα mRNAs were simultaneously microinjected in hippocampal neurons. The labeled mRNA formed granules which showed a rapid movement in a microtubule dependent bidirectional manner revealing that neuronal transcripts are differentially sorted to either dendrites (CamKIIα) or remain with in the cell somata (MAP2) (Tubing, Vendra et al. 2010). This sorting was abolished in the absence of the signal sequence in the respective mRNA underscoring the importance of the 3'UTR signal sequence in the sorting of mRNA in the cell.

Increasing evidence points to the importance of RNA localization in motoneurons physiology and transport within polarized cells. This is of clinical relevance. Regulation of the transport, the repression of translation of mRNA during transport, regulation of the translation initiation of specific mRNAs, its stability and degradation in response to specific external stimuli are some of the important steps involved in mRNA metabolism. Several of these processes are deregulated in the disease conditions. It is important to understand the molecular basis of various steps of mRNA metabolism for development of therapeutic strategies for some of the important diseases such as FXS (Fragile X syndrome) the motoneurons diseases ALS (Amyotrophic lateral sclerosis) and SMA (Spinal muscular atrophy).

Using microarray analysis of mRNA isolated from micro dissection of brain slices and by compartmentalized culturing of neurons, several hundreds of mRNA has been identified to be localized preferentially to various subcellular locations (Zivraj, Tung et al. 2010). In neural cells local and regulated translation of some of these localized mRNAs has been shown to be involved in neuronal processes like stimulus-triggered neurite outgrowth and/or collapse, axon guidance, synapse formation, pruning, activity-dependent synaptic plasticity, and injury-induced axonal regeneration.

Various cis-acting signal sequences in the 3'UTR and 5'UTR of the mRNA are known to be involved in the targeting of mRNA to specific locations and its translation in response to external cues. The RNA transport sequence for mRNA, the RTS(also referred to as the A2RE and the hnRNP A2 response element) or smaller segments of this sequence (A2RE11: GCCAAGGAGCC) have been demonstrated to be necessary and sufficient for trafficking of myelin basic protein RNA in oligodendrocytes (Barbarese, Brumwell et al. 1999). The RNA-binding protein HuR affects the stability of specific short-lived mRNAs that have an ARE (AU-rich element, with a AUUUA core sequence) in their 3´UTR. Translin (testis-brain RNA-binding protein, TB-RBP) is another protein that recognizes a small oligonucleotide, the Y-element CTGAGCCCTGAGCT. Blocking translin binding to this element disrupts the localization of the mRNAs encoding protamine 2, ligatin and CaMKIIα (Severt, Biber et al. 1999). One of the best studied targeted mRNA is β-actin. In case of β-actin, a 54nt sequence in the 3'UTR has been characterized to be responsible for the transport of mRNA to the periphery of the axons and chick embryo fibroblast cells (Kislauskis, Zhu et al. 1994).

3.2 Visualization of local translation

The initial discovery that mRNAs are transported to subcellular compartments of cells (Zhang, Eom et al. 2001) initiated research on the function of local protein translation and various attempts have been made in order to visualize the translation of locally transported mRNA (Aakalu, Smith et al. 2001). Especially the combination of recombinant expression constructs enabling the live imaging of local synthesis of fluorescent proteins such as GFP by microscopic approaches had a strong impact on the research field..

Aakalu et al 2001 used eGFP reporter constructs for CamKIIα, (5'eGFP3') where they fused both the 5'UTR and the 3'UTR of CamKIIα (Aakalu, Smith et al. 2001) to the coding region of eGFP. Recovery of fluorescence intensity after local laser-assisted bleaching of GFP fluorophores enabled to visualize local protein synsthesis in dendrites of isolated hippocampal neurons transfected with the eGFP reporter of CamKIIα. Due to the problem of free diffusion of soluble cytosolic eGFP, the authors developed a myristylation signal sequence for membrane-anchoring GFP reporter construct (Aakalu, Smith et al. 2001). With the help of this approach they were able to show that local protein production of CamKIIα at synapses is stimulated by application of BDNF.

A similar approach of membrane-anchored destabilized eGFP reporter construct was also used by Zhang et al. (Zhang, Eom et al. 2001) visualizing the local protein production of a β-actin reporter construct in rat DRG axons.

An alternative approach was devised by Yan et al. 2009. These authors used dendra, fusion construct as well as reporter construct for CEBP mRNA. Dendra is a photo convertible

protein.These experiments showed the involvement of DLK kinase in the stabilization and translation of localized mRNA in axons and synapses of *C. elegans* (Yan, Wu et al. 2009).

3.3 Regulation of local translation by external cues

Local protein synthesis is mandatory for the regulated dynamics of growth cone extension, collapse and turning (Campbell and Holt 2001; Wu, Hengst et al. 2005; Hengst, Deglincerti et al. 2009; Holt and Bullock 2009). The rearrangements of cytoskeletal elements in growth cones of Xenopus retinal axons are triggered by chemotropic gradients of the guiding factors Semaphorin 3A (Sema3a) and Netrin-1 (Campbell and Holt 2001). These factors act by triggering local protein synthesis and regulated changes in protein levels, not only via protein synthesis, but also by stimulated protein degradation (Campbell and Holt 2001). This mechanism is evolutionary conserved. Local translation of RhoA has been demonstrated to be involved in the Semaphorin3a mediated growth cone collapse in rat DRG growth cones (Wu, Hengst et al. 2005). an asymmetric translation of β-actin in the growth cones of Xenopus cortical neurons was shown in response to nitrin-1 signalling (Leung et al. 2006). This revealed that the growth cone steering in response to the external cue in neurons depends on the regulation of the local translation (Leung, van Horck et al. 2006).

Spatial and temporal regulation of localized mRNA in response to guidance cues may be achieved by regulation of the signalling to various RNA binding proteins. ZBP-1 (Zip code binding protein) is an RNA binding protein that binds to the ZIP code of the β-actin mRNA. It was demonstrated by Sasaki et al. 2010 (Sasaki, Welshhans et al. 2010) that the BDNF signalling causes phosphorylation of the ZBP-1 protein via Src kinase pathway and in turn up regulates the translation of β-actin mRNA.

Several other mRNA binding proteins and transport proteins have been identified that interact with β-actin mRNA. hnRNP-R is an RNA binding protein which was shown to interact with the 3'UTR of β-actin mRNA in the axons of mouse motor neurons (Rossoll, Jablonka et al. 2003). hnRNP-R binds to the 3'UTR of the β-actin mRNA in a complex which contains several proteins including SMN (survival of motor neurons). This finding led to the development of the hypothesis that the disease spinal muscular atrophy (SMA) is a consequence of a motoneuron-specific function of the SMN Protein and that the regulation of local protein translation is part of the disease pathophysiology ((Rossoll, Jablonka et al. 2003)).

3.4 Spinal muscular atrophy

Spinal muscular atrophy is caused by the mutation or deletion of the SMN1 gene encoding for survival of motor neuron (SMN) which is inherited in an autosomal recessive manner (Crawford and Pardo 1996). Depending on the severity of the disease and the age of onset,

SMA is categorized as type 1, type 2 and type 3 with type 1 being the most severe form of the disease due to infantile onset and death within 6 months of age (Crawford and Pardo 1996). Fig 1 describes the genetics of the SMN gene in humans.

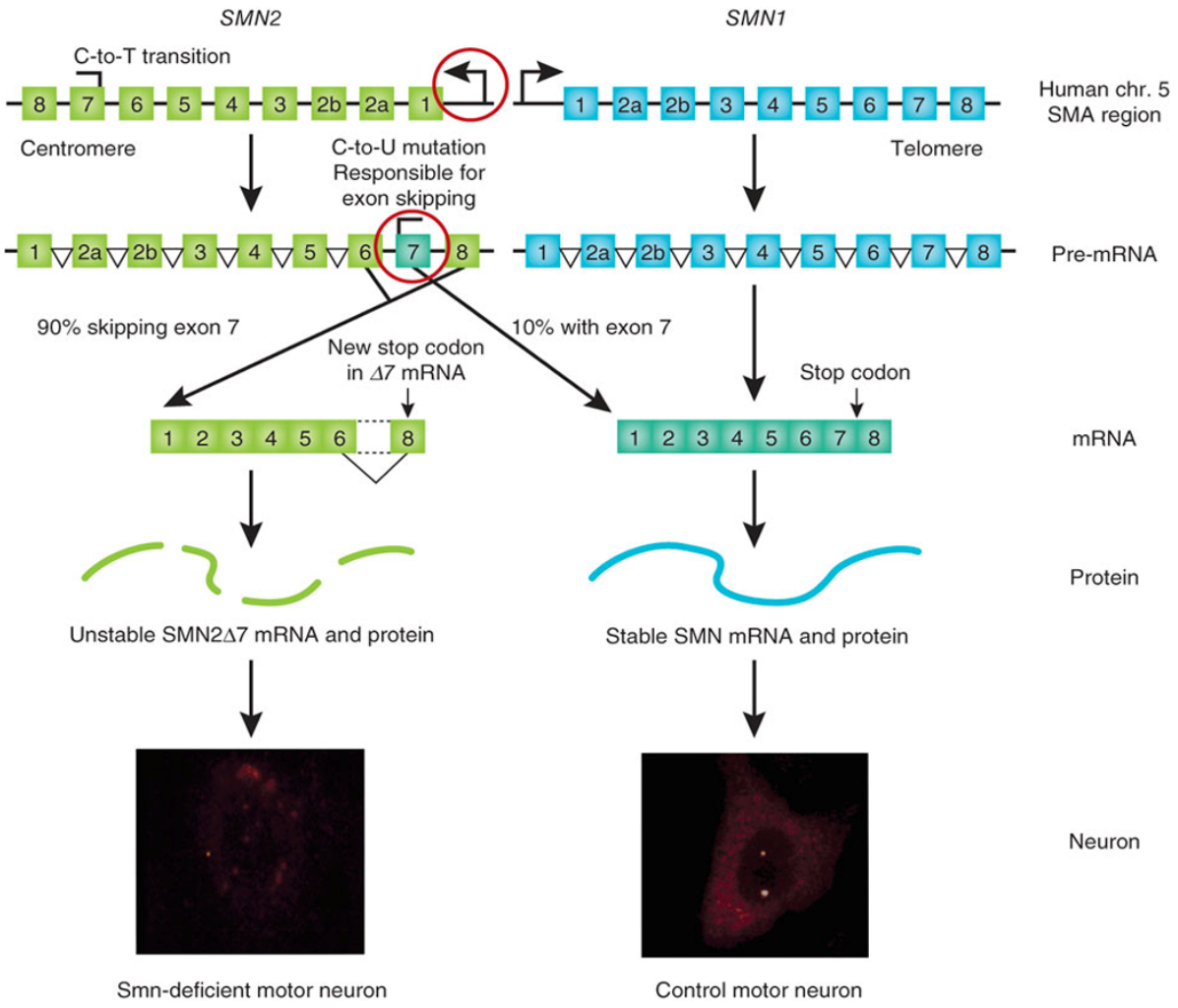

Fig1 Schematic diagram of the *SMN1* and *SMN2* genes on human chromosome (chr.) 5. In SMA, both copies of the *SMN1* gene are deleted or mutated. The *SMN2* gene is also expressed, but most of the resulting gene products give rise to a truncated SMN protein lacking the regions encoded by exon 7. This is caused by a C-to-T transition at position 6 of exon 7, leading to disruption of a splice enhancer site and generation of a new splice silencer site. Targets for therapy are marked as red circles. Increase of *SMN2* promoter activity gives rise to enhanced production of truncated *SMN2Δ7* mRNA, but also to enhanced production of *SMN2* full-length mRNA and SMN protein. Restoration of splicing and inclusion of exon 7 by means of antisense oligonucleotides forms a second target for therapy development. Bottom, SMN protein is normally found in both the nucleus and the cytoplasm of spinal motor neurons (right); the deficit in SMN expression (left) depletes SMN immune reactivity in both regions. From (Sendtner 2010)

Protein derived from the SMN2 gene is unstable and is not able to compensate for the loss of SMN1 gene but multiple copies of SMN2 gene may modulate the disease severity(Crawford and Pardo 1996; Battaglia, Princivalle et al. 1997). SMN is expressed in all tissues (Paushkin, Gubitz et al. 2002) with high levels within the central nervous system, especially

the spinal cord (Battaglia, Princivalle et al. 1997). SMN is known to be associated with a diverse set of ribonucleoproteins (RNPs) that are targeted to sub-nuclear structures, such as Cajal bodies (Carvalho, Almeida et al. 1999), and participate in spliceosome assembly and pre-mRNA splicing (Paushkin, Gubitz et al. 2002). The SMN complex is also known to contain Gemin2-7 (Battle, Lau et al. 2006) and localize in gems/Cajal bodies in which the pre-mRNA splicing factors are also localized and are thought to play a role in snRNP biogenesis and in the trafficking of snRNPs and snoRNPs (reviewed by (Battle, Kasim et al. 2006).

It is puzzling how the loss of the ubiquitously expressed SMN protein, which is a spliceosome assembling component in all cell types, is responsible for a specific degeneration of motor neurons. To address this questions, various model systems have been generated and studied in detail (reviewed in (Park, Kariya et al. 2010). The homozygous loss of SMN in mice is embryonic lethal (Schrank, Gotz et al. 1997), but heterozygous mice (*Smn*$^{+/-}$) are useful as a model for less severe forms of the disease. Two mouse lines, *Smn*$^{-/-}$*;SMN2* (Jablonka, Schrank et al. 2000; Monani, Sendtner et al. 2000) and *SmnΔ7(SMN2*$^{+/+}$*;Δ7*$^{+/+}$*;Smn*$^{-/-}$*)* (Le, Pham et al. 2005), have been vastly used as model for the most severe form of type I SMA.

Various studies on cultured embryonic motor neurons have indicated that SMN may have an additional motoneuron specific role (Jablonka, Rossoll et al. 2000). Investigations on the localization of the SMN protein in cells revealed that the SMN protein is present mainly in the nucleus and forms granules known as gems that are sites of mRNA splicing (Liu and Dreyfuss 1996). Later studies revealed that SMN is not only present in the nucleus but also in the cell cytoplasm and the axons of the motor neurons (Rossoll, Kroning et al. 2002; Zhang, Xing et al. 2006), specifically in the growth cones of motor neurons. SMN is found as part of a complex in growth cones which contains Gemin2 and Gemin3 to form a mRNP complex. This complex shows a bidirectional movement along the axons of motor neurons (Zhang, Xing et al. 2006). In a yeast two hybrid screen for interaction partners of SMN, the RNA binding protein hnRNP-R was identified (Rossoll, Kroning et al. 2002). Motor neurons isolated from *Smn*$^{-/-}$*;SMN2* embryos show shorter axon length when cultured on Laminin111 but over expression of hnRNP-R in a SMN deficient condition rescues the axon length phenotype (Rossoll, Jablonka et al. 2003). In the same study, it was seen that the SMN-hnRNP-R complex associates with β-actin mRNA in the growth cones of motor neurons (Jablonka, Beck et al. 2007). Further studies to understand the role of SMN and its interaction partner hnRNP-R in translocation of β-actin mRNA revealed its crucial role in its transport to the axon terminals (Glinka, Herrmann et al. 2010). Knock down studies of

hnRNP-R revealed that they also show reduced levels of β-actin mRNA in axons and axon terminals. *In vivo* studies to knock down the hnRNP-R protein using morpholinos in the zebra fish embryo showed increased branching and shorter axons (Glinka, Herrmann et al. 2010). This phenotype is similar to that of SMN knock down by morpholino in zebra fish (Winkler, Eggert et al. 2005).

β-actin mRNA was found to be enriched in the growth cones of embryonic cultured motor neurons isolated from the spinal cords of *Smn$^{+/+}$;SMN2,* whereas there was significant decrease in the β-actin mRNA in the growth cones of the motor neurons isolated from *Smn$^{-/-}$;SMN2* as seen by *in situ* hybridization against β-actin mRNA, indicating that the SMN protein may be involved in the transport of the β-actin mRNA. There is reduction in the size of the growth cones of *Smn$^{-/-}$;SMN2* motor neurons (Rossoll, Jablonka et al. 2003). This effect was accompanied by defective calcium channel clustering in growth cones, which caused reduced spontaneous calcium transients in growth cones of *Smn$^{-/-}$;SMN2* motor neurons. Interestingly the frequency and the amplitude of the calcium transients was increased in the *Smn$^{+/+}$;SMN2* when cultured on Laminin211/221 as compared to *Smn$^{+/+}$;SMN2* motor neurons cultured on Laminin111. This increase in spontaneous calcium transients was abolished in *Smn$^{-/-}$;SMN2* motor neurons. This indicated that the external cue of Laminin211/221 to stop growing and start differentiating is defective in *Smn$^{-/-}$;SMN2* motoneurons. Indeed, axon length of motoneurons isolated from *Smn$^{-/-}$;SMN2* is increased when cells are cultured on Laminin211/221 (Jablonka, Beck et al. 2007). Knock down of the protein PTEN (phosphatase and tensin homolog), a negative regulator of the mammalian target of rapamycin (mTOR) pathway, is able to rescue the axon growth defects and improves the survival of the motoneurons (Ning, Drepper et al. 2010).

3.5 Laminin isoforms as signalling cues in the extra cellular matrix

Laminin isoforms are a group of heterotrimeric molecules present in extracellular matrix of different tissues. The Laminin protein family consist of five α subunits, four β subunits and three γ subunits which form a heterotrimeric complex to give fifteen different isoforms of laminin (Li, Edgar et al. 2003).Extracellular matrix molecules are important signalling cues for guiding the axons to its target muscles and are also signalling components that regulate growth cone growth and differentiation into a presynaptic compartment (Nishimune, Sanes et al. 2004). Laminins also guide the mylenation of the extending axons of the peripheral neurons by Schwann cells (Chernousov, Yu et al. 2008). During development of the peripheral nervous system, Laminins, a family of extracellular matrix proteins play an important role in axon growth. Studies using adult mouse DRG neurons (Plantman, Patarroyo et al. 2008) showed that neurites grow faster and become longer on Laminin-1 (Laminin111) and Laminin-10 in comparison to the synapse specific Laminin-2 (Laminin211/221) and Laminin-8. Laminins are differentially expressed in various tissues.

Laminin111 is present in many basal laminae in peripheral nerves and skeletal muscle, whereas Laminin211 is present in the synaptic cleft at neuromuscular endplates (Sanes and Lichtman 2001). Laminin-4 (Laminin221), Laminin-9 (Laminin421), and Laminin-11 (Laminin521) are predominant in the small stretch of basal lamina that extends through the synaptic cleft at the neuromuscular junction (NMJ) (Patton, Miner et al. 1997). Mutant mice lacking Lamininβ2 that is common to the synaptic cleft specific Laminin221, -421, -521 show defects in differentiation of neuromuscular junctions (Noakes, Gautam et al. 1995).
Nishimune et al. (2004) demonstrated that the Lamininβ2 subunit present in the synaptic cleft interacts directly with the α subunit of voltage gated calcium channels in the presynapse. This then mediates the clustering of the calcium channels which promotes the differentiation of the presynaptic compartment (Nishimune, Sanes et al. 2004). Mutations in the Laminin isoforms cause several forms of muscular dystrophy and myelination defects (reviewed in (Chernousov, Yu et al. 2008). Mice lacking laminin β2 show marked defects in maturation of motor nerve terminals (Noakes, Gautam et al. 1995) and thus serve as a model system to understand the pathophysiology of defects at the neuromuscular junction apparent in several motoneuron degenerative diseases such as congenital muscular dystrophy (CMD). In view of these findings, the role of Laminins is of great clinical relevance.Previous studies demonstrated that axon growth in primary motoneurons is differentially influenced by various Laminin isoforms (Porter and Sanes 1995; Jablonka, Beck et al. 2007). Motor axons are shorter on Laminin221/211 and presynaptic differentiation is stimulated when these neurons are grown on Laminin221/211, the neuromuscular end plate specific Laminin, as compared to the more ubiquitously expressed Laminin111 that represents the predominant Laminin isoform from Schwann cells (Porter and Sanes 1995; Jablonka, Beck et al. 2007). This effect of different Laminins on axon growth is altered in motoneurons isolated from E13.5 embryos of *Smn*$^{-/-}$*;SMN2* mice. In this mouse model, the SMN (survival of motoneuron) protein is depleted and it serves as a model of type 1 spinal muscular atrophy (SMA) (Monani, Sendtner et al. 2000; Rossoll, Jablonka et al. 2003; Jablonka, Beck et al. 2007; McGovern, Gavrilina et al. 2008; Murray, Lee et al. 2010).

3.6 Embryonic stem cell differentiation into motor neurons

Primary motoneurons can be isolated from the spinal cord of chick(Arakawa, Sendtner et al. 1990), rat(Hughes, Sendtner et al. 1993), mouse(Wiese, Metzger et al. 1999) and even human embryos(Silani, Brioschi et al. 1998). They can be enriched and studied in culture at high purity in the absence of other neuronal and non-neuronal cells that influence specific properties such as survival(Wichterle, Lieberam et al. 2002) and axon growth(Porter, Weis et al. 1995). Such isolated embryonic motoneurons have been widely used to study mechanisms of motoneuron survival(Clarke and Oppenheim 1995; Sendtner, Pei et al. 2000), axon growth and guidance(Ming, Henley et al. 2001; Nguyen, Sanes et al. 2002), and

also for the analysis of disease mechanisms underlying amyotrophic lateral sclerosis(Camu and Henderson 1994) and spinal muscular atrophy(Rossoll, Jablonka et al. 2003). However, these neurons are normally only available at limited numbers, making it impossible to perform state of art analyses of disease mechanisms using for example Chromatin immune precipitation (ChIP) or microarray analysis for studying gene expression or pre mRNA splicing. Such techniques normally require high numbers of cells, usually in a range of several hundred thousands or millions(Tollervey, Curk et al. 2011). The number of motoneurons in the lumbar spinal cord of a mouse embryo at E13 to E14 ranges between 4.000-5.500(Oppenheim, Houenou et al. 1986) and only a portion of these primary motoneurons can be successfully isolated and cultured. Thus, the number of motoneurons that can be obtained from single embryos is by far not sufficient for such techniques that appear key to understand disease processes in spinal muscular atrophy and amyotrophic lateral sclerosis.

Embryonic stem cell technology is an important tool in neurobiology for creating defined populations of neural cells (Graf and Enver 2009; Cohen and Melton 2011; Hansen, Rubenstein et al. 2011) at high numbers. These techniques can be used to create defined populations of mouse and human neurons for studying function and disease mechanisms. They also can serve as a tool for therapy development. In the last five years, significant progress has been made in the field how to differentiate stem cells or to convert other types of cells into specific types of neurons such as midbrain dopaminergic neurons, cortical neurons or motoneurons (Hansen, Rubenstein et al. 2011; Peljto and Wichterle 2011). In principle, three strategies are used to produce specific neuronal populations: The directed differentiation of embryonic stem cells into neurons (Peljto and Wichterle 2011), the direct reprogramming of a specialized cell type into another by transdifferentiation, lineage switching, or lineage conversion (Heins, Malatesta et al. 2002; Heinrich, Blum et al. 2010; Vierbuchen, Ostermeier et al. 2010), or the generation of induced pluripotent stem cells (iPS) and subsequent directed differentiation towards distinct populations of neurons (Takahashi and Yamanaka 2006; Ebert, Yu et al. 2009).

These techniques appear as an attractive option to overcome the problem of low numbers of motoneurons that can be isolated from animal models. Several protocols have been developed to generate motoneurons from mouse (Wichterle, Lieberam et al. 2002; Plachta, Bibel et al. 2004; Wichterle and Peljto 2008) and human ES cells(Li, Du et al. 2005; Shin, Dalton et al. 2005), or to convert these cells from patient derived fibroblasts (Dimos, Rodolfa et al. 2008; Ebert, Yu et al. 2009). However, these protocols are limited in that the purity and homogeneity of the neurons in these culture is lower than with cultures of primary

motoneurons, for example from mouse spinal cord (Wiese, Metzger et al. 1999; Wiese, Pei et al. 2001; Wiese, Herrmann et al. 2010).

3.7 Objective of the present study

Several studies have shown the presence of β-actin mRNA in the growth cones of mouse motor neurons. Local translation of β-actin mRNA has been demonstrated in rat DRGs and Xenopus ratinal ganglion cells but local translation of β-actin mRNA in motor neurons was yet to be demonstrated. But a direct evidence was missing for the presence of local translation on β-actin mRNA in the growth cones of mouse motoneurons. The aim of the present study was to develop a suitable method to visualize local translation in cultured mouse motoneurons. Furthermore, the regulation of β-actin mRNA has been shown to be influenced by RNA binding proteins as well as by external cues for axon guidance. Laminin signaling is an important signaling cue derived by the growth cone of motoneuron axon from the extracellular matrix, it was of interest if and/or how this external cue affects the local protein synthesis. Using this assay system, we also addressed the question if and/or how this local translation is affected in motoneuron degenerative disease SMA (spinal muscular atrophy) is affected.

In order to analyse the disease mechanism at a biochemical level, it is important to have a uniform population of cells in a lorge quantity. The current available method of primary motoneurons does not provide sufficient quantities of enriched motoneurons. On the other hand, embryonic stem (ES) cell derived motoneurons are a good option but provide a mixed population of cells. In order to address this, we aimed to device a protocol for in vitro differentiation of ES cells into motoneurons and enriching the motoneuron for biochemical analysis.

4 Material and Methods

4.1 Chemicals and enzymes

All the chemicals and enzymes were procured from following companies unless otherwise mentioned: Amersham, Applichem, BD, Calbiochem, Chemicon, Fluka, Gibco, Invitrogen, Merck, R&D, Roche, Roth, Sigma-Aldrich, Qiagen, Biotherm, Fermentas, Invitrogen, Roche and Sigma, Worthington.

4.2 Animals

CD-1 wild type mouse line was used for the standardization of all the methods. *Smn*$^{+/-}$*;SMN2* with FVB back ground were used for breeding to obtain *Smn*$^{-/-}$*;SMN2* embryos and their heterozygous as well as wild type litter mates were used for control studies. The animals were bred in the animal facility of the Institut für klinische Neurobiologie, Uniklinik Würzburg.. They were maintained at 12hr/12hr light dark cycle and were on normal diet with full access to food and water.

4.3 Materials

4.3.1 Cell lines

HEK293T cells

HELA

Primary Motor neurons

4.3.2 Buffers, solutions and mediums

2xHBS:280mM

50mM HEPES

1.5mM Na_2HPO_4

Adjust pH to 7.1, filter sterilize and store at -20 ^oC

2.5M $CaCl_2$

10X TBST: 100 mM Tris

1,5 M NaCl

0,2 % Tween 20

ad 1 l dH_2O, pH 7,6

50x TAE: 242 g Tris

571 ml Acetic acid
100 ml EDTA (0,5 M)
to 1 L dH2O

TBE (10x): 108 g Tris Base
55 g Borat
40 ml EDTA (0,5 M)
add 1 l dH2O, pH 8,0

20x SSC: 175,3 g NaCl
88,2 g NaCitrate
ad 1l dH2O; autoclaved

4% PFA :40g Paraformaldehyde in 500ml dH_2O

4-5 drops of 5M NaOH

500ml of phosphate buffer (410ml 0.2MNa_2HPO_4 + 90ml 0.2MNaH_2PO_4) pH7.4.

Mouse tail lysis buffer

0.1M Tris-HCl (pH 8.5)
5mM EDTA (pH 8.0)
0.2% SDS
200mM NaCl

ACSF (stored at 4°C)
127 mM NaCl
3.0 mM KCl
1.25 mM NaH2PO4 H20
23.0 mM NaHCO3
25.0 mM Glucose (added fresh on the day of performing live cell imaging)

4.3.3 For Primary mouse motor neuron culture

Neurobasal (Gibco, cat. no. 21103-041), opened bottles should be used within 4 weeks.

- Horse serum (Linaris, cat. no. SHD3250ZK) Thaw at 4 °C overnight, aliquot into 5 ml and heat-inactivation at 55 °C for 30 min, store inactivated aliquots at -20 °C and thaw directly only before use at room temperature (20-24°C). Do not freeze again. The batch of horse serum has to be tested for compatibility with motor neuron culture (see Wiese et al., 2010).

- 50x B27-Supplement (Gibco, cat. no. 17504-044) store 1ml aliqouts at -20 °C; Glutamax (Gibco, cat. no. 35050-038), 100x (i.e., 200 mM).
- ß-Mercaptoethanol (Sigma, cat. no. M7522) Stock solution 100 µM in sterile cell-culture tested water should be stored in the dark at room temperature.
- Trypsin TRL3 (Worthington cat. no. LS003707) for trypsinization of cell aggregates; 1 g trypsin in 100 ml HBSS; store at -20 °C after thawing at 4 °C; use within 2 weeks.
- Trypsin-Inhibitor (Sigma, cat. no. T-6522) add 1 g to 98 ml HBSS and 2 ml of 1 M HEPES pH 7,4; 1 ml aliquots should stored at 4 °C
- Poly-DL-ornithine hydrobromide (PORN) (Sigma cat. no. P8638) dissolve 50 mg in 1 ml 150 mM borate buffer pH 8.35; store aliquots at -20 °C
- **PORN** working solution of 0.5 mg / ml Poly-DL-ornithine hydrobromide in 150 mM borate buffer pH 8.35.
- Laminin (Invitrogen, cat. no. 23017-015), working concentration 2.5 µg / ml in HBSS; store aliquots at -20 °C
- **Depolarization solution** Sterile filtered solution of 30 mM potassium chloride, 0.8 % (w/v) sodium chloride and 2 mM calcium chloride in water. Store at room temperature.
- p75 Antibody (MLR2, Biosensis, cat. no. M-009-100)
- **p75-antibody dilution solution** Prepare 10 mM Tris/HCl pH 9.5. Store at room temperature.
- **Coating of plates and coverslips** Cover the surface with sufficient PORN solution overnight at 4 °C. On the next day wash three times with water and air-dry coverslips. Thaw laminin aliquots at 4 °C and prepare 2.5 µg / ml laminin solution in HBSS. Cover the surface of PORN coated plates or coverslips. Incubate over night at 4 °C or at least 2 h at room temperature; laminin coated coverslips can be stored at 4 °C and used within 1 week.
- **Preparation of panning plates** Dilute p75 antibody 1 to 5000 in sterile antibody dilution solution. Cover the surface of a culture dish with sufficient antibody solution over night at 4 °C or for minimum of 2 hours at room temperature. Before dishes can be used for panning they should be washed 3 times with HBSS and covered with prewarmed Neurobasal medium containing GlutaMAX (Gibco). Store panning plate at room temperature until use.

- Neurotrophic factors, e.g. CNTF stocks 10 µg / ml or BDNF stocks 10 µg / ml (made in-house).

4.3.4 Antibodies and Labeling dyes

chicken anti GFP	Abcam
mouse anti Map2	Sigma

rabbit anti Tau	
DAPI (4´,6-diamidino-2-phenylindol)	Sigma Aldrich
Mouse monoclonal anti Sonic hedgehog	Institute of Neurobiology Uni. Wuerzburg
Mouse anti p75 Mouse anti p75	Abcam
Rabbit anti Islet	Sigma
goat anti chicken Alexa488	Invitrogen
donkey anti mouse DyLight549	JacksonImmunoResearch
donkey anti rabbit DyLight649	JacksonImmunoResearch
goat anti mouse HRP	JacksonImmunoResearch

4.3.5 Plasmids, Vectors and Lentiviruses made during the course of work.

The clones are deposited in the central plasmid facility with the vector map and sequence details.

Clone number	Vectorbackbone	discription
002-RR	pEGFP-C-1	Mouse Beta actin orf+3'utr
003-RR	pmCherry C-1	Mouse Beta actin orf+3'utr
004-RR	pLVXDsRed	Mouse Beta actin orf+3'utr
005-RR	pLVX Puro	EOS wt mouse Beta actin orf+3'utr
006-RR	pLVX Puro	MyrEOS wt mouse Beta actin orf+3'utr
007-RR	pLVX Puro	EOS td
008-RR	pLVX Puro	EOS td mouse beta actin orf+3'utr
009-RR	FuVal Ubiquitin promotor	MyrDestabilized DsRed fusion with 3'UTR of mouse beta actin
010-RR	FuVal Ubiquitin promotor	MyrDestabilized DsRed
011-RR	FuVal Ubiquitin promotor	MyrDestabilized DsRed fusion with 3'UTR of mouse beta actin

012-RR	FuVal Ubiquitin promotor	MyrDestabilized DsRed
013-RR	FuVal Ubiquitin promotor	MyrDestablized EGFP
014-RR	FuVal Ubiquitin promotor	MyrDestablized EGFP 3'UTR mouse beta actin
015-RR	FuVal Ubiquitin promotor	LCK Myr EGFP
016-RR	pcDNA3	LCK Myr signal sequence tagged at N-terminus of EGFP 3'UTR mouse beta actin
017-RR	pcDNA3	LCK Myr signal sequence tagged at N-terminus of EGFP
018-RR	FuVal Ubiquitin promotor	LCK Myr signal sequence tagged at N-terminus of EGFP 3'UTR mouse beta actin
019-RR	pcDNA3	LCK Myr signal sequence tagged at N-terminus of EGFP ZIP mouse beta actin
020-RR	FuVal Ubiquitin promoter	LCK Myr signal sequence tagged at N-terminus of EGFP ZIP mouse beta actin
008-RR A	Topo	Eosmono mouse beta actin orf+3'utr
004-RR A	Topo	Mouse Beta actin orf+3'utr
003-THRR	pTZ19R	3'UTRwith polyA tail (mouse beta actin)

002RR was made my Thomas Premsler

003THRR was made by Thomas Herrmann

015RR and 016RR was prepared by Steven Havlicek and Robert Blum

4.3.6 Primers for cloning

Amplicon	Forward primer	Reverse primer
Eos	GGGCCCATGAGTGCGATTAAGCCAGACAAT	CTCGAGACCCTCTAGATAATCGTCGT CTGGCATTGTC
MyrEos	GGGCCCATGGGGAGCAGCAAGAGCAAGAGTGCG ATTAAGCCAGACAT	CTCGAGACCCTCTAGATAATCGTCGT CTGGCATTGTC
Myr-Destablised DsRed	TACGGATCCGCCACCATGGGCTGTGTCTGCAGCT CAAACCCTGAAGATGACGGCGGATCTGGCGGAG CCTCCTCCGAGGACGTCAT	ATCAGCGGCCGCCTACACATTGATCC TAGCAGAAGC
3'UTR(β-actin) for 011RR	ATGTGCGGCCGCGGACTGTTACTGAGCTGC	ATGAATTCGTTTGTGTAAGGTAAGGT GTGC
Myr-EGFP-short	TACGGATCCGCCACCATGGGCTGTGTCTGCAGCT CAAACCCTGAAAGATGACGTGAGCAAGGGCGAG GAGCTGTT	ATGAATTCTTAGCTAGCCTTGTACAGC TCGTCCATGCC
Myr-EGFP-long FOR 015RR	TACGGATCCGCCACCATGGGCTGTGTCTGCAGCT CAAACCCTGAAGATGACGGCGGATCTGGCGGAG TGAGCAAGGGCGAGGAGCTGTTTGCTAGCTAAGC GGACTGTTACTGAGCTGCGTT	ATGAATTCTTAGCTAGCCTTGTACAGC TCGTCCATGCC
3'UTR(β-actin) FOR 018RR	TGCTAGCTAAGCGGACTGTTACTGAGCTGCGTT	ATGAATTCGTTTGTGTAAGGTAAGGT GTGC
ZIP(β-actin) FOR 020RR	TGCTAGCTAAGCGGACTGTTACTGAGCTGCGTT	
3'UTR(α-actin) FOR 024RR	TGCTAGCTAAGCGCACTCGCGTCTGCGTT	ATGAATTCTTGGAGCAAAACAGAATG GCTGG

4.3.7 PRIMERS FOR RT

Amplicon	Forward	Reverse
WPRE	GGAGTTGTGGCCCGTTGTC	AGTTCCGCCGTGGCAATAG
WPRE	TGTTGGGCACTGACAATTC	CCGAAGGGACGTAGCAGA

α-ACTIN	TAGACACCATGTGCGACGAAGA	GCCTCATCACCCACGTAGGAG
Γ-ACTIN	ATCGCCGCACTCGTCAT	GCCGTGTTCGATAGGGTACTTC
β-ACTIN	CATCCGTAAAGACCTCTATGCC	AACGCAGCTCAGTAACAGTCC
β-2LIKE-ACTIN	CAACACACCTGCCATGTATGTAG	GACATCCCGCACGATCTC
α-ACTIN-3'UTR	CAGGACGACAATCGACAATC	GAATGGCTGGCTGGCTTTAATGCT
β-ACTIN-3'UTR	CTGCGTTTTACACCCTTT	GTAGAACTTTGGGGGATGTT

4.3.8 Primers for smn Genotyping

Amplicon	Forward	Reverse
Smn wt	CTGGAATTCAATATGCTAGACTGGCCTG	AATCAATCTATCACCTGTTTCAAGGGA GTTG
SMNKO	CTGGAATTCAATATGCTAGACTGGCCTG	GATGTGCTGCAAGGCGATTAAGTTG

4.3.9 LNA probes for Insitu hybridization

Recognised mRNA	Probe sequence
Actb2L	/5Biosg/TCAAGACACATTATTAGGCTGA/3Bio/
α-3'UTR	/5Biosg/TGAGGTAAAACGAGTCAATCT/3Bio/
β-3'UTR	/5Biosg/AAACGCAGCTCAGTAACAGT/3Bio
γ-3'UTR	/5Biosg/AGTGACCGAGCCACATGAACTA/3Bio/
eGFP	/5Biosg/ATGTTGTGGCGGATCTTGAAGT/3Bio/
Scrambled	/5Biosg/AAGATGTGGCGGATCATGATGT/3Bio/

4.3.10 Ladders

100bp ladder	fermentas
1Kb ladder	fermentas

4.3.11 Kits

Endofree Plasmid Maxi Kit (10)	QIAGEN
Topo cloning kit	Invitrogen

Minipräp Plasmid Kit	SeqLab
Big dye terminator kit	Applied bioscience
NucleoBond Xtra Midi	Macherey-Nagel
QIAEx II Gel Extraction Kit (150)	QIAGEN
QIAquick PCR Purification Kit (50)	QIAGEN
LightCycler FastStart DNA Master SYBR Green I	Roche
SuperScirpt First-Strand Synthesis system for RT-PCR	Invitrogen
Invitro transcription Messageamp kit	Ambion
Neuromag Magnetofection Kit	OZ biosceneces

4.3.12 For Production of active sonic hedgehog

***E. coli* Culture Medium** prepare 5 l of Magic Medium (Invitrogen) according to the manufacturers instructions

Lysis buffer (500 mM NaCl, 20 mM Imidazole, 20 mM Sodium Phosphate buffer pH 7.5, 1 mM DTT, 1 mM EDTA, 1 tablet of Protease Inhibitor, 50 µl Benzonase, Lysozyme)

Buffer A (binding buffer) To make buffer A prepare fresh from stock solutions directly before use (500 mM NaCl, 20 mM Imidazole, 20 mM Sodium Phosphate buffer pH 7.5, 1 mM DTT)

Buffer B (elution buffer) To make buffer B prepare fresh from stock solutions directly before use (500 mM NaCl, 500 mM Imidazole, 20 mM Sodium Phosphate buffer pH 8.2)

Buffer C (Factor Xa buffer) To make buffer C prepare fresh from stock solutions directly before use (1 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris-HCl pH 8.0)

Buffer D (Palmitoylation buffer) To make buffer D prepare fresh from stock solutions directly before use (40 mM Sodium Phosphate buffer pH 7.0, 25 mM DTT, 150 mM NaCl)

Buffer E (final buffer) To make buffer E prepare fresh from stock solutions directly before use (5 mM Sodium Phosphate buffer pH 5.5, 150 mM NaCl, 1 % β-octylglucopyrinoside, 0.5 mM DTT)

4.3.13 For ES cell differentiation into motor neurons

- **ES medium 1** For 20 ml set up (17.56 ml DMEM, 200 µl NEAA (100 x), 40 µl ß-Mercaptoethanol of 50mM (final concentration 0.1mM), 2 ml KOSR (final concentration10 %), 200µl Pen/Strep.
- **ES medium 2** For 20 ml set up 19.5 ml DEMEM/F12 (with 4500 mg / ml Glucose), 40µl ß-mercaptoethanol of 50 mM(final concentration 0.1mM), 200 µl ITS (100 X), 4 µl Progesterone of 100µM (final concentration 20 nM), 3 µl Putrescine of 500mM (final concentration 60µM), 200 µl Pen/Strep of 100 X stock.
- **ES medium 3** For 40 ml set up (36 ml Neurobasal, 4 ml KOSR (10 %), 200 µl Pen/Strep (100 x), 80 µl ß-mercaptoethanol (50 mM))
- **ES cell culture differentiation medium** Mix ES medium 1, 2 & 3 in a ratio of 1:1:2 to make differentiation medium. Prepare fresh before use.
- **Coating of ES cell culture dishes** Coat culture dishes with 0.2 % gelatine solution for at least 10 min in the incubator.

- **Motoneuron culture medium** Neurobasal full medium: Neurobasal supplemented with 10 % horse serum, 1 x Glutamax, 1% B27 supplement.

4.3.14 Microscopes and instruments

Leica sp5 confocal microscope equipped with 488,561,649and UV lazer with 50mWatt power for illumination.
Nikon TE2000 epifluroscence microscope with
Roche real time light cycler PCR machine
Eppendorf gradient master cycler instrument

4.3.15 Software

Adobe Photoshop 7.0

Oligo 6

ApE – A plasmid editor

ImageJ software (WS Rasband, ImageJ, US National Institute of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/. 1997-2011)

Confocal Software (Leica, LAS AF Lite)

NIS element (Nikon TE2000)

4.4 Methods

4.4.1 Primary motor neuron culturing

The dissection of the lumbar spinal cord and culturing of motor neurons was done as described in Wiese et al. 2010. E15 embryos were dissected out from pregnant mothers. Lumbar spinal cords were dissected from the embryos and collected in 180µl HBSS. The head of the embryo was collected in 300µl of lysis buffer. The spinal cord was trypsinised by adding 1% trypsin to 180µl HBSS containing the spinal cord and incubating at 37 °C for 15min. The cells were triturated well and enriched by transferring them in a p75 panning 12 well cell culture plate with 1 ml neurobasal medium. The cells were incubated at room temperature for 45min and gently washed 3 times with 1ml pre-warmed neurobasal medium. The cells were depolarized with 100 µl of depolarization solution and eluted with neurobasal full medium. The cells were collected in a 2 ml reaction tube and centrifuged at 1200rpm for 4min. The supernatant was removed and the cells were resuspended in 500µl of neurobasal full medium. The cells were counted and 10000 to 15000 cells were used for plating on PORN + laminin111 or laminin211/221 coated live cell imaging microdishes after infecting with the virus. For virus infection, the cells were incubated with 1-3µl of corresponding measurements, 1500 to 2000 cells were plated on each coverslips coated with PORN and Laminin.

4.4.2 Genotyping of mouse embryos

The head from E14 embryos were put in 300µl lysis buffer and 20µl Proteinase K (20mg/ml).
Incubate head samples at 60^0C for overnight in a thermomixer. Isolate DNA from the dissolved head.
Add 450µl of 5%SDS and 150µl 3M NaCl and vortex. Add 750µl Chloroform and vortex and centrifuge at 4^0C for 10min at 14000rpm.
Take the upper phase in a 2ml reaction tube and add 750µl Chloroform and vortex and centrifuge at 4^0C for 10min at 14000rpm.
Take upperphase in a 1.5ml reaction tube (approximately 600-650µl) add 100% ethanol to to a final volume of 1ml.
Mix and centrifuge at 4^0C for 10min at 14000rpm.
Wash with 500µl 70% ethanol and centrifuge at 4^0C for 10min at 14000rpm.
Dry the pellet and dissolve in 100µl of TE buffer for over night. Use this as DNA sample for the PCR.

Reaction Conditions: following reaction was set up for the genotyping with the help of PCR amplification. Standard PCR reaction was set up to make 50µl reaction volume with 1 µl DNA, 5 µl 10 x PCR buffer,1 µl dNTP (10 mM), 1 µl each forward and reverse primer (10 pmol/µl) with 0.3 µl Taq polymerase (5-prime) and make up the volume to 50µl with H_2O

Cycle Conditions:	3´ @ 94°C		
Melting condition :	30´´ @ 94°C	⎫	
Anneling condition	30´´ @ 59°C	⎬	34X
Extension condition	1´ @ 72°C	⎭	
	5´ @ 72°C		
	∞ @ 15°C		

The product size for the genotyping is 600bp for *Smn$^{-/-}$;SMN2* and 879bp for *Smn$^{+/+}$;SMN2*

4.4.3 Cloning

4.4.3.1 Restriction enzyme digestion and analysis of DNA fragment.

Cloning was done using standard laboratory manual of Sambrook et al. For restriction digestion of plasmids for cloning, 2.0µg DNA was used with 2-5units of restriction enzyme and appropriate buffer depending on the restriction enzyme used. The reaction volume was 50µl. For the cloning of PCR products, the entire volume of the PCR (50µl) was purified using PCR purification kit (Qiagen) and used for the restriction digestion. The reaction was incubated at 37°C for 2-6 hours and analyzed by gel electrophoresis on 0.8% to 2% agarose gel in 1XTAE buffer depending on the size of the expected DNA fragment to be analyzed using UV detector.

4.4.3.2 Polymerase Chain Reaction (PCR)

For amplification of the 3'UTR of β-actin, pEGFP β-actin plasmid was used as a template after linearising it with appropriate restriction enzyme. A standard PCR reaction was done as followed:

25ng DNA,1.5 µl each of forward and reverse primer (10pmole), 5 µl 3prime PCR buffer, 1 µl eppendorf triple master mix proof reading DNA polymerase, 1 µl 10mM dNTPs and made up the voule to 50µl with H_2O

cycle conditions:	5 min	95°C		
Melting conditions	30 sec	95°C	⎫	
Anneling conditions	30 sec	primer specific	⎭	35x
Extension condition	2.5 min	72°C		

10 min	68°C
hold	15-22°C

The amplified product was analysed by gel electrophoresis and nanodrop was used for measuring the concentration of the DNA. The product was purified using a PCR purification kit before further use. For production of the reporter construct for α-actin 3'UTR, cDNA was synthesized from RNA isolated from the spinal cord of E14 mouse embryos (see below) and used a s a template.

4.4.3.3 Ligation of restriction enzyme digested DNA

The vector backbone and the insert are analysed on agarose gel and the concentration of the DNA is estimated with the help of the marker. Around 25ng of vector was used and 125ng of insert was used for the ligation reaction.

25ng Vector DNA

125ng Insert DNA

1µl T4-DNA-ligase

2µl 10x ligase buffer

filled up to a total volume of 20µl with water

At the same time a parallel control only containing vector DNA and no insert is performed to test the vector religation. The reactions were incubated overnight at 16^{o}C.

4.4.3.4 Transformation of chemical competent *E.coli*

Either TOP10 or HB101 chemically competent E.coli cells were used for the transformation of the ligated DNA. 2-5µl ligation reaction was added to 50µl of competent cells and incubated on ice for 30 min. The cells were given a temperature shock by incubating them at 42^{o}C in a water bath for 90 sec and then transferred to ice for 2mins. Prewarmed 500µl of SOB medium was added and bacteria were allowed to recover for 60min at 37 ^{o}C. 50µl of the transformed bacteria were plated on LB-agar plates with appropriate antibiotic as a selection marker, incubated overnight and colonies were selected and grown in 5ml LB medium with antibiotics overnight and DNA was isolated by mini prep to screen for positive clones. Mini preps were performed by alkali lysis method using SeqLab Miniprep plasmid kit. The resultant DNA was screened by restriction digestion profile followed by sequencing of the clone to confirm the identity of the positive clone.

4.4.3.5 Isolation of DNA

200ml LB medium containing appropriate antibiotic was inoculated with the positive clone and Endofree maxi kit (Qiagen) was used to isolate DNA suitable for transfection. In general, the bacterial cells were grown overnight at 37^{o}C in a shaker incubator. The bacterial pellet was obtained by centrifuging the bacteria at 6000rpm for 20min. the medium was removed and the pellet was subjected to alkali lysis method of DNA isolation by resuspending in buffer P1 containing RNAse to which, 10ml of lysis buffer P2was added and gently mixed by inverting the tube up and down several times to lyse the bacterial cells. The reaction was neutralized by neutralization buffer P3 to precipitate the protein. The supernatant was passed through a cartage to clear the solution. 2.5ml of buffer EB was added to the solution and incubated for 30min of ice to make it endotoxin free. The DNA was column purified and then precipitated with isopropanol. The DNA was pelleted by centrifuging at 15000rpm at 4^{o}C for 30min. The DNA pellet was washed with 70% endofree ethanol, air dried and dissolved in 100µl of endofree TE buffer. The concentration and purity was determined using the nanodrop device by checking the OD at 260 and measuring the OD260/280 ratio respectively.

4.4.3.6 Sequencing of the DNA

DNA sequencing was performed by Sanger sequencing (Sanger et al., 1977) using terminator dye (Applied Biosystems). This causes the four used didesoxynucleotides (ddNTP), lacking the 3´-OH group to get incorporated and labeled with a different fluorescent dye. The fragments of the PCR reaction thus generated are of various lengths depending upon the incorporation of the didesoxynucleotieds. These fragments are separated by size by capillary electrophoresis. At the end the sequence is identified by scanning the fluorescent signals of chains separated by size. The sequencing was done by following protocol:

PCR reaction:	500ng	DNA	
	2µl	Termination-mix	
	4µl	5x sequencing buffer	
	15pmol	primer	
	fill up to a total volume of 20µl with HPLC water		
Cycle conditions:	10 sec	96°C	25X
	4 min	60°C	
	5 min	60°C	
	hold	22°C	

The PCR product was precipitated by adding 50µl of 100% ethanol and 2µl of 3M sodium acetate. The DNA was pelleted by centrifugation at 14000rpm for 20min at 4°C. the pellet was washes twice with 70% ethanol, air dried and dissolved in 20µl of Hi-Di formamide and used for sequencing cappliary loading.

4.4.4 Isolation of RNA

Spinal cord were isolated from E14 mouse embryos used either freshly or stored at -80^{o}C for RNA isolation using RNAeasy kit (Qiagen) as per the manufacturers instruction.

4.4.5 cDNA synthesis from RNA(reverse transcription).

RT reaction from the mRNA isolated was performed using Invitrogen Superscript III reverse transcriptase according to the manufacturer's instruction. Following reaction was set up

25ng RNA

1.0 µl oligo dT primer (200-500ng)

1.0µl Random hexamere primer (50-250ng)

1 µl 10mM dNTPs

filled up to 13µl with water

Heat mixture to 65^{o}C for 5 min incubate on ice for at least 1min, add

4µl 5XFirst-Strand Buffer

1µl 0.1MDTT

1µl RNaseOUT

1µl SuperscriptIII RTenzyme

Incubate at 50^{o}Cfor 30-60min inactivate the reaction by heating at 70^{o}C for 15 min. 1:10 dilution in EB buffer was used this as cDNA.

4.4.6 Quantiative PCR

Gene specific primers were used for quantitative PCR for quantifying the mRNA in the sample using Roche SYBR green kit. Following is the schematic representation of the principle of the lightcycler quantification by SYBR green.

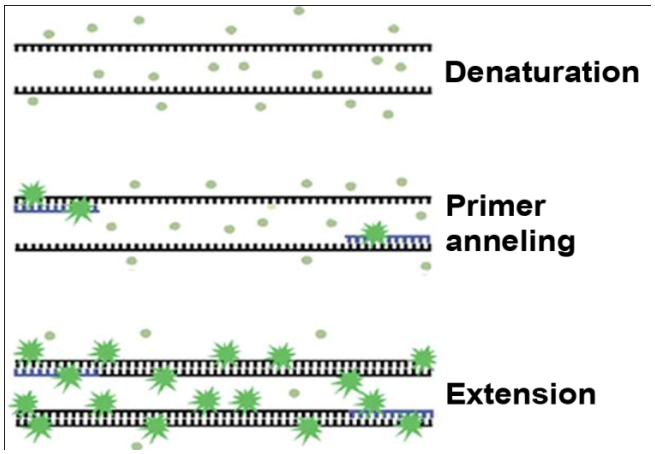

Fig 2 principle of the SYBR green mediated quantification. Adapted from Fraga et al. 2008 Current Protocols Essential Laboratory Techniques.

Primer optimization was done using following conditions and primer specific PCR cycle conditions for each of the gene specific primer.

	1	2	3	4	5	6	7
For Primer	2	3	4	2	3	4	2
Rev Primer	2	3	4	2	3	4	2
$MgCl_2$	1.6	1.6	1.6	2.4	2.4	2.4	1.6
SYBR	2	2	2	2	2	2	2
cDNA	2	2	2	2	2	2	2
H_2O							
	20	20	20	20	20	20	20

cycle conditions for GAPDH mRNA:

10 min	95°C	
0 sec	95°C	50X
5 sec	59°C (primer specific)	
6 sec	72°C (primer specific)	
5 sec	83°C (primer specific)	
0 sec	95°C	
15min	65°C	
0 sec	95°C	

4.4.7 Microinjection

The microinjection was done in 3DIV motoneurons grown on live cell imaging dishes in Leibovitz medium. The microinjection was done with the microinjection apparatus from Eppendorf fixed on the live cell imaging adaptor stage of Nikon TE2000 inverted microscope. The labeled mRNA along with Alexa 568 labeled 10000kD anionic dextran was filled in the injection needle with point diameter of 0.2µm and microinjected with the hold pressure of 40hPa and injection pressure of 80hPa for injection time of 0.3 second.

4.4.8 *In vitro* transcription and fluorescence labeling of mRNA

The plasmid was digested with EcoRI, which cleaves the plasmid at the end of 3'UTR+ polyA and linearizes the vector. The linearized vector was phenol chloroform purified and used as a template for *in vitro* transcription in presence of Alexa 488 labeled UTP. *In vitro* transcription was done using Ambion Megascript kit with the following set up using 3'UTR+polyA PTZ19R clone (clone no. 003THRR).

2µl	ATP
2µl	CTP
2µl	GTP
1µl	unlabeled UTP
1µl	Alexafluro488labeled UTP
2µl	10X reaction buffer
2µl	DNA template (0.5µg linearised DNA)
2µl	Enzyme mix
6µl	nuclease free water
20µl	

Total volume of 20µl was incubated at 37^{o}C for 7hrs. This was then subjected to spin column purification on spin 30 PEG columns from Biorad as per manufacturer's instructions. The yield of such a reaction was 544.5ng/µl of mRNA with the dye incorporation of 25pmol/µl. This was stored at -80^{o}C for storage or was used directly for microinjection.

4.4.9 Calcium precipitate method for transfection of motor neurons

Primary motor neurons were transfected with 5µg of pEGFP C-1 with full length mouse beta actin 3'UTR including the ORF or pEGFP C-1 vector as described (ref.) with minor modifications. 40,000 cells were plated on laminin coated special microdishes (ibidi) and allowed to grow for 3 DIV in NB+2%HS+2%B27 with BDNF and CNTF. Polyornithine was added to the cells at final concentration of 10µg/ml and incubated for 1 hr. The transfection mix was prepared as follows.

5µg expression plasmid
5µg carrier DNA
6.2µl 2M CaCl2
Xµl water to make up the volume to 50µl

Vortexed and added 50µl of 2XHBS pH7.0 and incubated at room temperature for 30 min. Added the mix to the cells and incubated them for 5 hrs. Removed the medium and shocked the cells by adding 30% DMSO in NB without serum and washed the cells thoroughly with HBSS. Added fresh NB+2%B27+2%HS with BDNF and CNTF. 100µg/ml cycloheximide was added to the cells and incubated for 1 hr prior to imaging in case of cyclohemaide control experiment. 20µM final concentration of Rapamycin and 40µM concentration of Anisomycin was added to the cells and incubated for 1 hr prior to imaging.

4.4.10 FRAP on NikonTE2000

The imaging was done in Leibovitz medium on the Nikon eclipse TE-2000E inverted microscope with BFI optilas camera. Bleaching was done using Melles Griot laser with 488 exitation with maximum intensity for 3 seconds. The growth cone and 2/3rd of the axon was bleached as shown in the fig. and recovery was observed by taking images at time interval of 5 min, 10min, 15min & 30 min and additionally for some experiments at 45min and 60min of bleaching.

4.4.11 Magnetofection of motor neurons

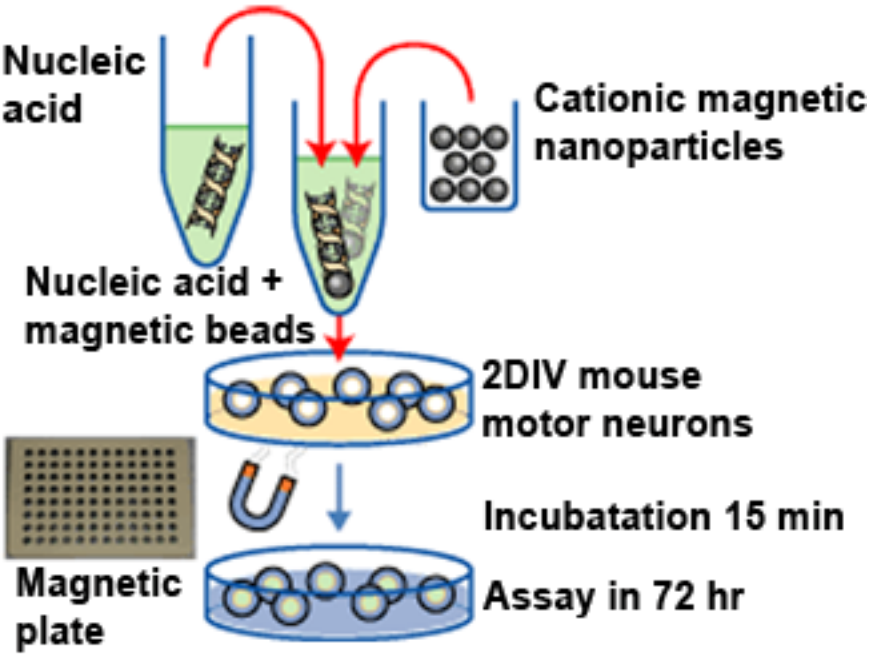

Fig3 Outline of the magnetofection. Adapted from origene website.

The magnetofection was done according to Fallini et al., 2010 (Fallini, Bassell et al. 2010). For magnetofection 2DIV motor neuron cells were cultured with NB full medium with BDNF and CNTF. On the day of magnetofection, the medium is changed to NB+2%B27with BDNF and CNTF for 1 hour to starve the cells for serum. 0.5µg DNA was mixed with 1.75µl of Neuromag in 100µl MEM medium were mixed and incubated for 15min. the mixture was added dropwise on the culture and incubated for 15min on the magnetic plate. The cells were removed from the magnetic plate and incubated for 1hr. The culture was washed with HBSS and NB full medium. The cells were examined after 72 hours.

4.4.12 Production of lentiviruses

A HIV based lentiviral expression system has been used in these study. The Δ8.9 and VSVG vectors were used as helper plasmid for packaging FuVal expression vector. HEK293T cells were used for packaging. The cells were propagated in DEMEM + 10%FCS in 3, 125 cm flasks up to 90% confluency. On the day of packaging, following reaction was set up in two 50ml reaction tubes:

Tube1		Tube2	
9ml	Opti-MEM	9ml	Opti-MEM
45µg	Δ8.9	216µl	Lipofectamin 2000
30µg	VSVG		
18µg	expression plasmid		

The above reaction was incubated for 5min and then the two reactions were mixed and incubated for 30min. The cells were washed with 10ml PBS and the cells were harvested by trypsination with 0.1% trypsine and incubated the cells for 10 min at 37^{o}C and then centrifuged at 14000 rpm for 5 min and the pelleted cells were resuspended in 30ml optimem with 10%FCS. This was then mixed with the transfection mix making the volume to 48ml. this was then distributed in 6, 10cm petri dishes with 8ml each and incubated in the cell culture incubator over night.

The cells were washed with PBS and the medium was changed to Neurobasal with 1% glutamax and 2%B27 packaging medium 12 ml in each petri dish and were incubated for 48hours. The transfection efficiency was estimated by microscopic examination.

For harvesting the virus, the supernatant medium was collected and centrifuged at 5000 rpm for 15min at room temperature and filtered the supernatant with 0.2µm filter. The supernatant was then layered on 10% sterile sucrose solution and centrifuged with ultra centrifuged at 250000rpm for 1.5 hr at 4^{o}C using a swing bucket rotor (SW28). The pellet was then slowly resuspended in 100µl of TBS buffer overnight on ice. The suspension was then gently mixed by pipetting up and down and 10µl aliquots were prepared and stored at -80^{o}C until further use.

4.4.13 Lentiviral infection of motor neurons

In order to see local protein synthesis in the mouse motor neurons, CD-1 E14 mouse embryos were dissected for spinal cord and motor neurons were cultured as published by Wiese et al. 2010 with BDNF and CNTF. 10000 cells were infected with either control MyreGFP or MyreGFP3'UTR reporter (mouse β actin) for 5min and then plated on Schwann cell specific laminin (laminin111) per live cell imaging dishes (ibidi).

4.4.14 FRAP on SP5

These infected cells were used for live cell imaging at 5&6DIV. On the day of imaging cells were washed with equilibrated ACSF and brought to the SP5 Leica inverted microscope in ACSF containing 10ng/µl of BDNF and CNTF. The stage of the microscope was adapted for maintaining the temperature and constant CO2 supply was given in order to maintain the pH of the ACSF. 63X oil objective (n.a. = 1.6) was used for the imaging and the FRAP wizard was used to program the bleaching of the defined area of the cell. The maximum laser power was kept at 80% and the used laser power was 100% for the bleaching and around 20% for imaging. The pinhole was kept completely opened and images were taken with camera settings at 700Hz and bidirectional scanning. Per bleach, 10 images were taken with a minimized time interval. 50 bleach images with a minimized time interval were taken. 10 immediate post bleach images with a minimized time interval were taken and 120 post bleach images were taken at interval of 30 sec to see the recovery to the total time of 3750 sec (1hr).

4.4.15 Data analysis

The measurement of the recovery after bleaching was done with the LAS lite software from Leica. The regions of interest were defined in a give series of images and the intensity as measured by the software was transferred to excel sheet. The 10 immediate post bleach image intensities were averaged and used for normalizing the change in the intensity over a period of 1hr. The normalized intensity was then plotted against time.

4.4.16 *In situ* hybridization

The cells were washed shortly with HBSS and fixed with 4%PFA for 15min at 37^0C. Washed the cells 2X5min with PBS and permiabilized with PBS+0.1%TritonX-100 at RT for 10min. This was followed by treatment with pepsin (0.1%in 10mM HCl) for 1 min at 37^0C.

The cells were washed 2x shortly with water and dehydrated through 70%, 90%, 100% ethanol and air dry.

Dilute the probe in *in situ* hybridization buffer to final concentration of 20-40nM and denature the diluted probe at 80^0C for 75sec and put on ice for 1min.

Hybridization was performed for 30min in an *in situ* adaptor at 62^0C (Tm-21). Stringincy washes were carried out by immersing the cover slip in 2xSCC,0.1%Tween-20 at room temperature and 3x5 min 0.1xSSC at 65^oC. Further dehydration was done through 70%,90%,and 100% ethanol. Air dry. The development of the signal was done by incubating with 1XTBST for 5 min and subsequent incubation with 1:100 dilution of Rhodamine avidin complex for 15 min. Then the cells were washed for 3X 5 min with TBST followed by washing with DEPC water briefly and mount with aquapolymount.

4.4.17 Immunocytochemistry

The cells were washed with HBSS and fixed with 4% PFA at 37^0C for 15 min. PFA was washed 2x with PBS. Permeabilization and blocking was performed with 1x blocking buffer (1xPBS, 10%BSA, 0.1% Triton X-100) at RT for 30min. Primary antibody was diluted and incubated for 2hr at RT. The cells were washed 4x with 1xPBS, 01%TritonX-100. Diluted secondary antibody in blocking buffer was incubated for 1hr at RT. Washed 4x with 1xPBS, 0.1% TritonX-100 followed by washing 2x with 1xPBS and 1x with water. The cells were subsequently treated with DAPI and washed with 1xPBS and then briefly with water. Dried and mounted with aquapolymount

4.4.18 Differentiation of ES cells into motor neurons

Gelatin coating of the dishes was done by covering the flask with a 0.2 % gelatin solution for at least 10 min in the incubator. Inactivated feeder cells were grown on these gelatin coated flasks. The feeder cells were thoroughly washed with medium and allowed to settle at least 1 hour in the incubator in R1 medium before adding ES cells. A vial of about 3 x 10^6 J1 ES cells quickly were resuspended in 10 ml R1 medium with 500 µl of LIF and centrifuge for 5 min at 180 g at room temperature. The cell pellet was resuspended in R1 medium and plated in the flask with feeders. The ES cells were propagated by splitting them 1:5 every two days by washing the cells with 5 ml PBS and trypsinizing with 1 ml trypsine for 7-10 min at room

temperature. The ES cells were cultured without feeders on gelatin coated plates for at least two passages by splitting the cells with a ratio of 1:3.
Trypsinized ES cells were then resuspended in ES cell culture differentiation medium (see materials for medium composition). 10^5 cells were plated onto bacteriological Greiner Petri dishes in 2.5ml ES cell culture differentiation medium without differentiation factors. Incubate at 37C for 2 days. Change ES cell culture differentiation medium after 2 days of aggregation by transferring the entire cell aggregate suspension into a 15 ml Falcon tube. The cell aggregates were washed twice with 5ml ES cell differentiation medium and resuspended in 2,5 ml of pre-warmed ES cell culture differentiation medium with Sonic Hedgehog (final concentration of 600 nM) and retinoic acid (final concentration of 2 µM). Change the ES cell culture differentiation medium after 4 and 6 days of aggregate formation (including retinoic acid and Sonic Hedgehog as above).

4.4.19 Enrichment of Differentiated Motor neurons by a p75 Antibody Panning Step

For enrichment and subsequent plating, the 2.5 ml differentiation medium including the cell aggregates were transferred into a 15 ml_Greiner reaction tube and allowed them to settle down. The cell aggregates were washed twice with 5 ml HBSS at RT to remove culture medium and serum and trypsinised in 200 µl HBSS containing 0.05% trypsin about 3 min at 37 °C. 20 µl trypsin inhibitor (1%) was added to stop trypsinization and triturated cell aggregates with a yellow tip until no cell aggregates are visible.

Enrichment was done by p75 antibody panning. For this the cells were transferred onto (prepared one day before) a panning plate covered with 10ml Neurobasal medium (including Glutamax) in a 9 cm culture plate and kept at room on a surface without vibration for 45 min. The panning plate was washed very gently three times with 5 ml pre-warmed neurobasal medium (containing glutamax) to remove cell fragments and p75 negative cells not expressing the p75 receptor. Add 1ml of depolarisation solution and after 30 seconds, add 5ml of pre-warmed neurobasal full medium (containing glutamax, horse serum and B27 supplement). The cells were collected in a reaction tube, counted using a hemocytometer and plated on laminin111 coated dishes or coverslips depending on the experiments.

5 Results

5.1 Microinjection of labeled β-actin mRNA to visualize transport of mRNPs.

In order to visualize the transport of the 3'UTR of β-actin mRNA in living cells, labeled mRNA was microinjected after in vitro transcription.

To standardize the microinjection procedure and to control that the cells were viable and healthy after the microinjection procedure, they were microinjected with 0.5µg/µl of pEGFP N1 plasmid. Motoneurons cultured for 3 days in vitro (3DIV) were microinjected using the microinjection settings described in the methods section. The microinjected cells were incubated for 72hr in the NB full medium with factors and examined for the expression of eGFP at 6DIV. As can be seen in the figure 5.3.1, the cells were alive and were able to express the eGFP protein, indicating that the methods for microinjection were suitable for keeping the cells alive and observe the subcellular distribution transport of mRNA.

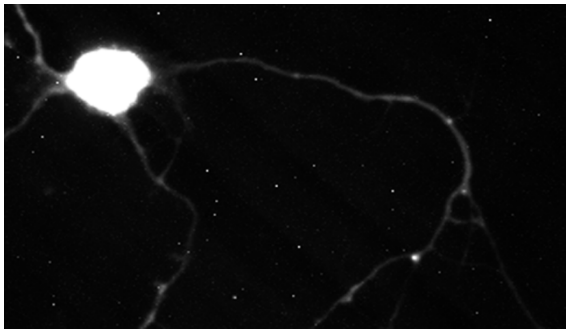

Figure 4peGFPC-1 plasmid microinjected motor neuron expressing eGFP

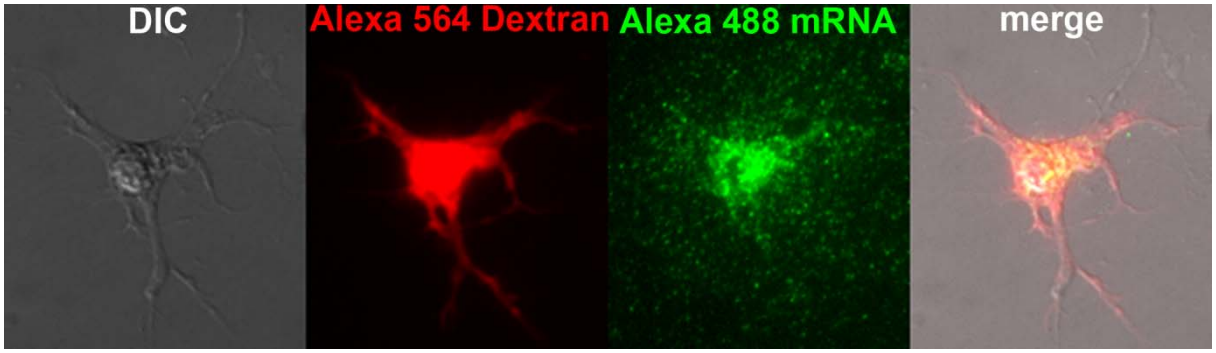

Figure5 Motor neuron co-microinjected with Alexa 488 labeled 3'UTR (β-actin)+polyA invitro transcribed mRNA and Alexa 564dextran.

These microinjection settings were subsequently used for the microinjection of 500 to 550ng/µl of mRNA of 3'UTR+polyA with incorporation of 25pmole/µl in 3DIV motoneurons.

As can be observed in the Figure 5.3.3, the cells which received the labeled mRNA showed a punctuate pattern of distribution soon after the microinjection. The cells were either imaged directly or incubated for 30min at 37^0C in a CO_2 incubator prior to imaging. Images were taken continuously over a period of 1hr to see the movement of the particles. We were not able to observe any movement of the particles over a period of 1.hour. Further optimization will be needed in imaging and/or labeling protocol in order to observe the movement.

5.2 Local translation of β-actin

The 3'UTR of β-actin has been shown to be responsible for the transport of the mRNA along the axon to the growth cone of DRG neurons (Bassell, Zhang et al. 1998). The subcellular accumulation of the β-actin mRNA in axons terminals of motoneurons has been shown by *in situ* hybridization by Jablonka et al. (2007). In order to investigate if the localized β-actin mRNA is translated, we established fluorescence recovery after photo bleaching (FRAP) to address this question.

For this purpose, primary wild type CD-1 mouse motoneurons were plated at high density on poly ornithine and Schwann cell specific laminin (laminin111) coated live cell imaging micro dishes (ibidi). The cells were transfected with mammalian expression plasmid expressing full length β-actin open reading frame along with the 3'UTR as an N-terminal eGFP fusion protein or eGFP alone (as a control) under the CMV promoter. For transfection, a calcium precipitate method was used on 3DIV mouse motoneurons. The plasmid was cloned in the lab by Thomas Premsler. Figure 6 shows the schematic map and the confocal image of the HELA cells transfected with the construct.

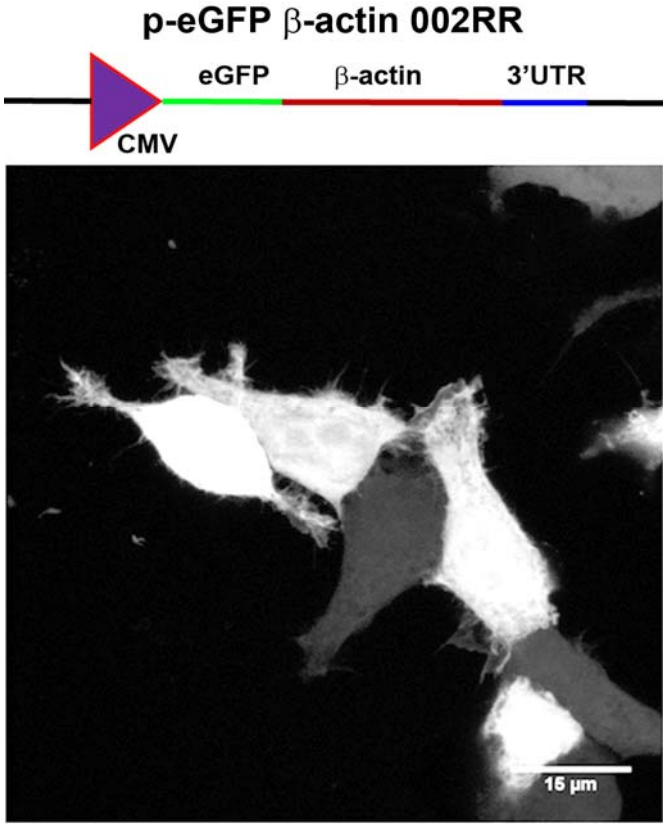

Figure 6 Schematic representation of p-eGFP β-actin fusion construct and confocal image of the HELA cells transfected with p-eGFP β-actin 002RR fusion construct.

The growth cones of the transfected motoneurons were examined for eGFP signal 24hr after transfection. The FRAP experiment was done within 2 days after transfection. For imaging, the cells were washed and imaged in Leibovitz medium containing BDNF (10ng/µl) and CNTF(10ng/µl). The FRAP and imaging was done as described in detail in the methods section. After bleaching, recovery of the eGFP signal was observed in the growth cones of motoneurons transfected with eGFP β-actin 3'UTR expressing motoneurons. The intermediate axonal section of the motoneurons did not show any significant recovery. The marginal increase in the fluorescence in the medial portion of the axon is possibly due to lateral diffusion of eGFP. Figure 7a shows a representative cell after FRAP and recovery monitored for 30 min with 5 min., 10 min., 15 min., and 30 min time intervals. The fluorescence intensity was measured using the NIS software of the Nikon TE2000 microscope. The fluorescence intensity was normalized using the formula F/F_0*100. F is the fluorescence intensity measured at a given time point and F_0 the initial fluorescence intensity before bleaching. Fig 7b shows the quantification of the recovery of fluorescence in the

growth cone for 30min. As shown in Fig7A, there is significant recovery in the cells with β-actin 3'UTR.

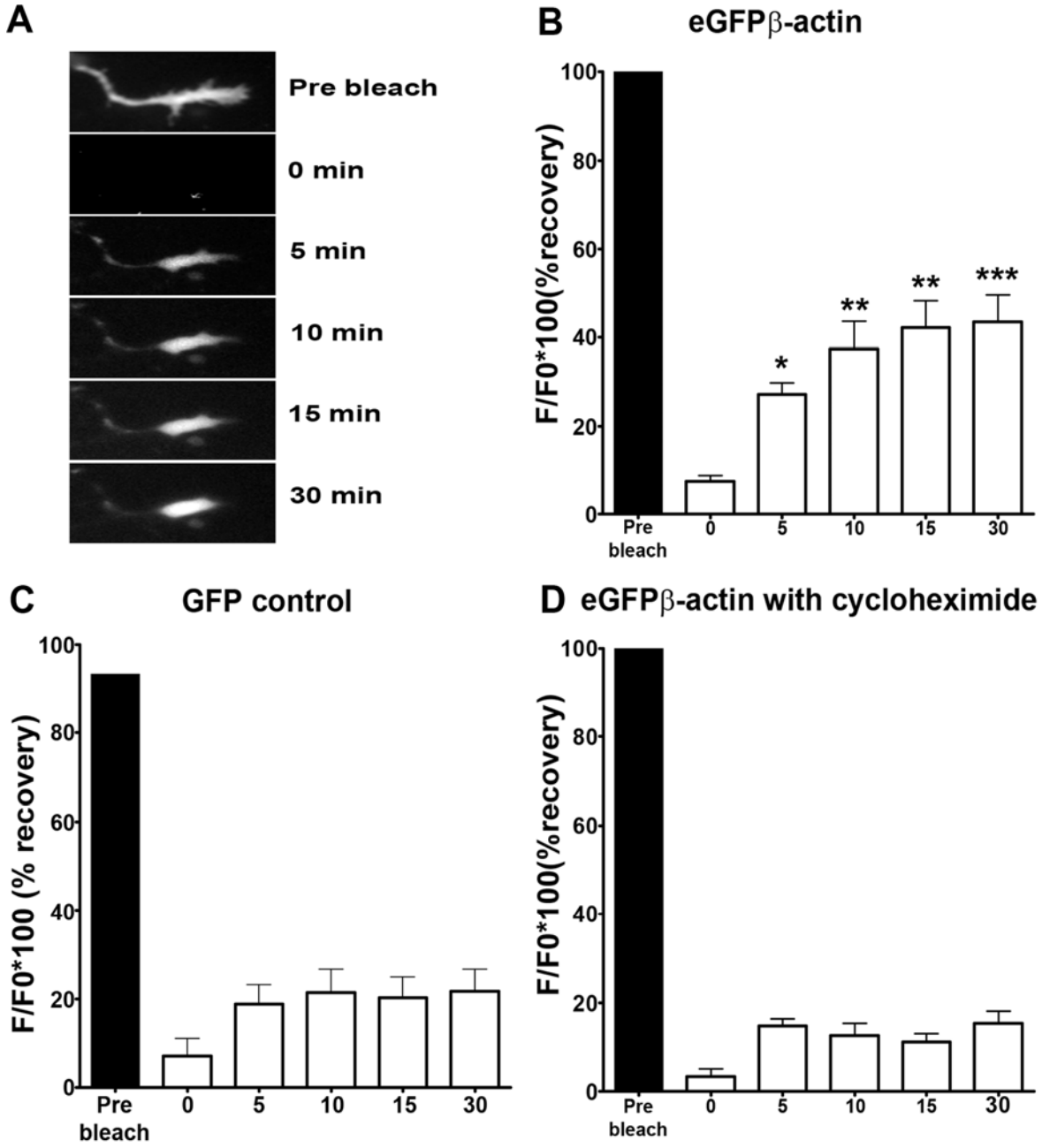

Figure7A Fluorescence recovery of bleached eGFP-β-actin transfected motoneurons. B) The graph shows the quantification of the fluorescence intensity after bleaching over 30 min in growth cones of p-eGFPβ-actin transfected motoneurons. C) Quantification of fluorescence recovery in growth cones of eGFP-β-actin transfected motoneurons. D) Quantification of the fluorescence intensity after bleaching over 30 mins in growth cones of p-eGFPβ-actin transfected motoneurons pretreated with cycloheximide.

To see if the recovery was specifically attributed to the translation of the localized mRNA of β-actin, we transfected the primary motoneurons with eGFP C-1 vector for control, the mRNA of which, is not expected to be translocalized to axon terminals. As shown in figure 7C, there is no significant recovery of the fluorescence in the growth cones of the cells transfected with eGFP alone.

Cycloheximide was added to the cells transfected with eGFP β-actin 3'UTR construct to block the translation and see if this affected the recovery after photobleaching. Pre incubation of cells with cycloheximide showed a significantly lower (***=P < 0.001) recovery compared to untreated cells within the same time interval as can be seen in the figure 7D.

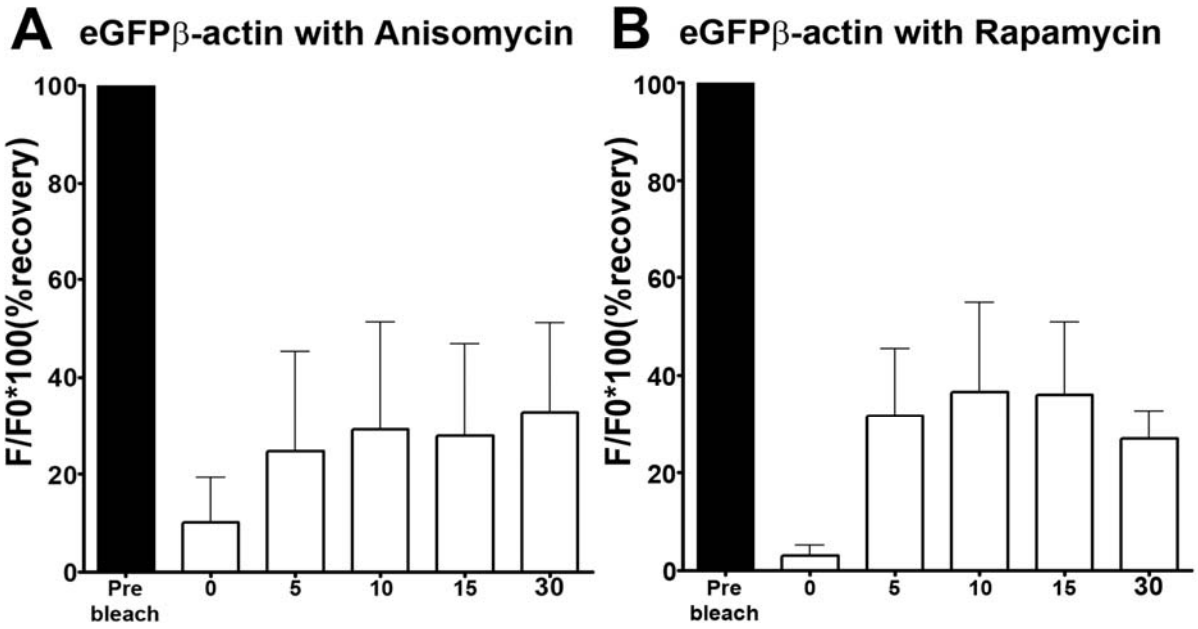

Figure 8A quantification of the fluorescence intensity after bleaching over 30 mins in the growth cone of p-eGFPβ-actin transfected motoneurons pre treated with anisomycin and B) Rapamycin

Anisomycin was also used as a translational blocker to confirm that the recovery of fluorescence is due to local translation of the transported eGFP β-actin mRNA (Figure 8A). To investigate if the mTOR pathway in involved in the regulation of the local translation, Rapamycin was used (Figure 8B). There was reduction of recovery showing the involvement of the mTOR pathway but the number of cells was too low to investigate the significance of the effect. Fig 8A shows the quantification of the recovery effects in Anisomycin and Rapamycin treated cells.

5.3 Optimization of constructs for expression of the 3'UTR of the β-actin to examine local translation in axonal growth cones of motoneurons

In order to examine the influence of various mRNP associated proteins such as hnRNP-R, IMP or SMN, it would be important to study the influence of either overexpression or knock down of these proteins. The vectors commonly used for overexpression or knock down of these proteins were available to generate eGFP fusion or reporter constructs. For this reason it was not suitable for us to use eGFP as a fluorescent protein for investigating local translation. Therefore we cloned the full length β-actin open reading frame (ORF) along with its 3'UTR as a fusion construct with mCherry.

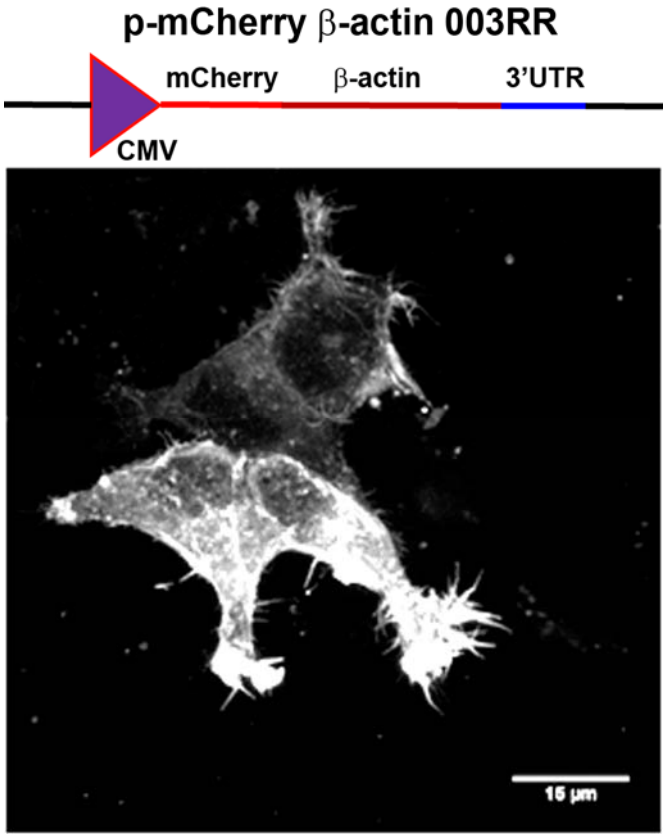

Figure 9Schematic representation of pmCherry β-actin fusion construct and confocal image of the HELA cells transfected with pmCherry β-actin 003RR fusion construct

This should enable us to examine local translation in combination with the overexpression or knock down of various mRNP proteins. The ORF of mouse β-actin along with 3'UTR was excised from a pEGFP C-1 β-actin clone, using BamHI and XhoI and inserted between BamHI and XhoI restriction sites of pmCherry C-1 vector. The positive clones were screened with the help of NheI and KpnI restriction sites. Overexpression was confirmed by western blot analysis of extracts from HEK293 cells expressing the construct. The expression pattern of the construct was examined under confocal microscope by transfecting HELA cells. Figure 9 shows the schematic map of the pmCherry β-actin and the confocal image of the

transfected HELA cells.Since the transfection efficiency of the calcium precipitate method was too low and there was high cell death of transfected cells, there was a concern if the cells we were examining were a selected subpopulation of motoneurons. Therefore it was important to device an alternative method for expressing proteins in motoneurons. For this reason, we decided to express β-actin in a lentiviral vector system. The ORF of β-actin with the 3'UTR was cloned in pLVX DsRed vector as a fusion construct which can be packaged as lentiviruses. XhoI and BamHI were used for opening the vector and the XhoI, BamHI double digested fragment from pEGFP-β-actin clone was inserted. Figure 10 shows the schematic map and the expression pattern of HELA cells transfected with the pLVXDsRed β-actin fusion construct.

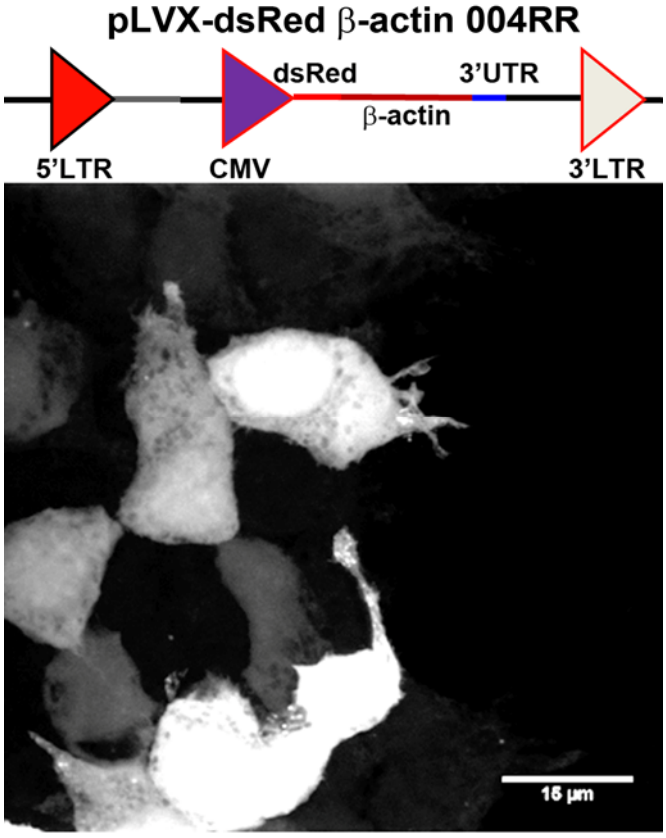

Figure 10 Schematic map of pLVXDsRed β-actin and confocal image of HELA cells transfected with pLVXDsRed β-actin 004RR construct

EOS is a photoconvertable protein which when exited with UV light is converted from a green fluorescent to a red fluorescent protein state. In order to avoid the recovery due to diffusion of free cytoplasmic protein, it was important to immobilize the newly synthesized protein. For this a myristoylation signal sequence was added to the construct in order to membrane anchor the EOS protein avoiding the recovery due to diffusion. For this purpose, the following strategy was implemented. The EOS tandem (td) repeat was purchased from Mobitech. The EOS td has a mutation which makes it possible to express it as a fusion protein.

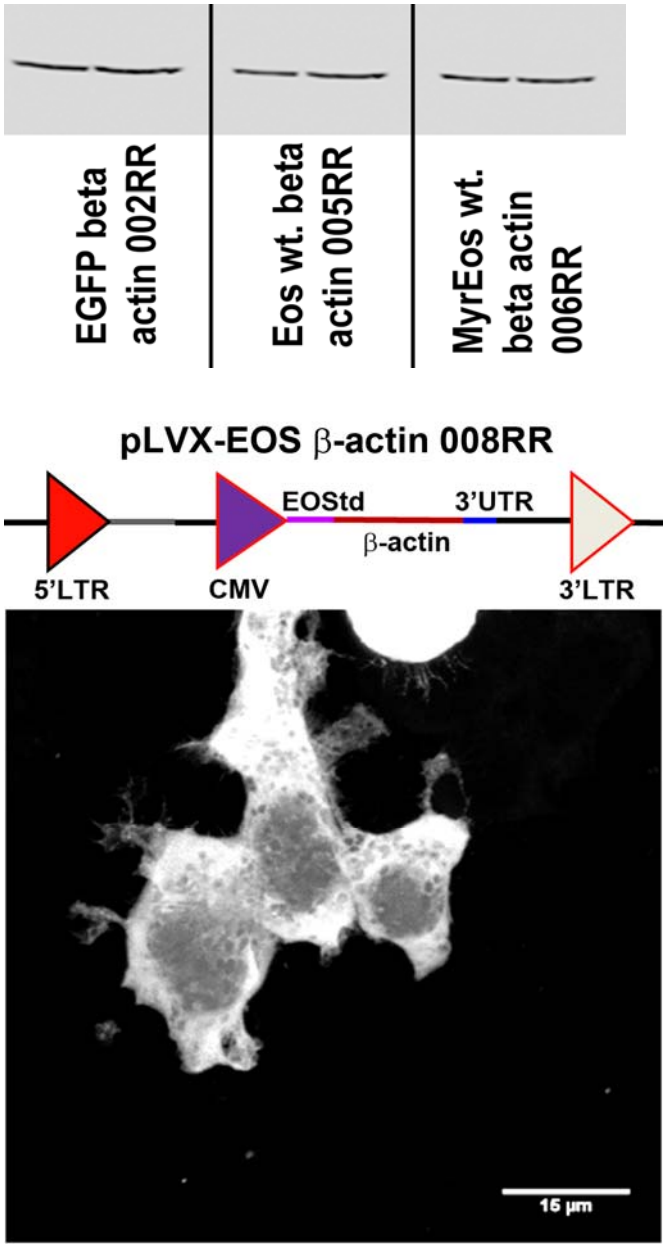

Figure 11a) anti β-actin western blot from transfected 293HEK cells expressing eGFPβ-actin (002RR) (lane1&2), EOSWTβ-actin (005RR) (lane 3&4) and Myr EOSWT β-actin (006RR) (lane 5&6). 11b) Schematic map of pLVXEOStd β-actin construct and confocal image of HELA cells expressing pLVX EOStd β-actin 008RR.

The wild type EOS used for the cloning produced the appropriate fusion protein as shown by western blot (Figure 11a)but was not fluorescent. This was possibly due to steric hindrance of correct folding of the protein. The ORF of the EOS td was then PCR amplified, introducing the restriction sites XhoI and ApaI. This fragment was brought into the Topo PCR2.1 vector. The ORF of β-actin was cloned in the Topo PCR2.1 vector containing the EOS td ORF by BamHI and XhoI. The resultant vector was a EOS/MyrEOStd β actin 3'UTR fusion construct. This was then excised with a ApaI-BamHI double digestion and was then inserted in the pLVX puro vector which is a lentiviral vector. Figure 11b shows the schematic map of the vector and the confocal image of HELA cells transfected with the pLVX PuroEOStd β-actin construct.

Destabilized GFP has a halflife of 1hr as compared to 8hr of the stable eGFP. This would allow us to monitor only the newly synthesized protein as compared to the interference of the previously existing and diffusing eGFP. But since all the knock down viruses we wished to investigate had an eGFP tag, we decided to either use the destabilized DsRed construct with 3'UTR of β-actin without the open reading frame as a reporter construct. The alternative would have been to clone all the viruses with tomato/cherry as a reporter and have 3'UTR of β-actin tagged with DsGFP. Therefore the destabilized dsRed was cloned into the FuVal vector for a lentiviral expression, as a reporter construct with 3'UTR(β-actin) (Fig.12).

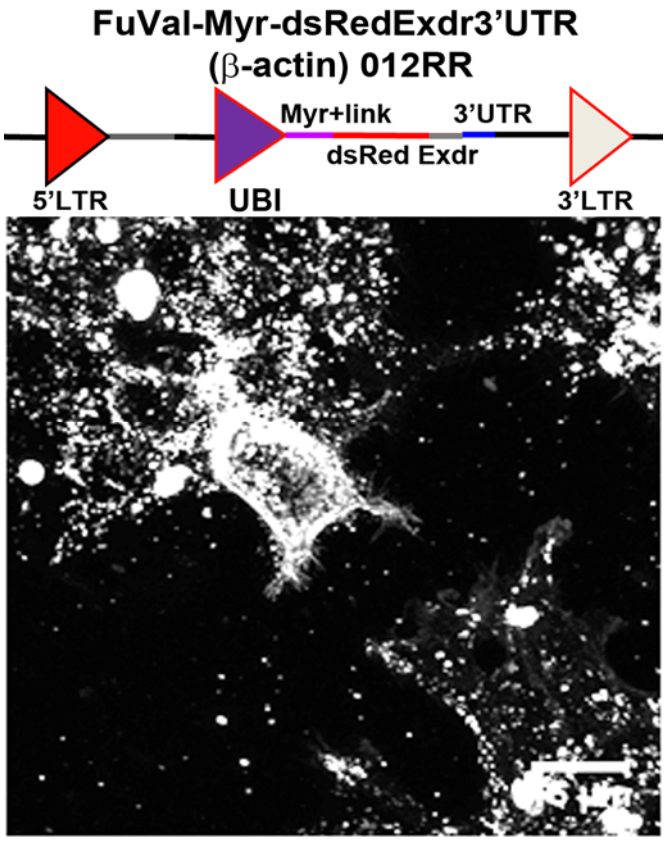

Figure12 Schematic map of Myr dsRedExdr3'UTR (β- actin). The lower pannel shows a confocal image of the expression pattern of Myr dsRedExdr3'UTR (β- actin) 012RR in transfected HEK293 cells.

The destabilized DsRed sequence was PCR amplified from the commercial destabilized DsRed obtained from Clonetech. The clone was constructed by inserting a BamHI restriction site into the forward primer and the myristylation signal sequence with a linker. NotI restriction enzyme was used in the reverse primer, and the PCR amplicon was inserted in the FuVal lentiviral vector backbone. The 3'UTR of the β-actin was PCR amplified with the NotI in the forward primer and EcoRI in the reverse primer, and inserted in the MyrDsRedExdr containing FuVal vector to obtain the MyrDsRedExDr3'UTR construct. Figure 12 shows the schematic representation of the vector and the expression pattern of the construct in transfected Hek293 cells.

As can be seen here, the protein formed aggregates and appeared not suitable for the expression in motoneurons and for the FRAP experiments.

Therefore, Dr. Rudolf Goetz cloned the myristoylated destabilized GFP 3'UTR in the FuVal.The schematic map of the MyrDsGFP 3'UTR of β-actin and the expression pattern of the transfected HEK293 cells is shown in figure 13.

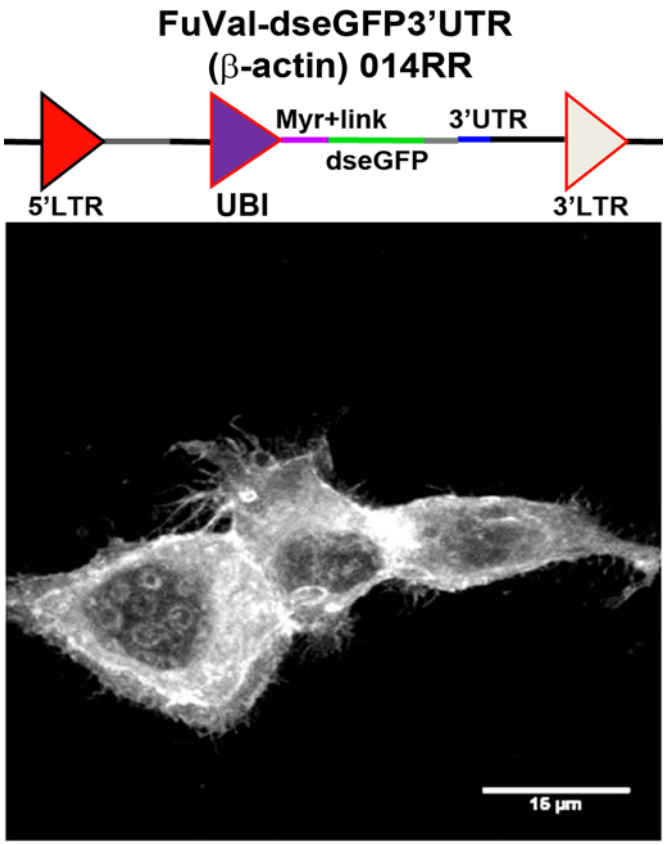

Figure 13 Schematic map of Myr dseGFP3'UTR (β-actin) and expression pattern of Myr dseGFP3'UTR (β-actin) in transfected HEK293 cells

The eGFP from this construct was localized in the membrane, and therefore the construct was used for generating lentiviruses. HEK293 T cells were transfected with VSVG and Δ8.9 helper plasmids, but no infectable viruses were obtained. To analyze the problem encountered, small scale viral packaging was performed. We found that the initial transfection efficiency was very good and nearly 95% of the HEK293 cells were transfected and showed fluorescence after 24 hours of transfection. However,as shown in figure 14, there was a rapid downregulation of the expression by 48 hours of transfection of the Myr DsGFP3'utr FuVal construct whereas the control vector continued to fluorescence and good infectious viruses were obtained.

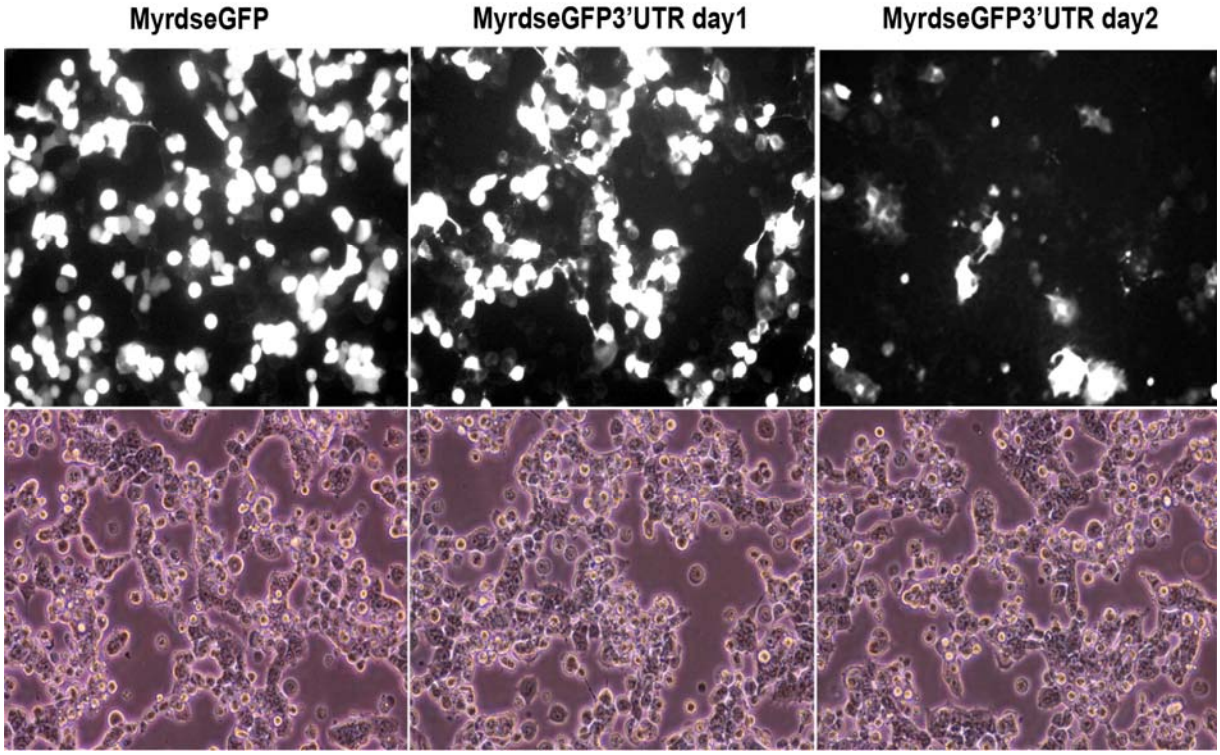

Figure 14 Fluorescent (upper panel) and DIC images (lower panel) of transfected HEK293T cells for viral packaging. MyrdseGFP and MyrdseGFP3'UTR constructs show efficient initial transfection but MyrdseGFP3'UTR fluorescence was rapidly downregulatedwithin48 hours after transfection.

Figure 15 shows confocal images of HEK293 cells after exposure to supernatants of harvested viruses after packaging after postlabelling with anti-eGFP antibody. As can be observed, there no signal could be detected in cells infected with MyrDsGFP3'UTR construct whereas MyrDsGFP and control FuGW viral supernatant showed excellent virus mediated GFP expression in HEK 293 cells.

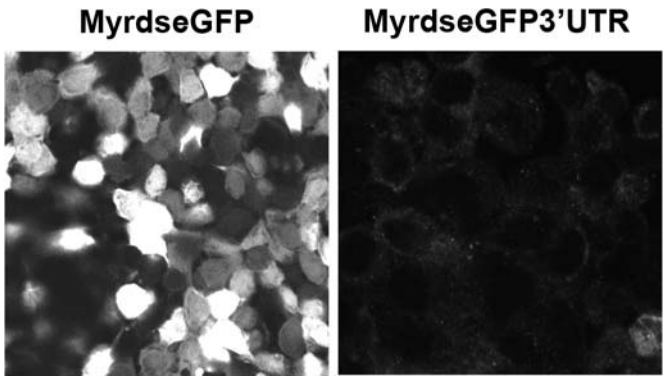

Figure 15 Confocal image of 293 cells infected with either MyrdseGFP or MyrdseGFP3'UTR virus after anti eGFP staining shows no eGFP expression in the cells.

To further investigate whether the downregulation of the expression of MyrDsGFP3'UTR construct in the HEK293 cells was at the transcriptional or at the translational level, we performed quantitative RT PCR. For this, HELA cells were infected with the corresponding virus and the mRNA was extracted using RNAeasy RNA isolation kit. mRNA was also extracted from the viral supernatant in order to compare if the viral packaging took place or not. Real time RT PCR was done against the viral WPRE sequence inorder to detect the viral mRNA. The real time RT PCR results showed that there was no difference in the packing of the 3'UTR containing virus as compared to the FuGW virus in the viral supernatant as well as in the transduced HELA cells extracted mRNA. This indicates that the viral particles were present but the expression was inhibited. Figure 16 shows the crossing point of the WPRE containing transcripts. cDNA was reverse transcribed from the HELA cells mRNA either infected with FuGW virus or MyredsGFP virus or MyrdseGFP3'UTR.

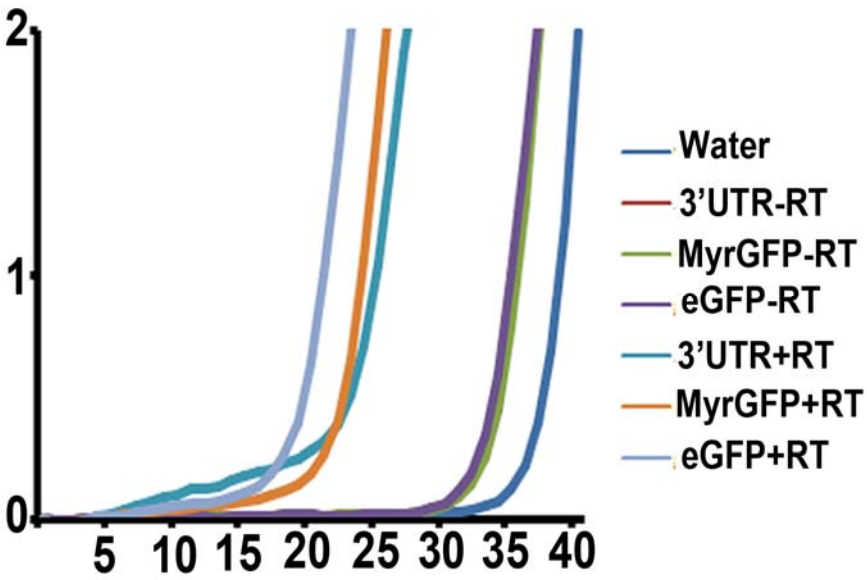

Figure 16 Crossing point for WPRE containing transcript with and without reverse transcription.

The samples without RT show the specificity of the primers, but that also some contamination with DNA might have occurred. However, there is no difference in the cycle numbers for the crossing points between the FuGW or MyrdseGFP or MyrdseGFP3'UTR. This indicates that there was no difference in the transcription of these constructs but thatdownregulation of expression probably occurred at the translational level.

Because the transient transfection of the vector gave proper localization and expression pattern, we decided to express it with the help of magnetofection.

Magnetofection is a method of transfecting the MN using magnetic nanoparticles coated with DNA. We optimized magnetofection in order to express the MyreGFP 3'UTR construct from Dr. Goetz since it was not possible to express it as a recombinant virus. Therefore, 3DIV MN were transfected transiently with either eGFP or MyreGFP3'UTR constructs. Figure 17 shows the magnetofection transfected motoneurons with the MyreGFP3'UTR construct at low magnification, and phaseconstrast as well as confocal image.

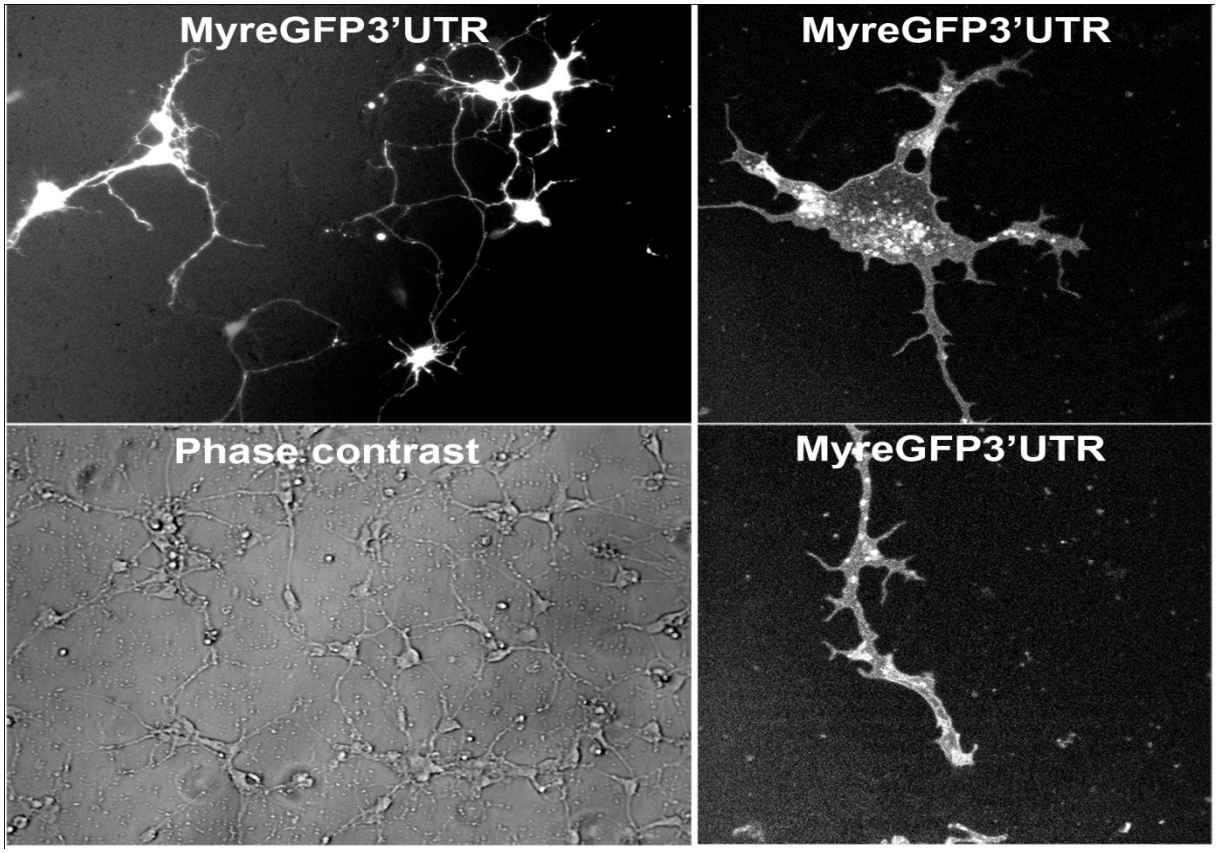

Figure17 Low magnification fluorescence (upper left) and phase constrast (lower left panel) image of the motoneurons expressing MyrdseGFP3'UTR transfected with magnetofection. Confocal image of the cell body (upper right) and growth cone (lower right)of motoneurons expressing MyrdseGFP3'UTR by magnetofection.

In parallel, we tried to clone again the 3'UTR of β-actin in the lentiviral vector with a different strategy. Inorder to investigate the problem of inhibited translation or transcription of the 3'UTR containing lentiviral vector, we investigated alternatives for the pest sequence of the destabilized GFP. For this purpose, we cloned MyreGFP with or without a linker between the Myr signal sequence and the stable eGFP ORF. The fragment was first cloned in a pcDNA3 vector with BamHI in the forward primer which contained a Kozack sequence, the Myr signal sequence and the linker sequence besides the amplification sequence for the eGFP. The reverse primer contained the stopcodon „TAA" flanked by NheI and EcoRI restriction sites. The 3'UTR was cloned in the shuttle vector between NheI and EcoRI and then the enitre sequence was cloned in FuVal between BamHI and EcoRI. Figure 18shows the schematic map and the expression pattern of the Myr eGFP3'UTR (β-actin) 018RR construct. Inorder to analyze the role of ZIP code of the 3'UTR of β-actin we also cloned the ZIP code alonewhich consisted of the first 54nt of the 3'UTR of β-actin. This sequence is known to be necessary and sufficient for the localization of the β-actin mRNA. The cloning strategy and expression pattern of MyreGFPZIP was the same as that for the MyreGFP3'UTR FuVal construct.

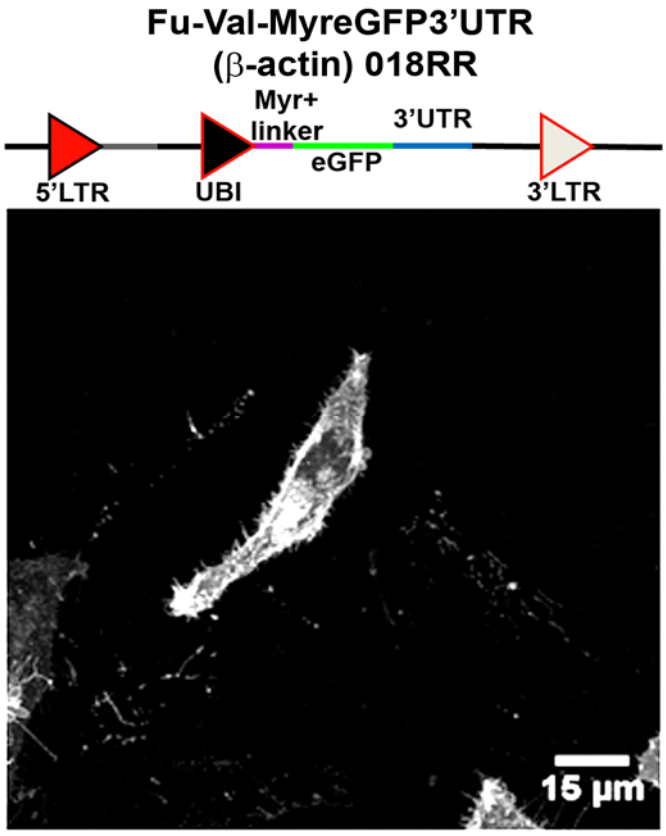

Figure 18 Schematic map of FuVal-MyreGFP3'UTR (β-actin) construct and confocal image of HELA cells expressing FuVal-MyreGFP3'UTR (β-actin) 018RR.

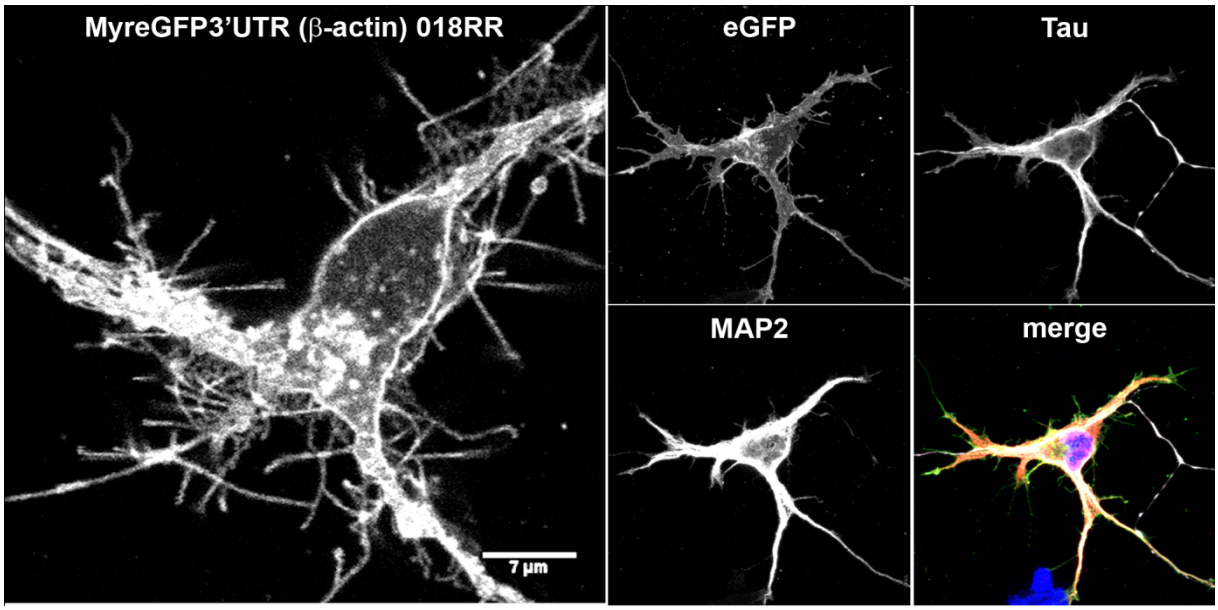

Figure 19 Membrane localization of eGFP expressing construct MyreGFP3'UTR (β-actin) in a living motor neuron and a fixed motor neuron stained for GFP, Tau and MAP2.

As can be observed from the confocal images, this construct was nicely expressed in the membrane of HELA cells. Therefore this construct was then used for the viral packaging. The viral supernatant was harvested and used for transducing motoneurons. The construct efficiently transduced motoneurons and it was possible to observe expression at DIV3. Figure 19 shows the expression pattern of the MyreGFP3'UTR construct in a living motor neuron and the expression in a motor neuronthat had been fixed and stained with antibodies against GFP, Map2 and Tau

5.4 Local protein synthesis in axons of mouse motoneurons

The 3'UTR of β-actin is known to be responsible for the localization of its mRNA to the leading edge of migrating chicken fibroblasts (Kislauskis, Zhu et al. 1994) and in the growth cones of cortical cells (Bassell, Zhang et al. 1998), where local protein synthesis has been observed. In mouse motoneurons, Jablonka et al. 2007 have observed that there is less β-actin mRNA and protein in the growth cones of *Smn*$^{-/-}$*;SMN2* motoneurons as compared to motoneurons from control litter mates. Therefore, inorder to investigate the translation of the localized β-actin mRNA, we performed FRAP experiments in living cells. Figure 20A shows the bleached area and the regions of interests that were analysed. As shown in figure 20B, significant recovery in growthcones of CD-1WT motoneurons was observed after infection with MyreGFP3'UTR (β-actin) as compared to the MyreGFP construct lacking the 3'UTR. Figure 20C shows the quantification of fluorescence recovery for 1hr. The MyreGFP mRNA is not expected to translocalize to axons and therefore cannot show local translation in this subcellular compartment. As expected,MyreGFP expressing motor neuron growth cones did not show any significant recovery within 1hr after fluorescence bleaching.

Inorder to confirm that the recovery observed in the growth cones with MyreGFP3'UTR construct is due to local translation, we used translational inhibitors. The inhibitors were added under standardized conditions at various concentrations and incubation time for each of the translational inhibitors. 4DIV cultured MN were incubated with 10µg/ml cycloheximide and incubated overnight. Cycloheximide was also present in the ACSF during imaging. In case of Anisomycin and Rapamycin, cells were incubated with 10ng and 30ng, respectively for 2hr prior to imaging. These inhibitors were also present in the ACSF during imaging the FRAP experiment. As shown in figure 20D, the recovery after photobleaching was inhibited when translational blocker Anisomycin was used. This confirms that the recovery in the growth cones is due to local protein synthesis.

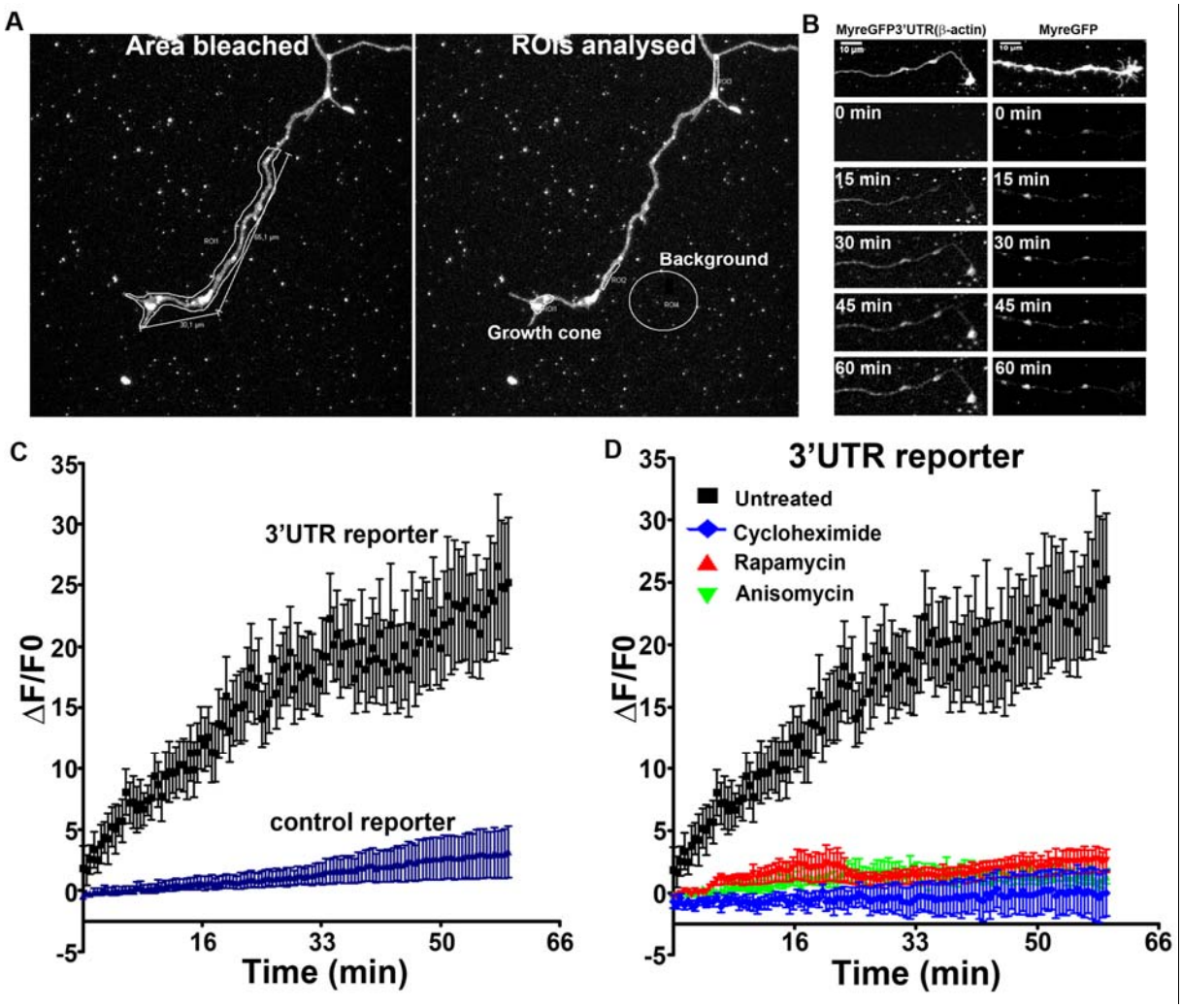

Figure 20 (A) axon of a motor neuron marked for the region that received bleaching (right) and the regionof interest analysed for recovery of florescence after bleaching (left). (B) Fluorescence recovery after photobleaching (FRAP) of the β-actin reporter construct in axons of isolated motoneurons. Distal axon including the axonal growth cone of a motoneuron that was bleached (upper panel). Fluorescence recovery of the MyreGFP signal is shown over a period of 60 min in motoneurons transduced with control MyreGFP virus (Control), (C) Quantification of the change in fluorescence normalized for the post bleach images in axonal growth cones of motoneurons treated with Myr-eGFP 3'UTR (β-actin) reporter(black n=15), Myr-eGFP control virus (blue n=8). (D) Recovery is abolished by inhibitors of protein translation, 10 µg/µl cycloheximide (blue n=8) added overnight, 30 ng/µl rapamycin (red n=6) added for 2 hours, or 10 ng/µl anisomycin (green n=8) added for 2 hours

5.5 Local protein synthesis in growth cones of embryonic mouse motoneurons is regulated by Laminin signalling

External signalling regulates the local transaltion for eg. Nitrin-1 induces β-actin local translation in retinal ganglion cells (Leung, van Horck et al. 2006) and local translation of RhoA is regulated by Semaphorin3a (Wu, Hengst et al. 2005). In order to understand the effects of different Laminin isoforms on local axonal translation of β-actin in mouse motoneurons, we cultured motoneurons on Schwann cell specific Laminin111 which is known to strongly promote axon extension (Porter and Sanes 1995; Jablonka, Beck et al. 2007) When motoneurons transduced with Myr-eGFP 3'UTR (β-actin) reporter were cultured on Laminin211/221, a neuromuscular endplate specific Laminin isoform, we observed significantly lower recovery in axonal growth cones (Fig. 21A). The quantification of FRAP of 3'UTR reporter-derived fluorescence of growth cones on Laminin111 vs. Laminin211/221 is shown in figure 21B

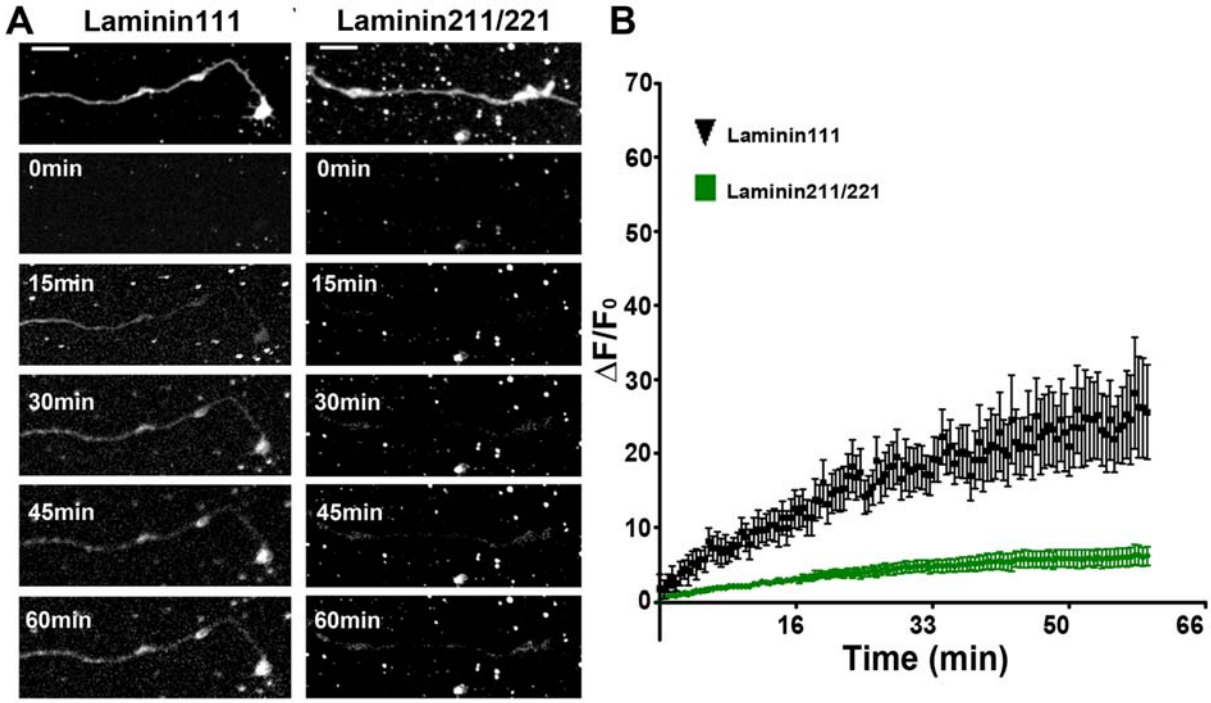

Figure 21 A Fluorescence recovery after photobleaching (FRAP) of the β-actin reporter construct in axons of isolated motoneurons cultured on Laminin111 (left) MyreGFP3'UTR (β-actin) cultured on Laminin211/221 (right) (B) There is reduced recovery when motoneurons infected with β-actin reporter are cultured on Laminin211/221 (G, green n=6). Shown are mean intensity values ± SEM.

5.6 Deregulation of local protein synthesis in *Smn$^{-/-}$;SMN2* cells

Previous studies have shown that β-actin mRNA levels are reduced in growth cones of mouse motoneurons isolated from *Smn$^{-/-}$;SMN2* E14 mouse embryo (Rossoll, Jablonka et al. 2003). Furthermore the *Smn$^{-/-}$;SMN2* motoneurons are known to have shorter axonon Laminin111 (Schwann cell specific) as compared to *Smn$^{+/+}$;SMN2* motoneurons. When plated on Laminin211/221, the predominant form of laminin at motor endplates, the *Smn$^{-/-}$;SMN2* cells show longer axon length as compared to *Smn$^{+/+}$;SMN2* motoneurons. The levels of β-actin mRNA and protein in the growth cones but not the cell bodies are lower in *Smn$^{-/-}$;SMN2* motoneurons, indicating that there is a defect in the transport of the β-actin mRNA containing transport mRNP. We investigated whether the reduction of SMN in the motoneurons isolated from *Smn$^{-/-}$;SMN2* E14 embryos leads to defects only in the transport or in local protein synthesis as well. For this, motoneurons from *Smn$^{+/+}$;SMN2, Smn$^{+/-}$;SMN2* and *Smn$^{-/-}$;SMN2*mouse embryos were prepared from E14 littermates, infected with MyreGFP3'UTR (β-actin) virus and cultured on Laminin 111 or Laminin211/221 coated micro-dishes. The FRAP experiment for analysis of local translation was done as described in the methods section. As shown in figure 22 b, there is markedly reduced local translation in the growth cones of *Smn$^{+/+}$;SMN2* mouse motoneurons when plated of Laminin211/221 as compared to Laminin111. This indicates that local protein translation of β-actin is influenced by an external signal from Laminin.

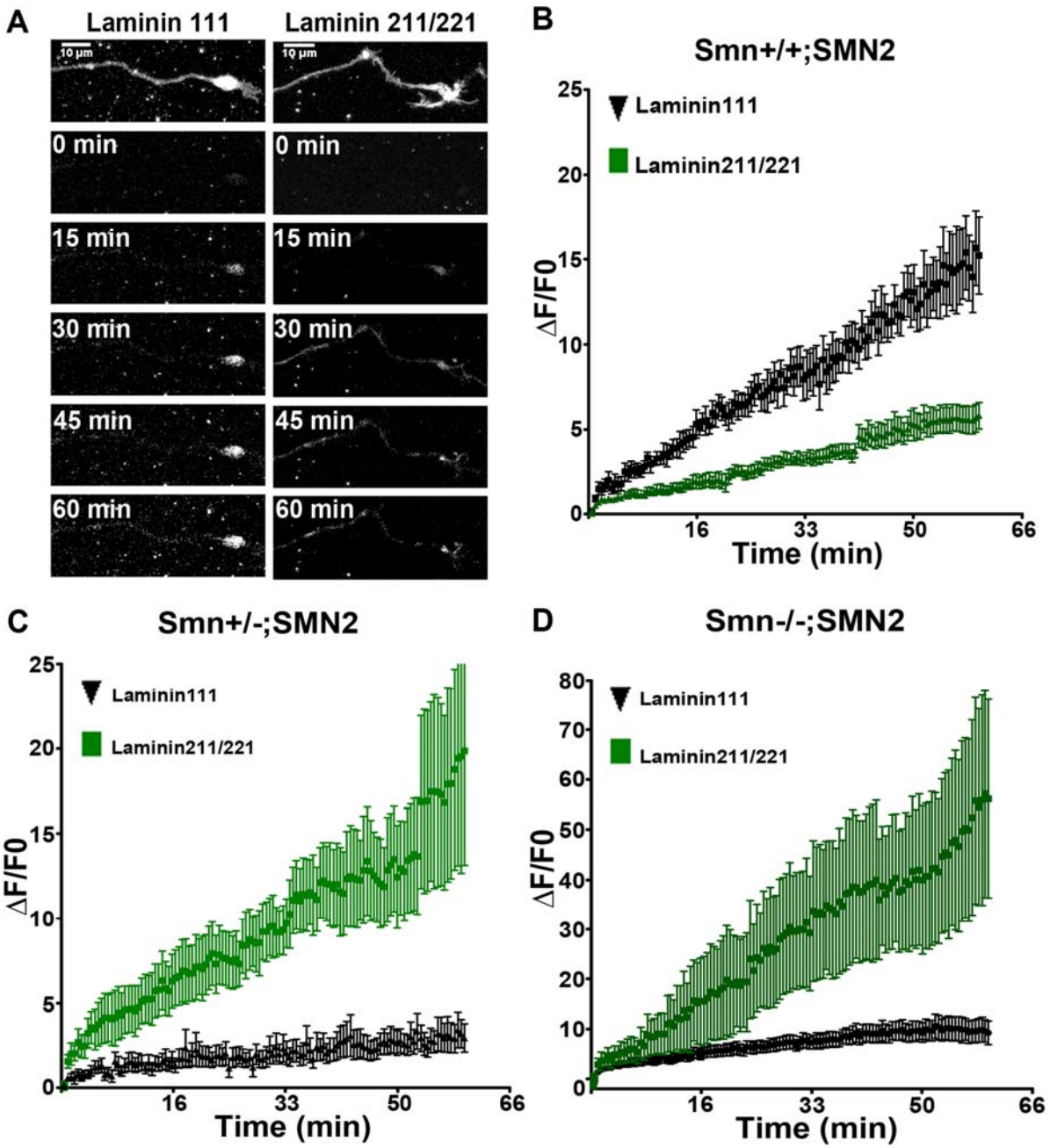

Figure 22 A) Fluorescence recovery of bleached MyreGFP3'UTR (β-actin) transfected *Smn$^{+/+}$;SMN2* motoneuron growth cone cultured on Laminin111. B) The quantification of the fluorescence intensity after bleaching and analysis of recovery over 60 min in growth cones of *Smn$^{+/+}$;SMN2* motoneurons cultured on Laminin111 (black, n=8) and Laminin211/221(green=8). C) The quantification of the fluorescence recovery after bleaching over 60 min in growth cones of *Smn$^{+/-}$;SMN2* motoneurons cultured on Laminin111 (black, n=8) and Laminin211/221(green , n=6). D) The quantification of the fluorescence recovery after bleaching over 60 min in growth cones of *Smn$^{-/-}$;SMN2* motoneurons cultured on Laminin111 (black, n=12) and Laminin211/221(green, n=4).

The question of how reduced SMN levels regulate the local translation of β-actin mRNA was addressed by studying local translation in *Smn*$^{+/-}$*;SMN2* cells. As can be seen from figure 22c, there is very minimal local protein synthesis taking place in the *Smn*$^{+/-}$*;SMN2* motor neuron growth cones plated on Laminin111 where as the *Smn*$^{+/-}$*;SMN2* motoneurons plated on Laminin211/221 show much higher recovery in the growth cone.

The recovery in the growth cones of *Smn*$^{-/-}$*;SMN2* motoneurons plated on LLaminin111 was much less as compared to the ones plated on Laminin211/221 as shown in figure 22d. Therefore, in case of the *Smn*$^{-/-}$*;SMN2* motoneurons plated on Laminin211/221, there was much higher recovery and the deregulation was much more severe as compared to *Smn*$^{+/-}$*;SMN2* motoneurons. It was difficult to estimate the recovery in the growth cones of *Smn*$^{-/-}$*;SMN2* motoneurons owing to the fact that they had very small growth cones and in several cases there was a rapid extension of the axon length within the time course of the measurement after photo bleaching.

This shows that the local protein synthesis in the growth cones of mouse motoneurons is a tightly regulated phenomenon and is sensitive to external signaling as well as to levels of SMN in the motoneurons. Inorder to understand the surprising results obtained for local protein synthesis and to gain better insight into the role of SMN in the local protein synthesis, we transduced the motoneurons with a lentiviral construct containing only the ZIP code (54 nt directly following the stop codon in the 3'UTR of β-actin mRNA). The ZIP code is known to be necessary and sufficient for the translocation of the mRNA to the periphery (Kislauskis, Zhu et al. 1994). Besides, the SMN-hnRNP-R protein complex is known to bind the 3'UTR of β-actin at a region other than the ZIP code. Thus the local protein synthesis of this construct would not be sensitive or less sensitive to alterations of SMN levels.

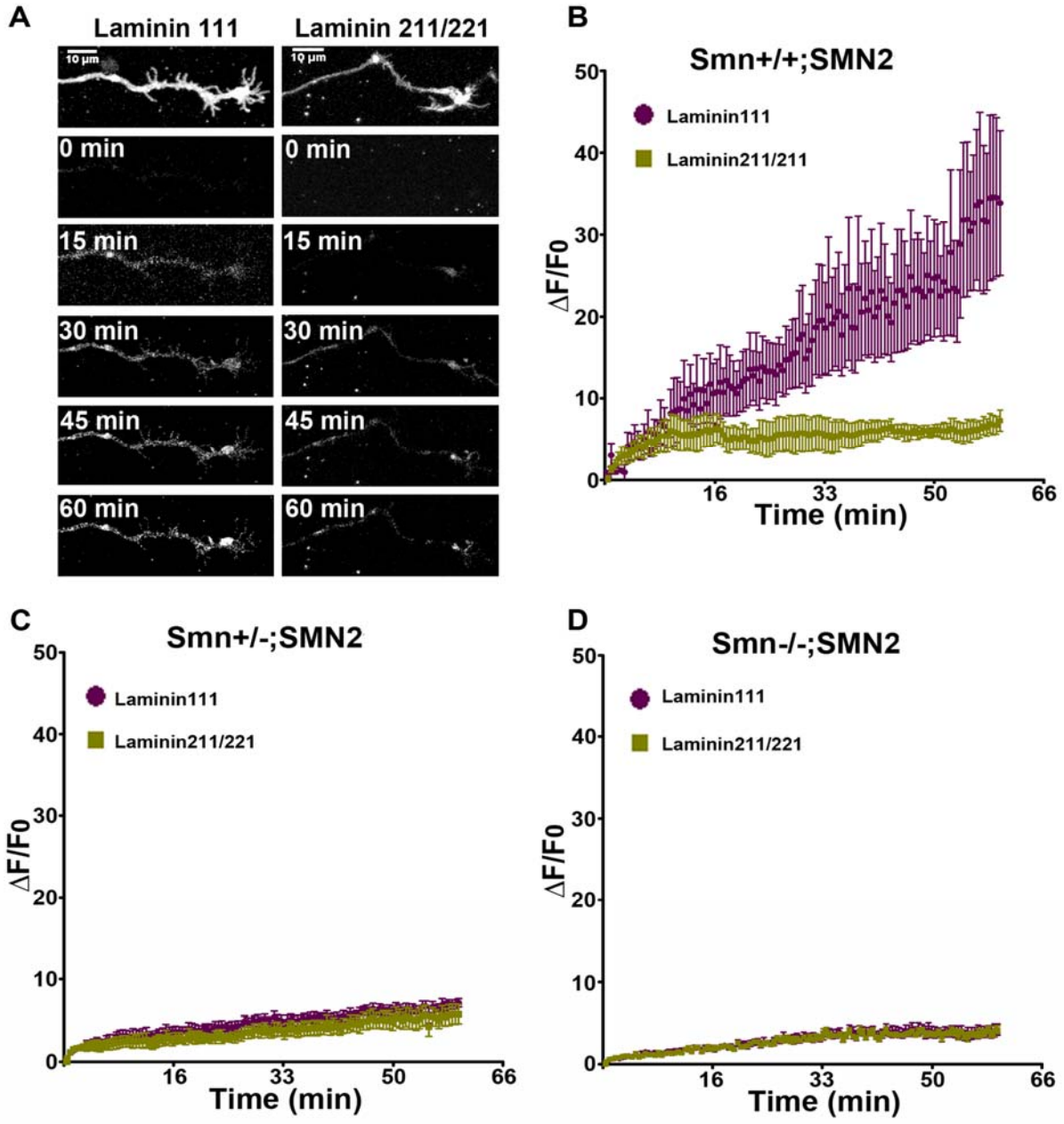

Figure 23 A) Fluorescence recovery of bleached MyreGFPZIP (β-actin) transfected *Smn$^{+/+}$;SMN2* motoneuron growth cones cultured on Laminin211/221. B) The quantification of the fluorescence recovery after bleaching over 60 min in growth cones of *Smn$^{+/+}$;SMN2* motoneurons cultured on Laminin111 (magenta, n=8) and Laminin211/221 (khakhi=8) . C) The quantification of the fluorescence recovery after bleaching over 60 min in growth cones of *Smn$^{+/-}$;SMN2* motoneurons cultured on Laminin111 (magenta, n=8) and Laminin211/221(khakhi , n=6). D) The quantification of the fluorescence recovery after bleaching over 60 min in growth cones of *Smn$^{-/-}$;SMN2* motoneurons cultured on Laminin111 (magenta, n=6) and Laminin211/221(khakhi, n=8).

FRAP was carried out with the standardized method in the motoneurons transduced with a MyreGFP-ZIP construct.Figure 23a shows a representative image sequence of the *SMN$^{+/+}$;SMN2* motoneurons cultured on Laminin211/221 and shows no significant recovery in growth cones. As can be observed in the figure 23b, the *Smn$^{+/+}$;SMN2* showed higher recovery when plated on Laminin111as compared to the recovery on Laminin211/221, whereas the recovery in the growth cones of *Smn$^{+/-}$;SMN2* motoneurons(Figure 23c) and the *Smn$^{-/-}$;SMN2* (Figure 23D) growth cones was nearly absent on either of the Laminins. This indicates that although SMN is important for the regulation of translation of the MyreGFPZIP(β-actin) construct, it is subjected to a different mechanism of regulation as compared to MyreGFP3'UTR(β-actin).

5.7 Standardization of *in situ* against eGFP with LNA probes.

In 2003, Rossoll et al. showed that there was a reduction in the amount of β-actin mRNA in the growth cones of the *Smn$^{-/-}$;SMN2* motoneurons as compared to the *Smn$^{+/+}$;SMN2* motoneurons (Rossoll et al., 2003). In order to investigate whether the local protein synthesis is also influenced by SMN protein levels, , we optimized techniques for *in situ* hybridization of eGFP RNA as a tool to investigate the localization of this mRNA. HEK293T cells were transduced with the lentiviral construct expressing eGFP under a CMV promotor and were grown for 48hr. The cells were washed and fixed with 4% PFA and then treated with 0.3% triton X-100 followed by pepsin treatment (see method section). As shown in figure 5.11a, specific signals were obtained for eGFP mRNA in HEK293T cells with an anti-eGFP probe, whereas there was no signal with the scrambled probe. Figure 5.11b shows the quantification of this experiment.

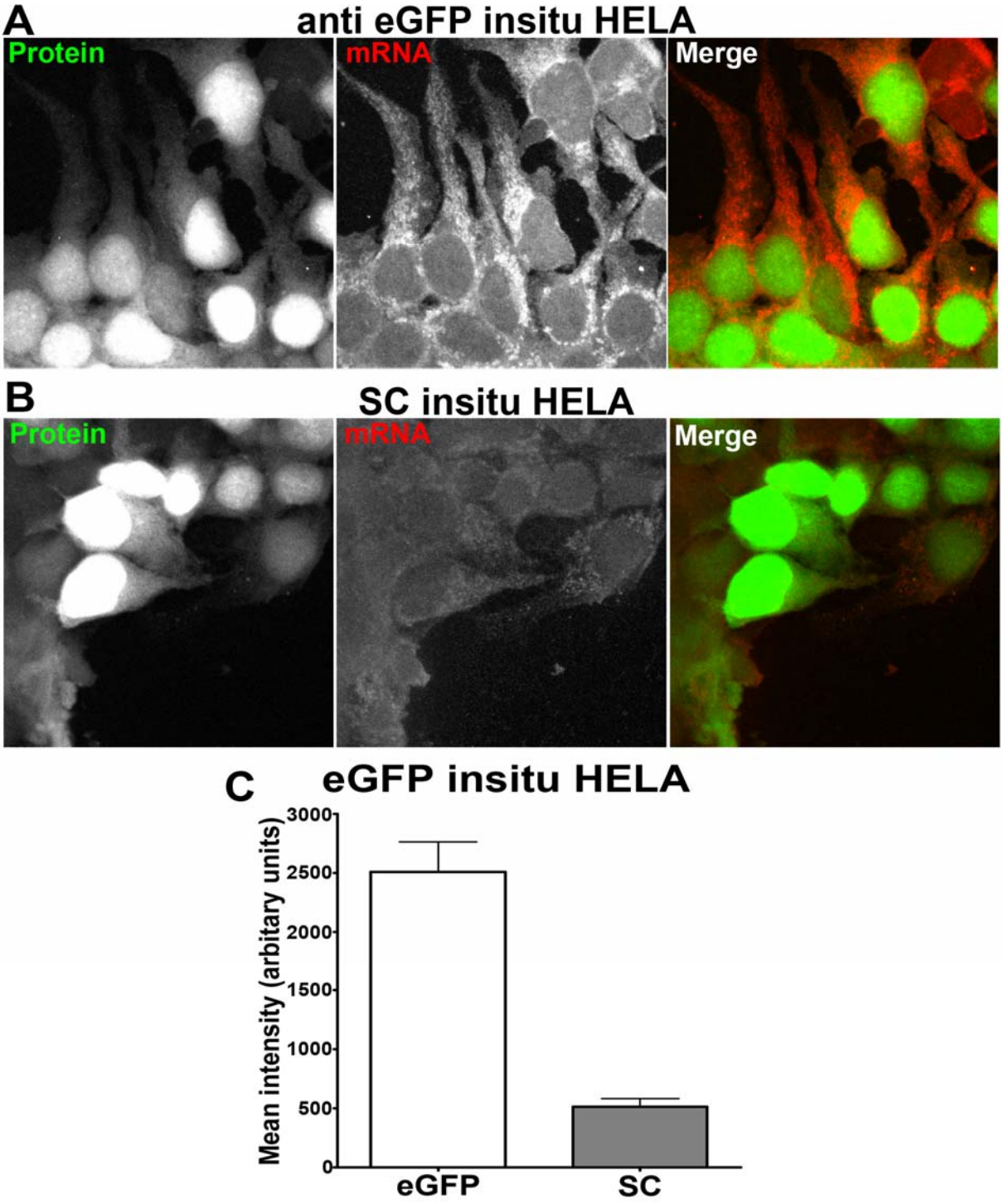

Figure 24 A) Anti eGFP *in situ*hybridization with LNA probes in HEK293 T cells transduced with eGFP expressing lentivirus under the CMV promoter (upper panel) and B)*In situ*hybridization with a scrambled probe in HEK293T cells transduced with eGFP expressing lentivirus (lower panel). Figure 24 C) Quantification of the anti-eGFP *in situ*hybridization signal vs the scrambled signal in HEK 293T cells.

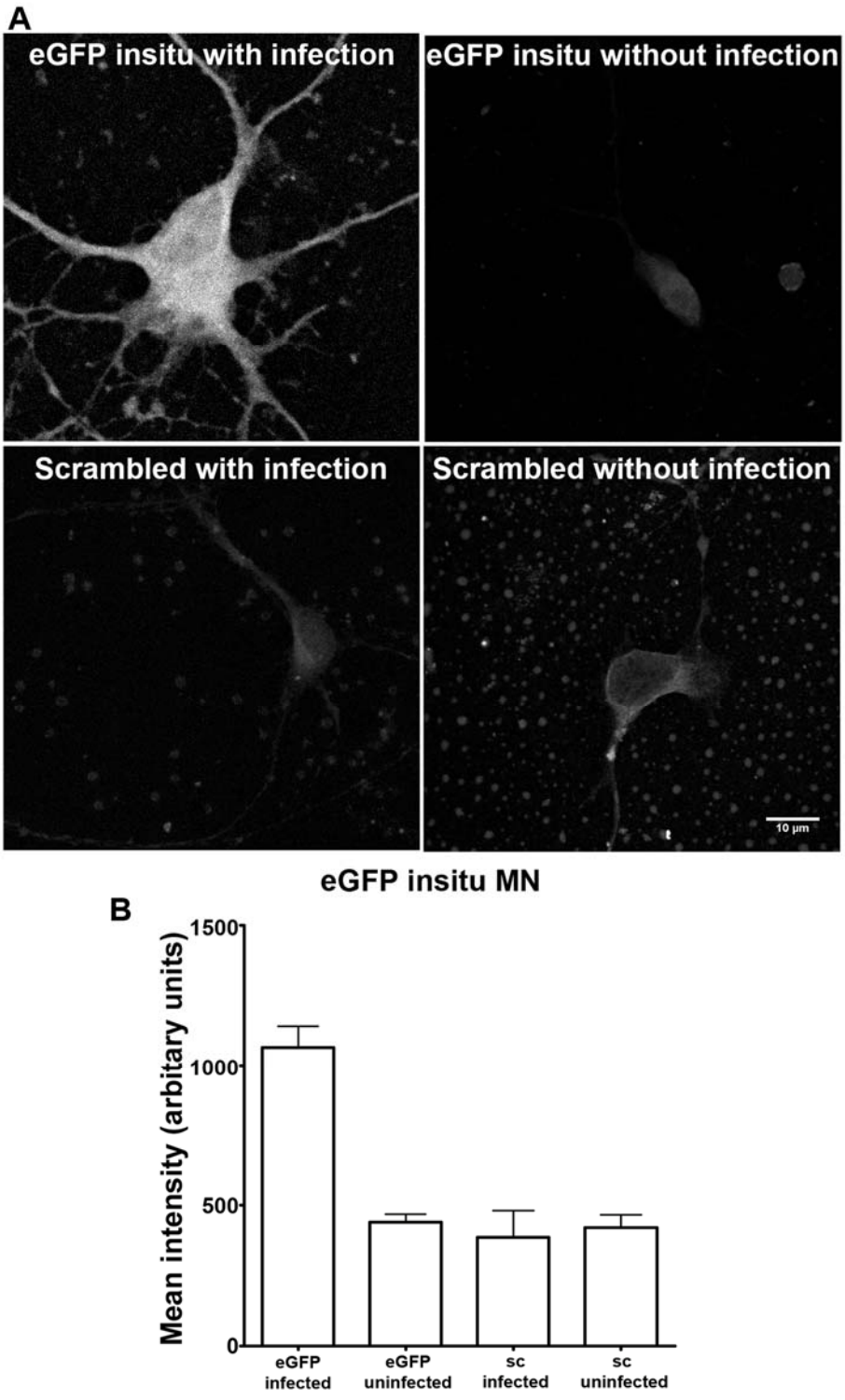

Figure 25A)*In situ*hybridization against eGFP using eGFP specific probe. This figure shows the signal in the cell body of CD-1WT motoneurons transduced with MyreGFP3'UTR (β-actin) (018rr) (upper left), eGFP scrambled probe in CD-1 motoneurons transduced with MyreGFP3'UTR (β-actin) (lower left), eGFP probe in CD-1 untransduced motoneurons(upper right) and scrambled probe in CD-1 untransduced motoneurons. B)Quantification of the *in situ*hybridization signal in the cell body .

Treatment with 0.3% Triton X-100 in combination with pepsin destroyed the axons of the motoneurons and therefore was not suitable for the experiment. CD-1 WT motoneurons transduced either eGFP expressing lentivirus cultured on Laminin111 for 5DIV were used for standardizing the *in situ*hybridization conditions. Subsequently,0.1% triton X-100 was used for the permeabilization in combination with pepsin. As shown in figure 25a, there is a specific signal for eGFP mRNA in the motoneurons expressing eGFP, whereas the untransduced neurons and neurons treated with scrambled control showlow signal intensity. Figure 25b shows the quantification of the mean intensity of *in situ*hybridization signals.

5.8 Localization of the eGFP containing mRNA

CD-1 WT motoneurons were transduced with MyreGFP3'UTR(β-actin), MyreGFPZIP(β-actin), MyreGFP or eGFPand were cultured on Laminin111 for 5DIV. The cells were then fixed with 4%PFA and *in situ* hybridization was carried out using eGFP specific probes or scrambled probe as a control. As shown in Figure 26, there is a distinct *in situ*hybridization signal present in the axons of motoneurons transduced with either MyreGFP3'UTR(β-actin) (figure 26a) or MyreGFPZIP(β-actin) (figure 26b), but there was only a low *in situ*hybridization signal in the axons of motoneurons transduced with MyreGFP (figure 26c) virus or eGFPcontaining virus (figure 26d). The quantification of signals in the cell body of motoneurons (figure 26e) revealed high expression of the eGFP under the CMV promoter whereas the ubiquitin promoter driven constructs showed lower expression in the cell body. Figure 27f shows the quantification of the *in situ*hybridization signal in axons.

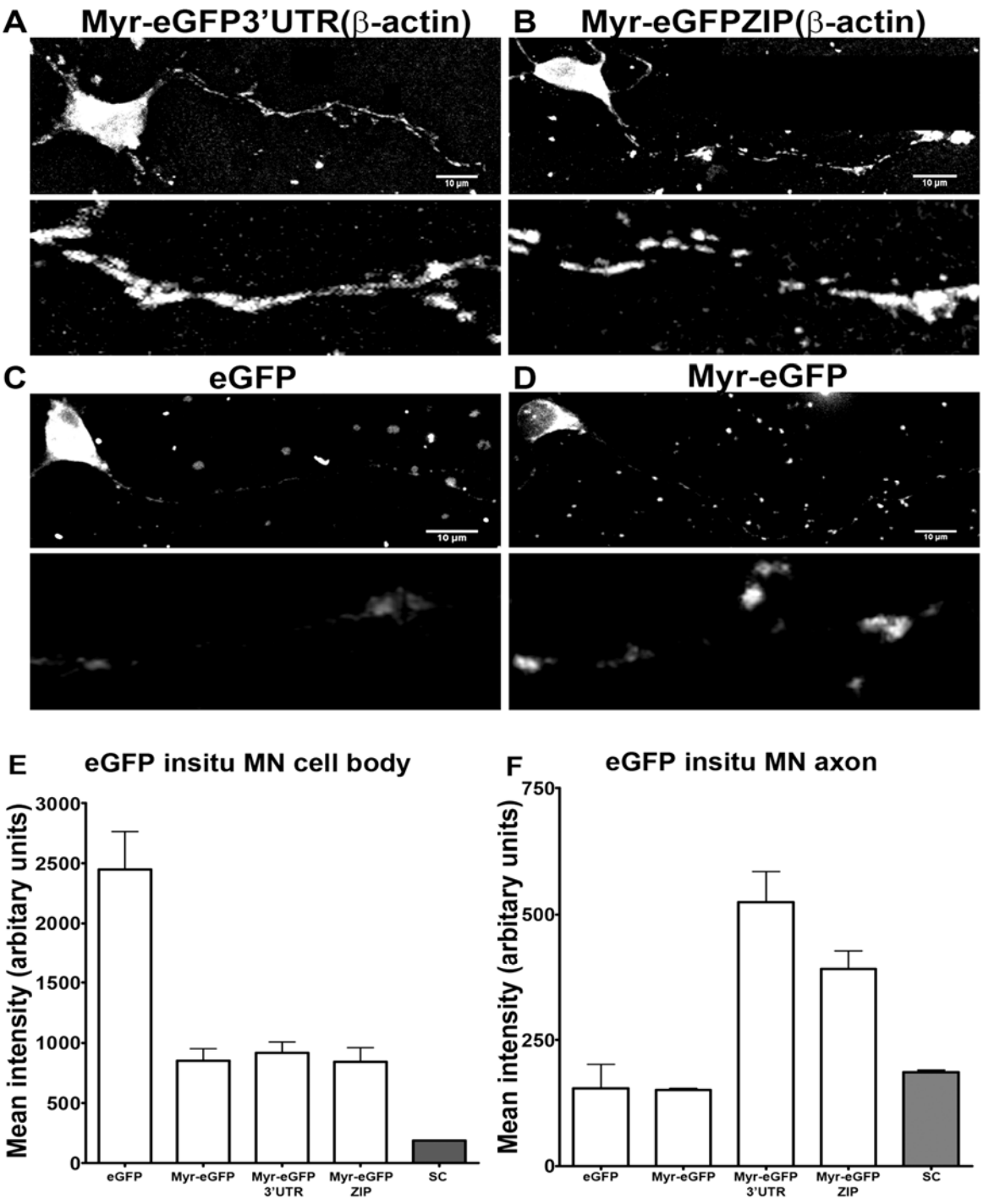

Figure 26 A) *In situ*hybridization against eGFP usingna eGFP specific probe for detection in cell bodies and axons of CD-1WT motoneurons transduced with MyreGFP3'UTR (β-actin). B) MyreGFPZIP (β-actin). C) MyreGFP D) eGFP (PGJ eGFP). E) Quantification of the insitu hybridization signal in the cell body and F) axons of CD-1 WT motoneurons with afore mentioned virally transduced CD-1 WT motoneurons.

5.9 Localization of mRNA is affected by SMN levels but not by Laminin signaling

To further investigate if SMN and Laminin signaling influence the localization of the β-actin mRNA, *Smn*$^{+/+}$*;SMN2* , *Smn*$^{+/-}$*;SMN2* and *Smn*$^{-/-}$*;SMN2* were plated on either Laminin111 or Laminin211/221 after transducing the motoneurons with MyreGFP3'UTR(β-actin). The cells were cultured for 5DIV, and then*in situ*hybridization against eGFP was performed.

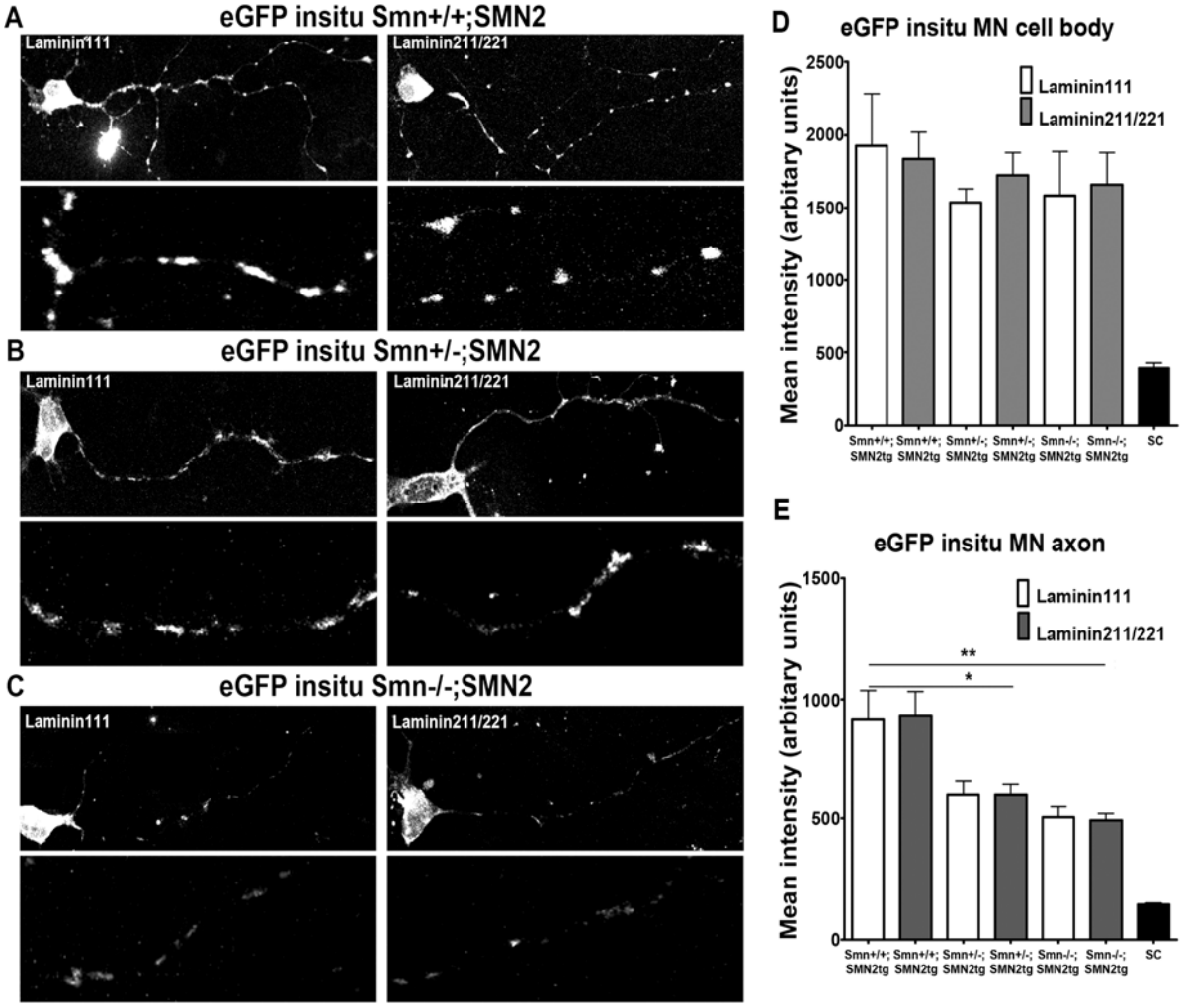

Figure 27 Transport of mRNA is dependent on SMN levels but not on the signaling from Laminin. A) Representative image of antieGFP *in situ*hybridization in *Smn*$^{+/+}$*;SMN2* B) *Smn*$^{+/-}$*;SMN2* and C) *Smn*$^{-/-}$*;SMN2* motoneurons cultured on Laminin111(left) and Laminin211/221. The lower panelsshowsimages of the axon at higher magnification. Quantification of eGFP *in situ*hybridization signal in D) the cell body and E) the axon of motoneurons with aforementioned genotype on Laminin111 and Laminin211/221.

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As shown in Figure 27a, 27b and figure 27c, there is decreased intensity of the MyreGFP3'UTR(β-actin) signal in the axons of the *Smn*$^{+/-}$*;SMN2* cells which is further decreased in the axons of *Smn*$^{-/-}$*;SMN2* cells as compared to *Smn*$^{+/+}$*;SMN2* cells. However, the axonal localization of the MyreGFP3'UTR(β-actin) mRNA is not dependent on the signal from Laminin. Figure 27d shows the quantification of the *in situ*hybridization signal intensity in the cell body of motoneurons cultured on Laminin111 and Laminin211/221. It can be observed that the expression levels are similar in the cell body irrespective of the genotype and the type of Laminin on which the cells were cultured. Figure 27e shows the quantification of the *in situ*hybridization signal intensity in the axons of the respective motoneurons. Therefore it can be concluded that the localization of the MyreGFP3'UTR(β-actin) mRNA is dependent on the SMN level in the cell but independent of the external signal coming from Laminin.

5.10 Axon length measurement

Smn$^{+/+}$*;SMN2* motoneurons plated on Laminin111 have longer axons as compared to *Smn*$^{-/-}$*;SMN2* motoneurons, In contrast, on Laminin211/221, the *Smn*$^{-/-}$*;SMN2* motoneurons have longer axons as compared to Smn+/-;SMN2tg. The status of axon length in the *Smn*$^{+/-}$*;SMN2* motoneurons is not known. To investigate this, *Smn*$^{+/+}$*;SMN2*, *Smn*$^{+/-}$*;SMN2* and *Smn*$^{-/-}$*;SMN2* cells were cultured on Laminin111 and Laminin211/221. The motoneurons were stained with anti-Map2 and anti-Tau antibodies and axon length was measured using the Leica SP5 10X objective. As can be seen in figure 5.13.1, there was no difference in axon length in the *Smn*$^{+/-}$*;SMN2* cultured on Laminin 111 or Laminin211/221.

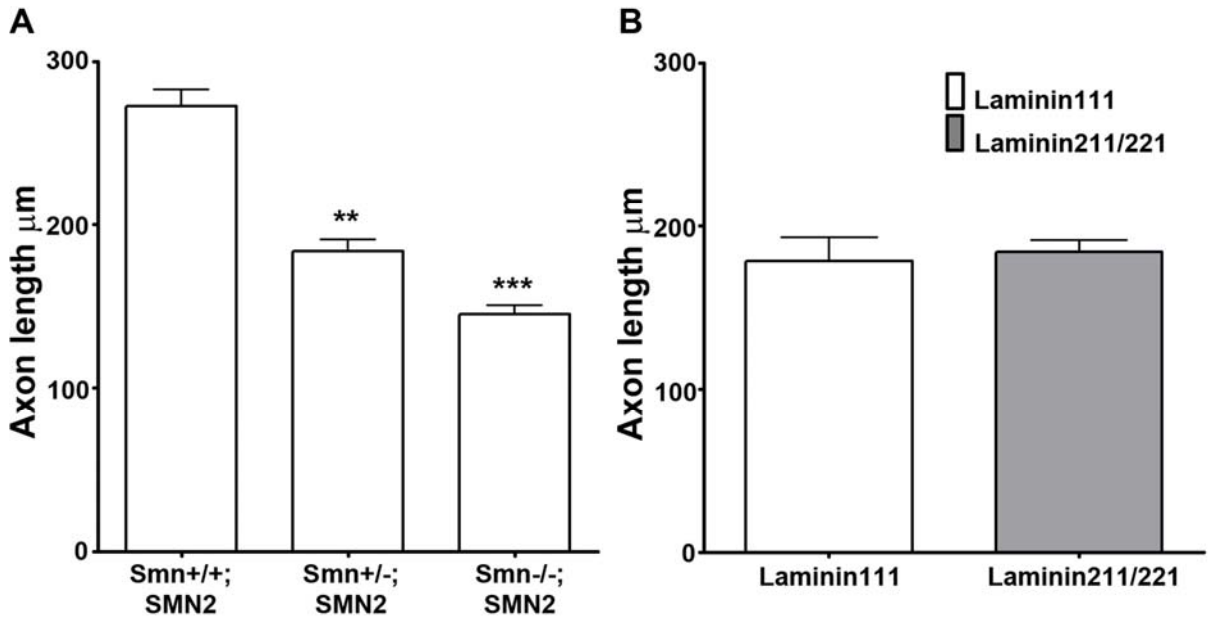

Figure 28 A) Axon length of *Smn*$^{+/+}$*;SMN2*, *Smn*$^{+/-}$*;SMN2* and *Smn*$^{-/-}$*;SMN2* cells cultured on Laminin111 B) Axon length of motoneurons cultured on Laminin111 and Laminin211/221 at 5DIV

5.11 Endogenous actin isoform expression

In 2011, Cheever et al. reported that the motor neuron specific knockout of the β-actin gene in mouse did not show any disease phenotype, indicating that β-actin is not essential for the function or regeneration of motor axons. This investigation prompted that the loss of β-actin could be compensated by transport and/or translation of other actin isoforms i.e. γ-actin or α-actin or the actinß2Like gene product. Inorder to further investigate this possibility, compartmentalized cultures of motoneurons were performed by Lena Saal and RNA was isolated from cell body and axonal compartments. The isolated RNA was linear amplified and a 1:10 dilution was used as a template to perform realtime quantitative PCR. For this, isoform specific and intron spanning primers were designed and the PCR conditions were standardized. Real time quantitative PCR was performed on whole cell cDNA using primer pairsfor α-actin (NM_009606.2),β-actin (NM_007393.3),γ-actin (NM_009609.2) and β2like actin (NM_175497.3). GAPDH was used as a control.

Figure 29a shows that the crossing point for the α-actinin the cellbody as well as in the axon is around 32nd cycle, which is an unexpected result since the α-actin mRNA is thought to be a highly expressed mRNA as can be seen from the *in situ*hybridization result and from literature (Kislauskis, Li et al. 1993). Also the presence of mRNA in the axonal compartment is an unexpected result since from the *in situ*hybridization results (figure 30), it is not detected in the axons of the motoneurons. We did agarose gel electrophoresis of the PCR amplicon and found that there was presence of multiple bands in the amplicon (Figure 29F). Upon sequencing, it was found that there were multiple amplicons containing the intron as well suggesting that there is genomic DNA contamination in the sample. In case of β-actin, the cell body cDNA shows a crossing point around 17th cycle which shows that it is expressed highly in the cell body. The axonal cDNA shows a crossing point at 32nd cycle showing the presence of the mRNA in the axonal chamber (figure 29B). In case of γ-actin the cell body crossing point is comparable to that of β-actin (figure 29C), but the water control also shows the crossing point at 32nd cycle along with the axonal cDNA, indicating that there might be unspecific amplification, but the sequencing results showed the identity of the PCR amplicon as γ-actin which is an unexpected result. In case of the β2Like actin isoform (figure 29D), the cell body showed the crossing point at 32nd cycle and the axon as well as here as the α-actin and β2Like actin cell body show crossing point around 32nd cycle, indicating that there is low expression of these two actin isoforms. Alternately, it may be due to the genomic DNA contamination. But the level of mRNA for β and γ actin isoforms and GAPDH is similar in the axonal compartment (figure 29E). This is an unexpected result since the β-actin mRNA in the axonal compartment is expected to be enriched and therefore the crossing point at

lower cycle number. In order to further analyse the results obtained by the Real time PCR, unamplified cDNA from the cell body and the axonal compartment needs to be performed.

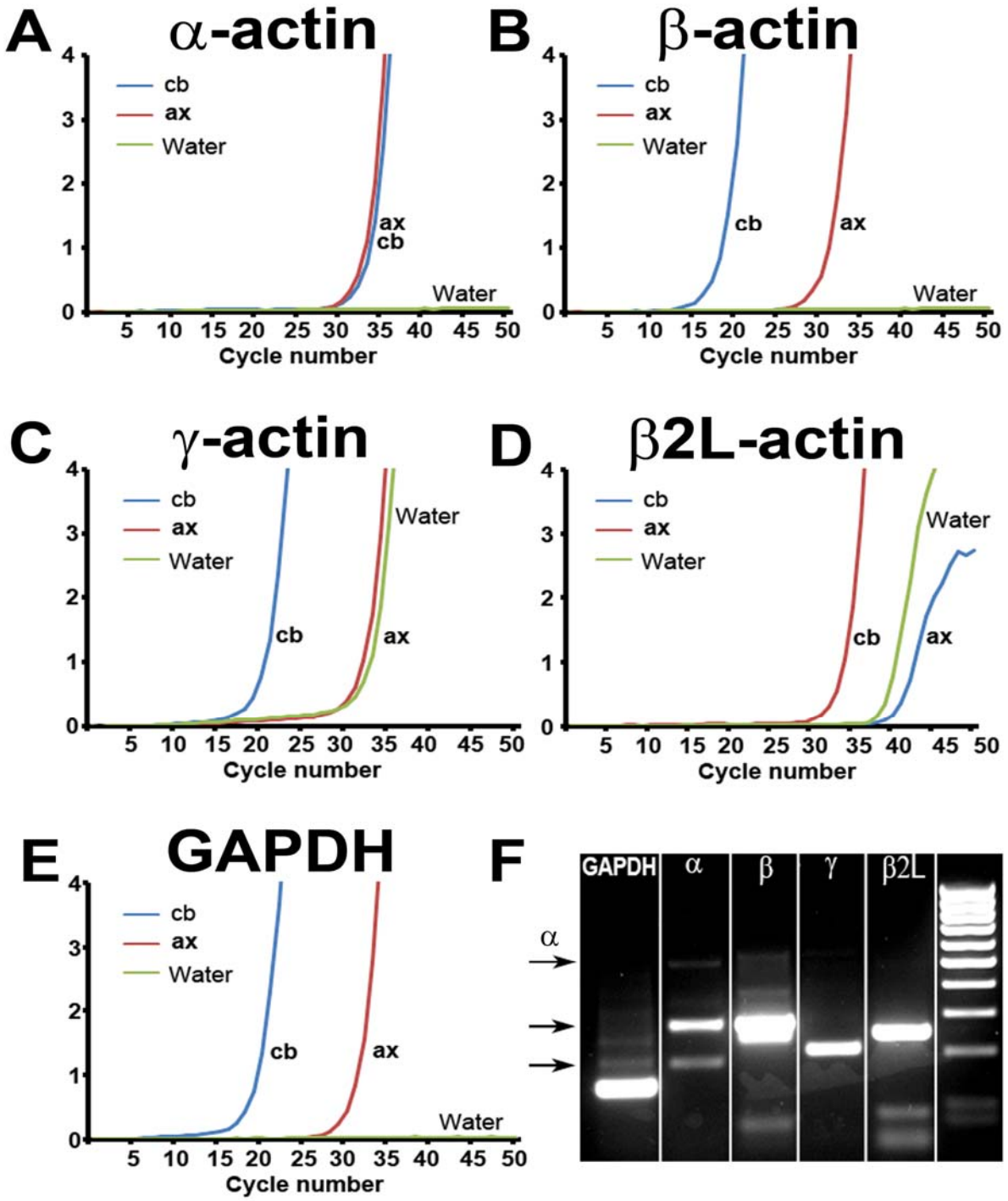

Figure 29Real time quantitative PCR of A) α-actin, B) β-actin,C) γ-actin D) β2Like actin and E) GAPDH with cDNA from cell body and axonal compartment of motoneurons cultured in compartmentalised Xona chambers after linear amplification.F) Agarose gel analysis of the amplicon after real time quantitative PCR used for sequencing. The arrow points at multiple bands obtained by PCR which was also detected in the sequencing as intron containing DNA.

5.11.1 *In situ*hybridizationfor analysis of subcellular distribution of endogenous actin isoforms

Inorder to see if the distribution of the different actin isoforms in motoneurons is altered due to SMN deficiency, we performed *in situ* hybridization against endogenous α-actin, β-actin and γ-actin isoforms. The LNA probes were designed from sequences in the 3'UTR of each of these isoforms because the coding region is highly similar for all actin isoforms. *Smn*$^{+/+}$*;SMN2*, *Smn*$^{+/-}$*;SMN2* and *Smn*$^{-/-}$*;SMN2* cells were cultured on Laminin111 for 5DIV and *in situ*hybridization was performed. As shown in Figure 30, the α-actin mRNA is localized in the cell body but not in axons of the *Smn*$^{+/+}$*;SMN2* motoneurons and the signal is not altered in *Smn*$^{+/-}$*;SMN2* cells and *Smn*$^{-/-}$*;SMN2* motoneurons.

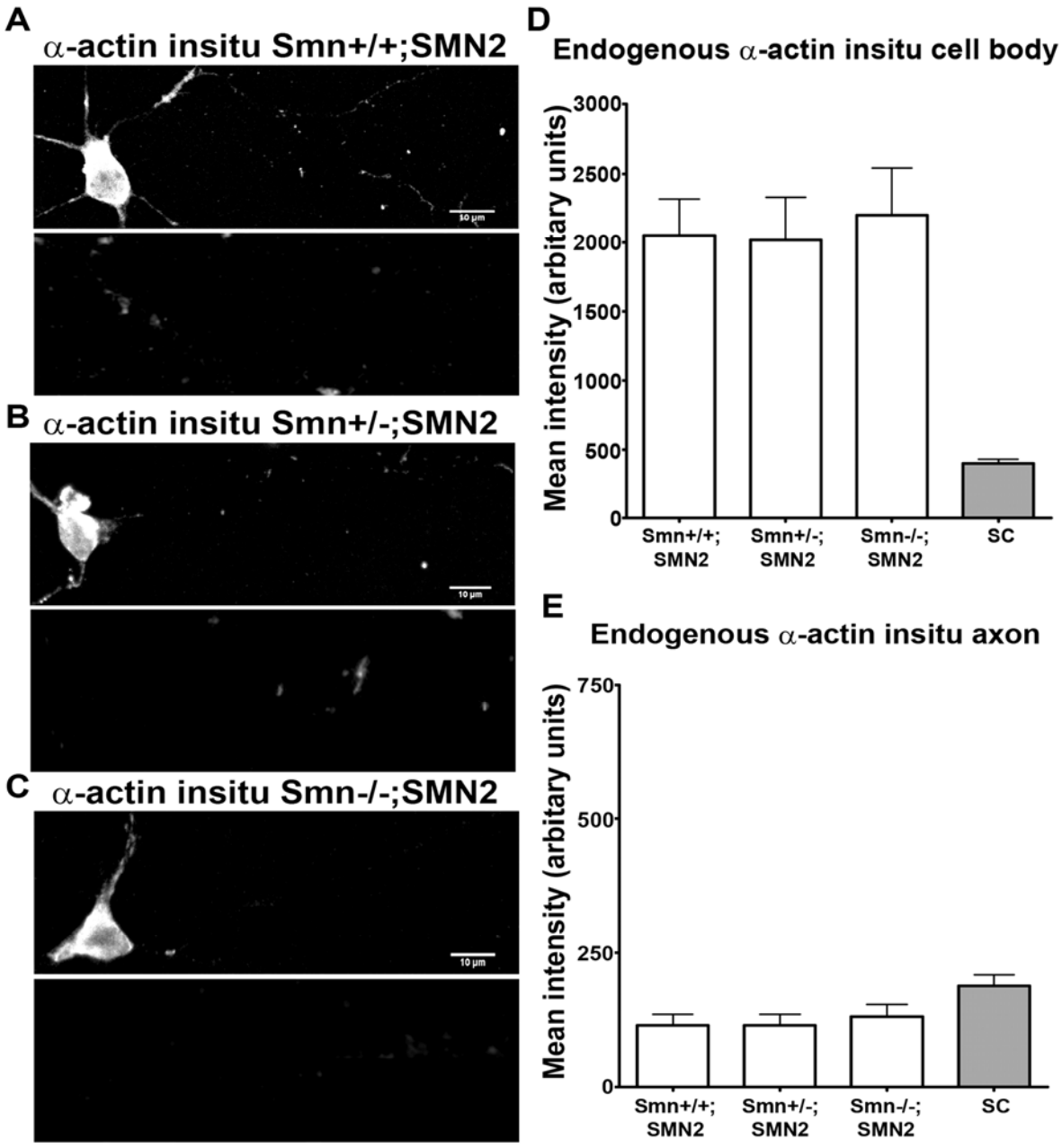

Figure 30 *In situ*hybridization signal of endogenous α-actin mRNA in A) *Smn$^{+/+}$;SMN2*, B)*Smn$^{+/-}$;SMN2* and C) *Smn$^{-/-}$;SMN2* motoneurons. D) Quantification of *in situ*hybridization signals in the cell body and E) axon.

In case of β-actin, as can be seen from Figure 31, the *in situ*hybridization signal is present in the cell body and axons of *Smn$^{+/+}$;SMN2* motoneurons. The signal intensity is reduced in axons of *Smn$^{+/-}$;SMN2* motoneurons and in *Smn$^{-/-}$;SMN2* axons, it is further reduced. Therefore, the level of β-actin mRNA in axons depends on the SMN levels in the cells.

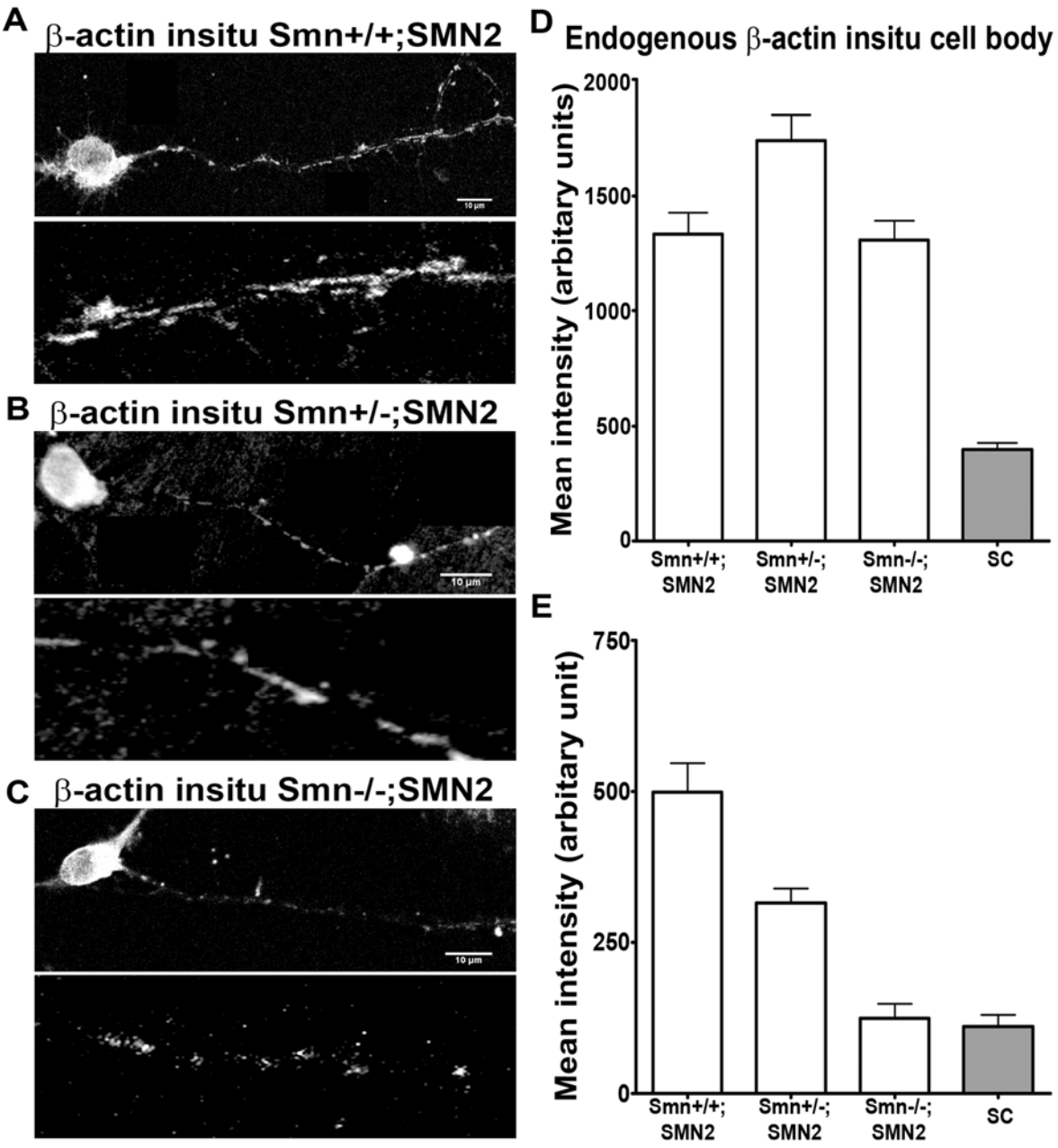

Figure 31*In situ*hybridization signal of endogenous β-actin mRNA in A) *Smn$^{+/+}$;SMN2*, B) *Smn$^{+/-}$;SMN2* and C) *Smn$^{-/-}$;SMN2* motoneurons. D) Quantification of the *in situ*hybridization signal in the cellbody and E) in the axon of motoneurons.

In case of γ-actin as with α-actin, the in situ hybridization signal was restricted to the cell body irrespective of the genotype of the motoneurons (figure 32).

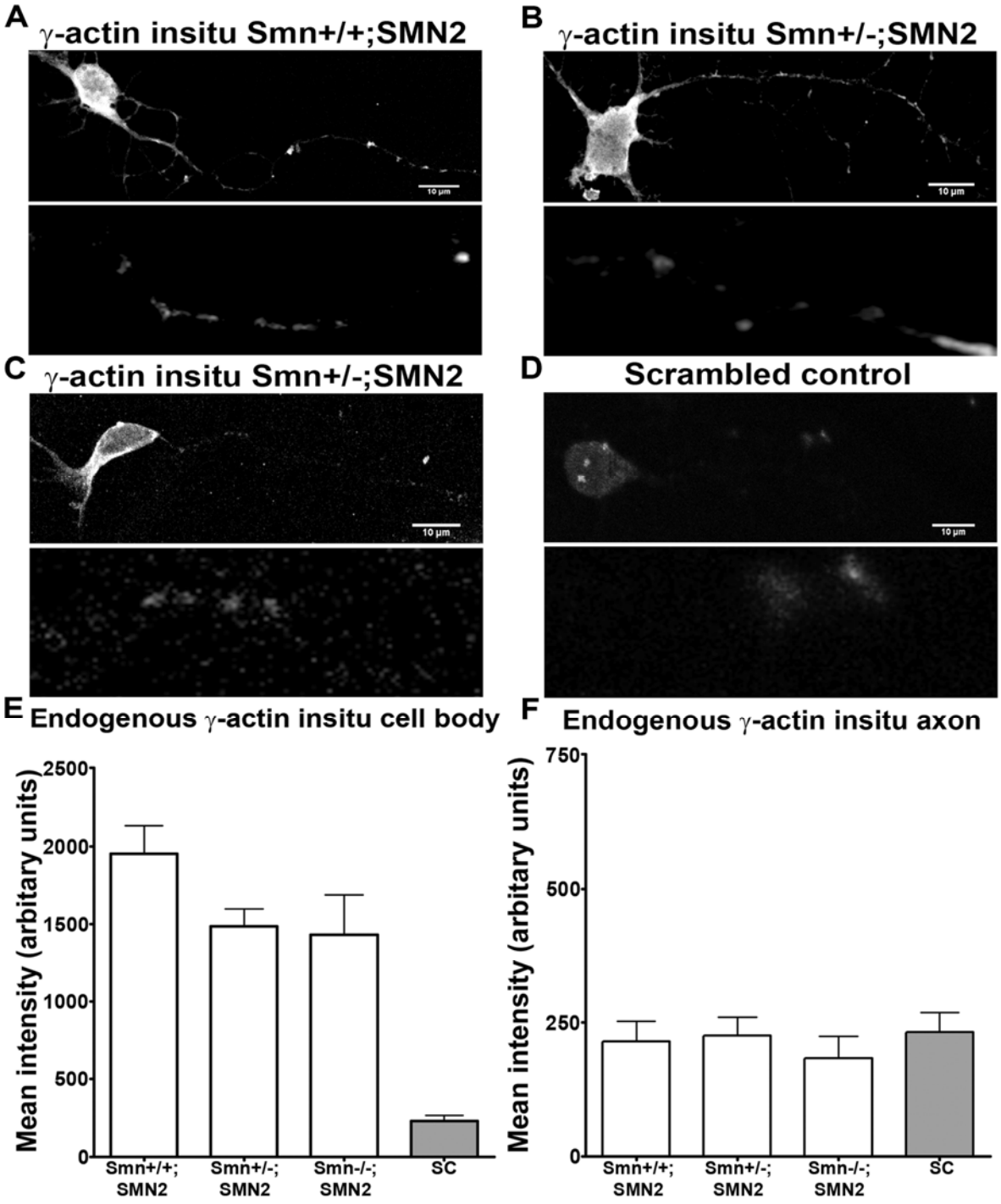

Figure 32*In situ*hybridization signal of endogenous γ-actin mRNA in A) *Smn$^{+/+}$;SMN2*, B) *Smn$^{+/-}$;SMN2* and C) *Smn$^{-/-}$;SMN2* motoneurons. D) Quantification of the *in situ*hybridization signal in cellbody and E) in the axon of motoneurons.

5.12 Cloning of a reporter construct for α-actin 3'UTR

Inorder to see if there is any local translation of α-actin mRNA, we cloned the 3'UTR of α-actin in a pcDNA3 shuttle vector containing MyreGFP. For this purpose, MyreGFP3'UTR(β-actin) construct in the pcDNA shuttle vectorwasdigested using NheI and EcoRI. The 3'UTR of α-actin was PCR amplified from the cDNA using NheI forward primer and EcoRI reverse primer and then ligated into the pcDNA3 shuttle vector. The MyreGFP3'UTR(α-actin) was the cut out with BamHI and EcoRI double digestion and then ligated to the FuVal vector. The final construct was sequenced and used for viral packaging. Figure 33 shows the schematic map and the expression pattern of the virally transduced HELA cells.

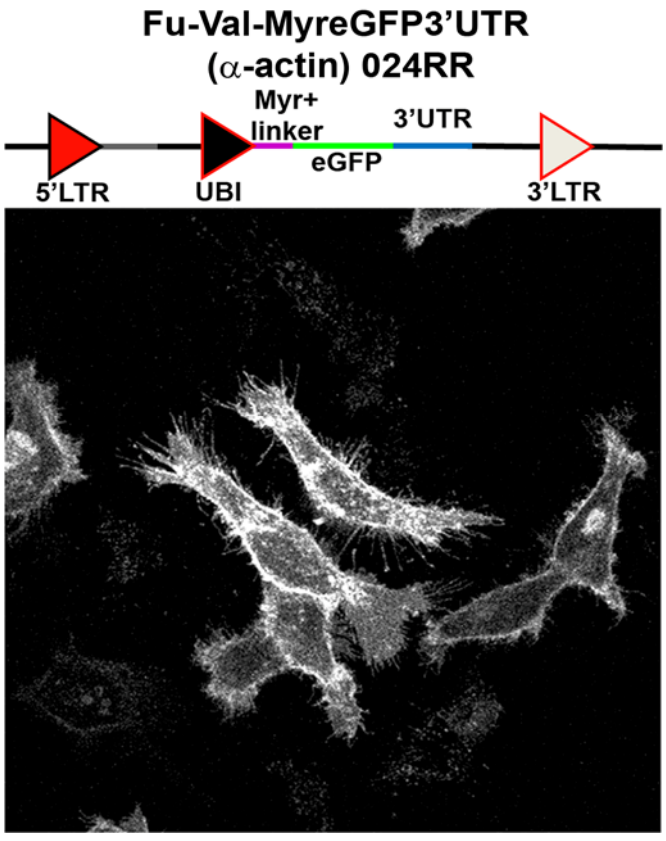

Figure 33 Schematic map of FuVal-MyreGFP3'UTR (α-actin) construct and confocal image of HELA cells expressing FuVal-MyreGFP3'UTR (α-actin) 024RR.

5.13 Production of active Sonic Hedgehog

Cell culture is an important tool in biological science to address fundamental questions of the molecular as well as implicational basis of the disease progression. Owing to the limited number of primary motoneurons and the relative heterogeneity of the cultures that limit biochemical analyses, embryonic stem cell derived motoneurons appear as a useful tool. For ES cell derived motoneurons, retinoic acid and sonic hedgehog are needed as differentiation factors. Therefore, sonic hedgehog was produced from recombinant *E.coli* expression of N-terminal fragment of sonic hedgehog. *In vitro* palmitoylation was done after purification inorder to have active sonic hedgehog. For this purpose, the *E. coli* strain BL21 with the pET-28-Shh expression plasmid was used with a His tag at the N-terminus for subsequent purification of the recombinant protein by Nickel affinity column purification. *E.coli* was grown overnight in Magic medium (Invitrogen) containing the antibiotics Chloramphenicol and Kanamycin. The cells were harvested by centrifugation and resuspended in lysis buffer. They bacteria were lysed by sonication and then centrifuged. The supernatant was loaded on Ni column and the purified protein was eluted. Figure 34A is a representative curve of the Ni column loading of bacterial lysate in the FPLC. Figure 34B is a representative HPLC run for the Ni column loading and elution.

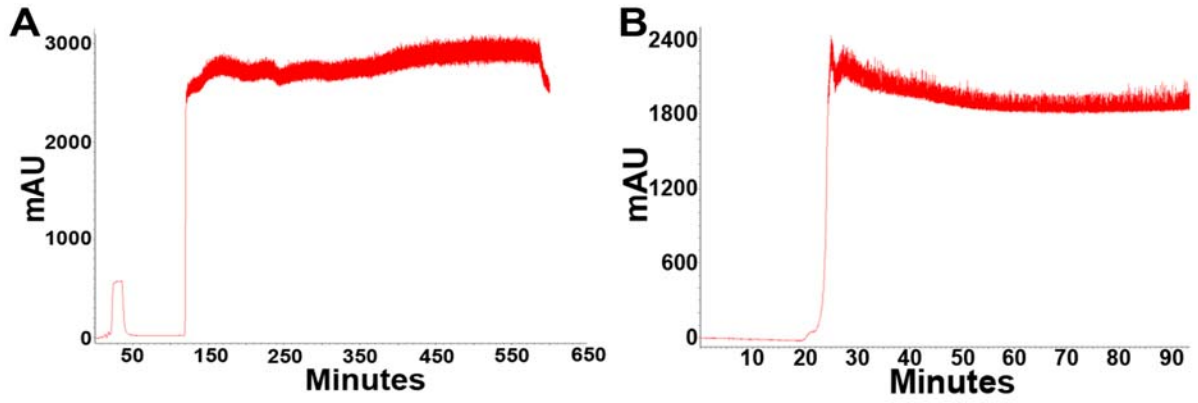

Figure 34A)Ni column loading of bacterial lysate containing Shh B)Ni column elution of purified His-tagged Shh

The purified protein was subjected to buffer exchange by gel filtration column in order to exchange the buffer suitable for the Factor Xa treatment to remove the His tag present on the N-terminal of the purified Shh. Figure 35A is a representative HPLC run for the buffer exchange.

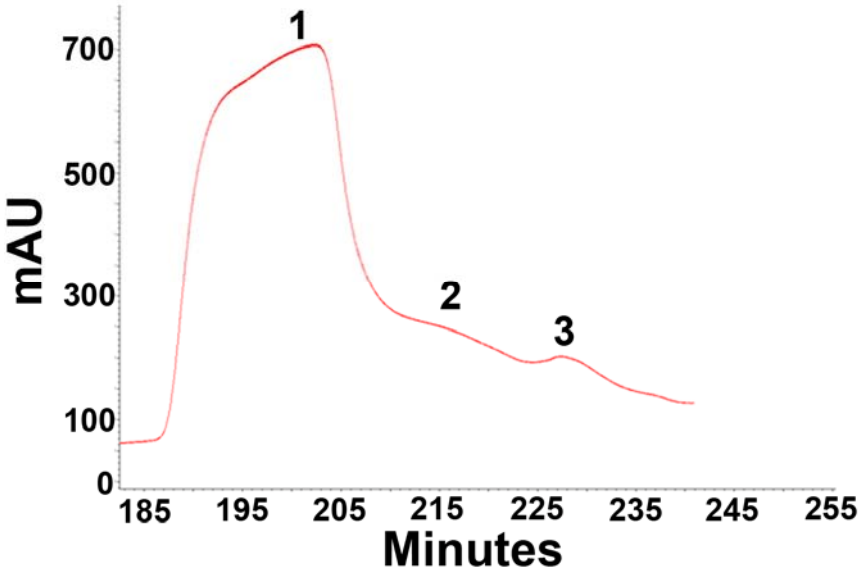

Figure 35HPLC run for the buffer exchange. Peak 1 is the purified Shh which was collected in various fractions. Peak 2 and 3 is the imidazole peak and Peak 3 contains salt

The protein was then treated with FactorXa. The protein was then dialysed against the buffer for palmitoylation. Palmitoylation was carried out by adding a 10 times molar excess of Palmitoyl-CoA and 1% β-octyl-glucopyranoside in the protein sample at 28^0C for 24hrs. A final Buffer exchange was performed and purified active sonic hedgehog was lyophilized for subsequent use. Figure 35B is a representative HPLC run for the final desalting.

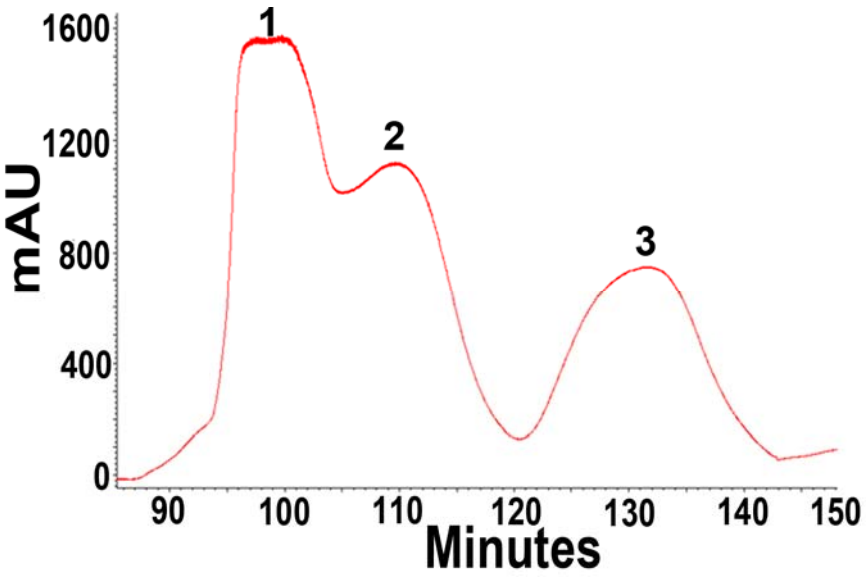

Figure 36 HPLC run of the final desalting of active sonic hedgehog. Peak 1 represents Shh that was collected as different fractions. Peak 2 containsβ-octyl-glucopyranoside and Peak 3 DTT

Samples were collected at each step of purification and loaded on SDS PAGE and stained with Coomassie to test the purity of each fraction. Figure 37shows a representative SDS PAGE with samples from various purification steps.

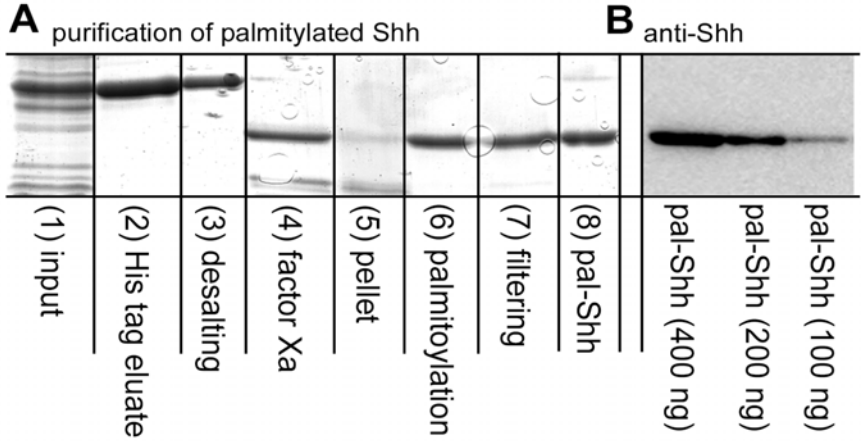

Figure 37 Representative SDS PAGE analysis after Coomassie staining of Shh at different stages of purification.B) anti-Shh western blotting to show the identity of he purified protein.(Figure prepared by Dr. RobertBlum).

5.14 Differentiation of embryonic stem cells into motoneurons

Embryonic stem cells were first propagated with feeder cells and propagated for 3 passages at high densities and then without feeder layer. The cells were then subjected to aggregate formation by propagating in ES cell differentiation medium without differentiation factors. The cell aggregates were then subjected to differentiation with retinoic acid and sonic hedgehog. The cell aggregates were then trypsinised and subjected to panning with a p75NTR antibody that specifically binds motoneurons. The cells were then plated on Laminin coated coverslips. Figure 38 shows the outline of the procedure for ES cell differentiation. For a detailed protocol, please refer the methods and material section 2.3.18. For immunostainings, 3000 to 5000 cells per coverslip (∅ 10mm) were plated in a volume of 100 µl and incubated at 37°C in an incubator. The cells were allowed to attach for 1 hour and Neurobasal full medium (containing neurotrophic factors CNTF and BDNF at 10 ng / ml) was added after cells had attached to the coverslip. The cells were cultured for 48 hours, washed with phosphatebuffered saline twice and fixed with 4 % paraformaldehyde. Antibody stainingtechniques were performed using standard techniques.

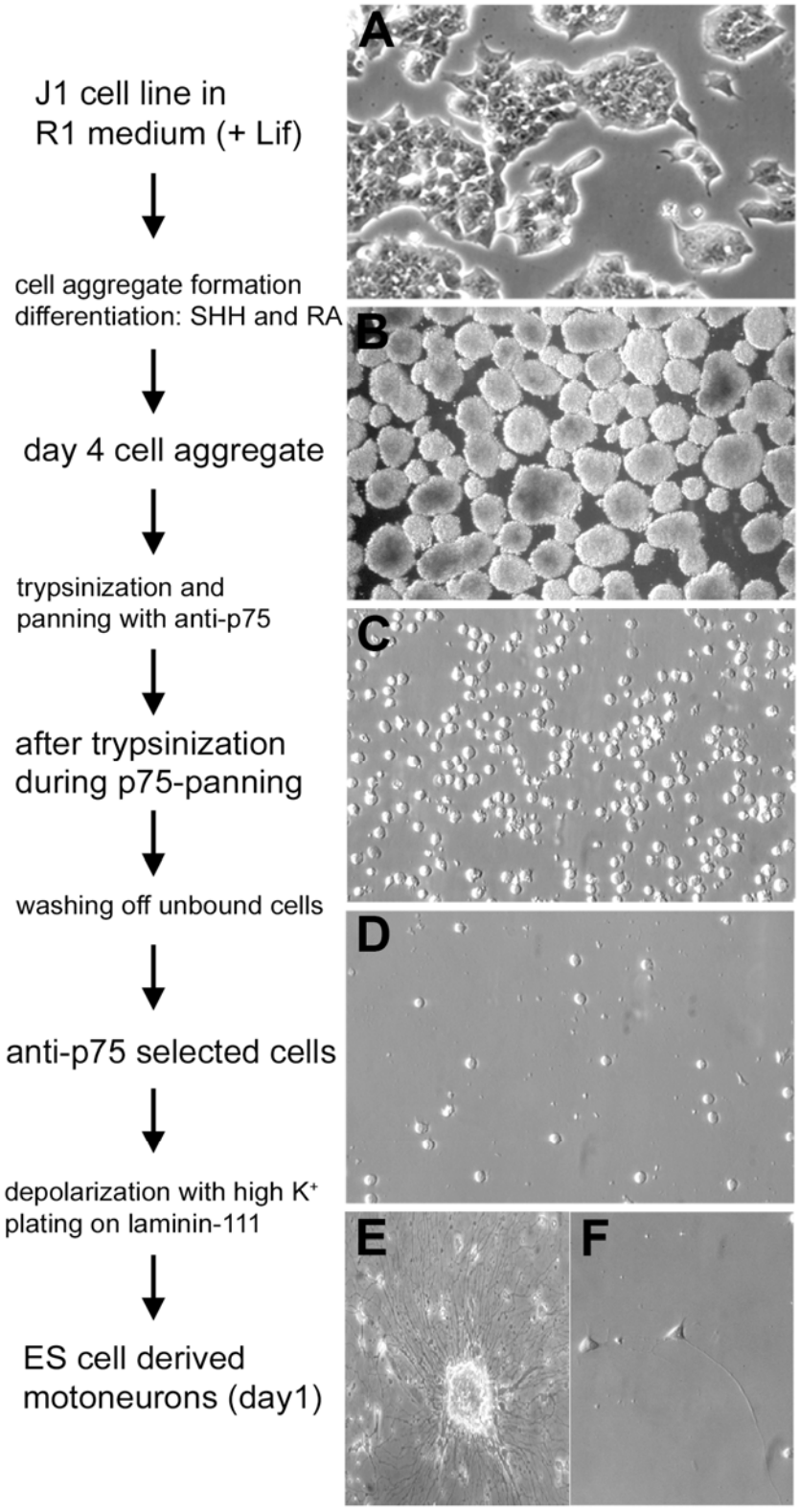

Figure 38Outline of ES cell differentiation into motoneurons. A)Morphology of ES cells 1 day after the third splitting without feeder cells. B) Cellular aggregates before dissociation. C) As shortly after dissociation. D) Panning and washing off the unbound cells. E) Differentiated cells without panning and F) differentiated cells with panning (Figure prepared by Dr. RobertBlum).

Typically, 20-35% of the cells are motoneurons when differentiated *in vitro*(Wichterle, Lieberam et al. 2002). The cell aggregates after trypsination, when put for panning can be compared with the cells obtained after washing the panning plate thoroughly. Only 10% of the cells remain attached to the panning plate .Figure 38 shows an outline of the procedure and highlights the difference in the number of cells on the panning plates after and before washing the panning plate. With the help of panning, we are also able to eliminate the cell aggregates which sometimes remain even after trypsinization and when plated on Laminin coated coverslips or culture dishes. These give rise to clusters of cells with long axons but also gives rise to a mixed population of cells (Figure 38E) and cannot be used for any further analysis.

p75 positive cells are present at a low concentration (about 20 %) if you omit the panning step (Figure 39B & C). However, when the panning step is included, nearly 90% of the cells are strongly p75 positive cells, which can be observed by immunostaining (Figure 39A & C). In addition, after panning there is 60% increase of the cells which are p75 and ISLET 1/2 double positive (Figure 39C).

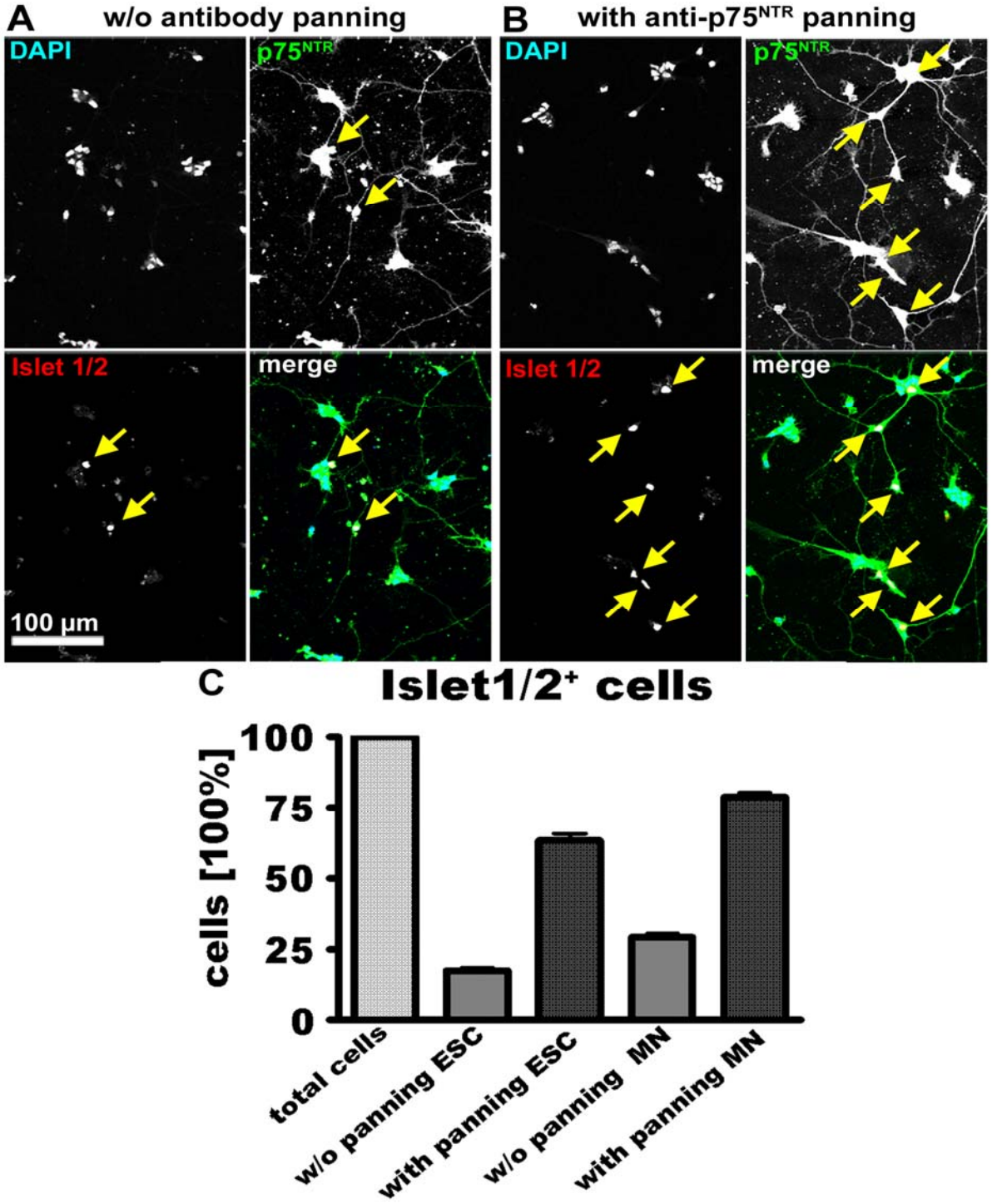

Figure 39A)Anti p75 and Islet 1/2 staining of ES cell derived motoneurons without panning and B) with panning step included. C) Quantification of the Islet 1/2 positive cell with and without panning of primary as well as ES cell derived motoneurons. (Figure prepared by Dr. Robert Blum, Quantification by Dr. Thomas Herrmann)

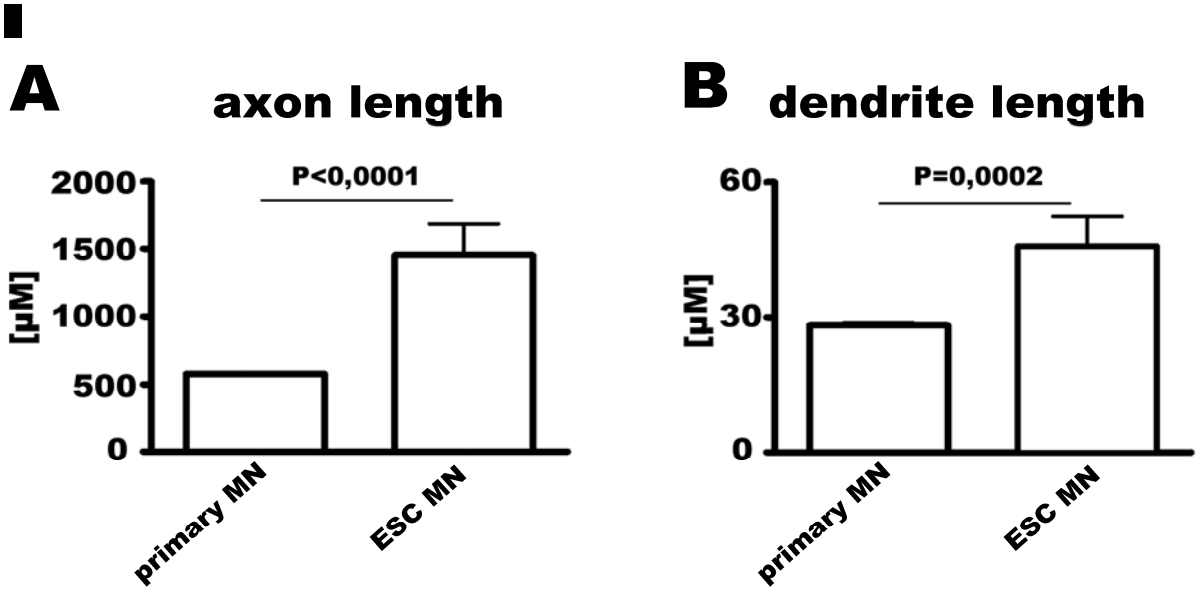

Figure 40A) Axon length and B) dendrite length of primary motoneurons as compared to ES cell derived motoneurons. (measurement done by Dr.Thomas Hermann)

These cells will give rise to a fairly homogenous population of motoneurons.Taking into consideration the comparison of ES cell derived motoneurons and primary motoneurons in our previously described protocol (Wiese et al., 2010), the Es cell derived motoneurons have a much longer axon length 24 hours after plating as compared to the 7 DIV motoneurons.($P<0.0001$)m, as shown in Figure 40A. Average dendrite length is also significantly longer ($p=0.0002$) than in primary motoneurons (Figure 40B). The primary motoneurons can survive for upto 7 days in culture whereas the survival of the ES cell derived motor neuron declines within 3 days in culture. motoneuronsmotoneurons

6 Discussion

6.1 Optimization of construct for studying local translation in motoneurons

Local translation of peripherally localized mRNA and its regulation in response to external cues is an important means of compartmentalizing signaling in neurons. To study this in motoneurons and how it is deregulated in degenerative motor neuron diseases is an important question for understanding the underlying disease mechanisms. Local translation of β-actin has been studied in various cell types using membrane anchored destabilized eGFP expressing constructs using standard transfection methods such as lipid micro vesicle based DNA delivery (lipofectamin), Calcium phosphate based delivery or sindbis virus based delivery (Wu, Hengst et al. 2005) . Motoneurons are especially susceptible to the toxicity due to these delivery methods. Based on this limitation, getting healthy motoneurons expressing the gene of interest was a challenge. Owing to this, local translation was not demonstrated in motoneurons so far. We devised and optimized a combination of suitable vector and gene delivery methods for motoneurons in order to demonstrate local protein synthesis. We expressed an eGFP reporter construct with 3'UTR of β-actin mRNA under the control of an ubiquitin promoter ensuring low levels of expression and therefore preventing the mis-targeting of the mRNA expressed. We also used a myristoylationsignal sequence at the N-terminus of the eGFP leading to membrane anchoring of the newly synthesized eGFP, minimizing the recovery of fluorescence due to diffusion. Analysis of various methods of gene delivery such as Calcium precipitation, lipofectamin 2000-mediated transfection and magnetofection revealed that these methods were toxic to the cells and that lentiviral based delivery is a viable option. The use of destabilized eGFP instead of stable eGFP for the study was also tested but it was found that although the cells received the destabilized eGFP expressing virus (see figure 14), there was rapid down regulation of the expression of the destabilized eGFP when delivered as alentiviral construct. Therefore, we have used a myristoylated eGFP reporter construct under ubiquitin promoter control as a lentivirus for the study of local translation in motoneurons.

6.2 Local translation in mouse motoneurons

As shown in the result section 3.4, by use of the reporter construct in combination with FRAP, we could investigatelocal translation in the growth cones of mouse motoneurons. There is recovery of the fluorescence intensity after bleaching in the growth cone of the MyreGFP3'UTR (β-actin) reporter expressing motoneurons where as the cells expressing MyreGFP did not show any significant recovery. The recovery was abolished in the presence of translational inhibitors such as cycloheximide, anisomycin and rapamycin, showing that the recovery in the fluorescence intensity is due to local translation at ribosomes. This

method was then used as a tool to see how the external signalling in motoneurons is affected in healthy as well as diseased motoneurons.

6.3 Transport of β-actin mRNA to axon growth cones and role of SMN

The SMN protein is part of a complex that plays a role in the assembly of spliceosomal snRNP particles. It is expressed in all cell types, but reduced levels due to homozygous mutation or deletion of the *SMN1* gene affects exclusively motoneurons. This raises a puzzling question in the field of SMA. The presence of SMN in the axon growth-cone along with β-actin mRNA and its interaction with other mRNP proteins such as hnRNP-R and Gemin2-3 in the axon growth cone hints towards a neuron specific role of the SMN. It has been demonstrated by Rossoll et al.2003 that there are reduced levels of β-actin mRNA in the axonal growth cones of *Smn$^{-/-}$;SMN2* motoneurons hinting towards the role of SMN in the transport of β-actin mRNA towards the axon growth cones.

Although the levels of β-actin mRNA are reduced in motoneurons of *Smn$^{-/-}$;SMN2*, they extend longer axons when cultured on Laminin211/221 as compared to the *Smn$^{+/+}$;SMN2* neurons(Jablonka, Beck et al. 2007). The external cue derived from the extra-cellular matrix component Laminin is impaired in the axonal growth cones of the Smn-/-;SMN2 motoneurons. *Smn$^{+/+}$;SMN2* cells, when cultured on Laminin211/221 have a higher frequency of spontaneous calcium transients as compared to *Smn$^{+/+}$;SMN2* cells cultured on Laminin 111. This increase in spontaneous calcium transients is abolished in *Smn$^{-/-}$;SMN2* cells. This indicated that the Laminin signalling is deregulated in the *Smn$^{-/-}$;SMN2* cells

6.4 Analysis of local translation in response to Laminin 1 and Laminin 2 and role of SMN in motoneurons

We have attempted to further characterize the pathophysiology of SMA in this study. We monitored the levels of local translation in axonal growth cones of mouse motoneurons from *Smn$^{+/+}$;SMN2*, *Smn$^{+/-}$;SMN2* and *Smn$^{-/-}$;SMN2* cells cultured on Laminin111 or Laminin211/221. The study revealed that there is deregulation of Laminin signalling depending on the levels of SMN in motoneurons, affecting the local translation rate of the β-actin mRNA. As can be seen from the results in the section 3.5, there is increased local translation of the β-actin mRNA in growth cones of *Smn$^{+/-}$;SMN2* motoneurons cultured on Laminin211/221 which is further exaggerated in the *Smn$^{-/-}$;SMN2* motoneurons. This was an unexpected finding since we observed that although there is reduced β-actin mRNA and increased translation in the growth cones of *Smn$^{-/-}$;SMN2* motoneurons cultured on Laminin211/221, there is decreased β-actin protein in the growth cones of *Smn$^{-/-}$;SMN2* cells cultured on either Laminin111 or Laminin211/221 according to Jablonka et al. 2007. One possible explanation for this observation was that there was a differential sorting of the reporter construct and that it did not behave like the endogenous β-actin mRNA distribution.

To test this, we performed *in situ* hybridization against eGFP mRNA. The *in situ* hybridization of MyreGFP3'UTR (β-actin) reporter mRNA reveals that there is decreased translocation of eGFP containing mRNA into the growth cones of the *Smn$^{-/-}$;SMN2*, which is consistent with the previous findings and therefore it can be ruled out that the reporter construct is sorted differentially as compared to endogenous β-actin mRNA. The decreased signal intensity of *in situ* hybridizations in *Smn$^{+/-}$;SMN2* motoneurons shows that the transport machinery of the cell is critically dependent on the levels of SMN protein.Here we find that the transport of the β-actin mRNA is not dependent on the external cue derived from Laminin since the levels of *in situ* hybridization signals is comparable on Laminin111 and Laminin211/221. However, the signal is markedly reduced in the axons depending on the levels of SMN in the cells.

6.5 Analysis of local translation of reporter constructs with ZIP code in motor control and SMA motoneurons

A study by Ghosh et al. 2008 (Ghosh, Soni et al. 2008)investigated the translocation of different species of β-actin mRNA into neurites in PC12 cells which either contained the full length 3'UTR of β-actin mRNA or only the ZIP code of the 3'UTR of β-actin mRNA. The translation of the longer full length version of β-actin mRNA was shown to be upregulated by miRNA binding to its 3'UTR whereas the translation of the shorter mRNP containing only the ZIP code was differentially regulated. Besides, it is known that SMN interacts with the 3'UTR of β-actin mRNA as a complex with hnRNP-R which binds to the mRNA at a site distinct from the ZIP code (Rossoll, Jablonka et al. 2003; Glinka, Herrmann et al. 2010). Therefore it was of interest to study the regulation of local translation of an mRNA which can be targeted but may not depend on the levels of SMN for its translational regulation. Our study with a MyreGFPZIP reporter showed that the mRNA derived from the ZIP code containing construct was transported to the axons but the local translation was abolished in *Smn$^{+/-}$;SMN2* as well as *Smn$^{-/-}$;SMN2* cells irrespective of the Laminin on which the motoneurons were cultured. This indicates that the regulation of translation of MyreGFPZIP in response to Laminin signalling is distinct from that of the MyreGFP3'UTR (β-actin). It would be of great interest to see if indeed a different species of β-actin transcript exists physiologically in motoneurons as well. The physiological role of such a transcript may help to better understand the molecular interplay which governs the axon extension and SMA pathology.

6.6 Transport and translation of other actin isoforms in SMA

In 2011 Cheever et al. (Cheever, Olson et al. 2011) reported that the motor neuron specific knockout of β-actin gene in mice did not show any disease phenotype indicating that β-actin is not essential for the neuronal function or regeneration of motor axons after injury. This observation could be due to the possibility that the loss of β-actin is compensated by transport and/or translation of other actin isoforms i.e. γ-actin or α-actin or the actinβ2Like

gene product.Our *insitu hybridization* experiments with 3'UTR specific LNA probes revealed perinuclear localization of α-and γ-actin mRNA in the mouse motoneurons whereas β-actin mRNA was present also in the axons of motoneurons. Previous findings ofHill et al. 1993(Hill and Gunning 1993), Bassell et al. 1998(Bassell, Zhang et al. 1998) and Willis et al. 2005 (Willis, Li et al. 2005)showed that γ-actin is not transported to the periphery or to neurites in neurons. Kislauskis et al. showed that α-actin mRNA is also not transported to the periphery(Kislauskis, Li et al. 1993). Our *insitu hybridization* results are consistent with the previous findings of Hill et al. 1993 as well as Kislauskis et al 1993. The sequencing analysis of real time quantitative PCR of α-actin showed the amplification of an additional amplicon including the intron. This indicates that there was genomic DNA contamination in the cDNA. The cycle number for the β-actin in the axonal compartment was also found to be the same as that of γ-actin and GAPDH which are not transported to the axons. In case of actinβ2Like quantification, it can be interpreted that the expression of this transcript is either very low or not expressed and the amplification was due to the genomic DNA contamination. Further experiments using different primers and unamplified cDNA needs to be done in order to interpret the results obtained by the real time quantitative PCR in this study.

β- actin complete knock out is embryonic lethal(Bunnell, Burbach et al. 2011) where as γ-actin knockout mice are viable but show delayed development and die 48hr postnatal (Belyantseva, Perrin et al. 2009; Bunnell and Ervasti 2010). These studies also highlighted that the β-actin$^{-/-}$ mice derived primary embryonic fibroblasts showed defects in the cell migration. An interesting observation from the same lab showed that motoneuron specific ablation of β-actin in mice are healthy and viable(Cheever, Olson et al. 2011). Total actin protein concentration was not found to differ in any of these models indicating that each actin isoform has a unique cellular functions which may not be compensated(Perrin and Ervasti 2010). Cheever et al. argued 2011that if the β-actin mRNA was critical for the neuronal path finding, recovery after injury and formation of a proper NMJ, then the loss of the β-actin in motoneurons should lead to SMA like degenerative phenotype, but these mice are healthy. These findings indicate that β-actin loss specifically in motoneurons may be compensated by other actin isoforms in vivo. Alternatively studies by Court et al 2008 showed that there is transfer of ribosomes from Schwann cell to axon of motoneuron in vivo (Court, Hendriks et al. 2008). An alternative explanation for the above observation may come from several new findings which give convincing evidence of cross talk between neighbouring cells(Twiss and Fainzilber 2009). Court et al. observed 2008that in a Walleriandegeneration mouse model of motor neuron degeneration following injury, there was increased transfer of fluorescently labeled ribosomes in desomatized axons from neighbouring Schwann cells *in vivo*, giving evidence of the transfer of the translational machinery from Schwann cells to axons(Court, Hendriks et al. 2008). Other studies have shown the presence of exosomes which contain

distinct sets of mRNA and proteins from that of the recipient cells and thus hint towards the possibility that proteins and mRNA can be derived from neighbouring cells (Valadi, Ekstrom et al. 2007) and this transfer of cellular components is not restricted to a certain cell type(Twiss and Fainzilber 2009). Transport of β-actin mRNA being an important component for regulation of various cellular processes, it can be speculated that the mRNA is derived from the ensheathing Schwann cells or target muscle cells in the Cheever et al 2011 study. Interestingly,(Park, Maeno-Hikichi et al. 2010) study on a motor neuron specific reduction in the SMN levels revealed that these mice (Olig2CreSMA mice) show an age-dependent attenuation of early functional deficits observed. The authors of this study argue that a cross talk exists between the terminal Schwann cells and target muscles with a “wild type” SMN level to the motoneurons which specifically have reduced SMN thus resulting in a homeostatic compensation at a later age.

6.7 Regulation of β-actin local translation by mRNA binding proteins

Previous studies on the transport of β-actin mRNA revealed that ZBP1 (ZIP code binding protein-1) is essential for the localization of β-actin mRNA (Oleynikov and Singer 2003) in axons. Interfering with the interaction of ZBP-1 and its recognition sequence ZIP code causes mis-localization of β-actin mRNA from the leading edge of polarized chick fibroblasts. Besides the involvement of ZBP-1 in the transport of β-actin mRNA to the periphery of the cells and maintenance of the cell polarity in chick fibroblasts, it has been shown to be involved in the regulation of the translation of β-actin mRNA in response to netrin-1 and BDNF signalling in neurons (Sasaki, Welshhans et al. 2010). Taking these findings together, it can be seen that ZBP-1 acts both for transport as well as regulation of the translation of β-actin mRNA. Another example is that of the FMRP (Fragile X mental retardation protein)which is associated with RNA trafficking granules and translation in dendritic spines(Bassell and Warren 2008). FMRP has been characterized as an mRNP component for targeting several mRNAs to dendritic compartments. It also n regulates the translation of CamKIIα mRNA in response to the mGluR5 stimulation. Mutation in fmr-1 gene is associated with two well-recognised syndromes that are a major cause of intellectual disability. In the light of these findings,it is not surprising that SMN also has a dual role i.e. in transport as well regulation of translation of the mRNA in response to external cues..

It has also been observed that the local translation of β-actin is dependent on activity in hippocampal neurons(Tiruchinapalli, Oleynikov et al. 2003) and on Ca2+ signalling for growth cone guidance(Yao, Sasaki et al. 2006). Further experiments involving the role of Ca2+ transients mediated by Laminins on β-actin local translation in *Smn$^{-/-}$;SMN2* motoneurons may reveal the convergence of signalling from different external cues to the Ca2+ dependent regulation of β-actin mRNA local translation.

6.8 Signalling pathways for regulation of local translation

Local protein synthesis is mandatory for the regulated dynamics of growth cone extension, collapse and turning (Campbell and Holt 2001; Wu, Hengst et al. 2005; Hengst, Deglincerti et al. 2009; Holt and Bullock 2009). Rearrangements of cytoskeletal elements in growth cones of Xenopus retinal axons are triggered by chemotropic gradients of the guiding factors Semaphorin 3A (Sema3a) and Netrin-1 (Campbell and Holt 2001). When Smn protein is reduced in PC12 neuronal cells, profilin IIa expression is increased and a mis-regulated activation of the RhoA/ROCK pathway affects cytoskeletal integrity and neurite growth (Bowerman, Shafey et al. 2007). In an intermediate SMA mouse model, increased profilin IIa expression causes a decrease in the actin bundling protein plastin 3 (Bowerman, Anderson et al. 2009) and this might participate in disturbed actin dynamics observed in SMA model systems (Rossoll, Jablonka et al. 2003; Bowerman, Shafey et al. 2007; Jablonka, Beck et al. 2007; Bowerman, Anderson et al. 2009).Based on our analyses, we can estimate the number of β-actin molecules that are generated per minute due to local translation versus transport, and correlate them to the number which may be needed for integration in the actin cytoskeleton. Such analyses have previously been performed with polarized chick fibroblasts to study the role of locally produced β-actin for crawling, These analyses show that local translation can provide only around 7% of the β-actin protein needed for polymerization (Condeelis and Singer 2005). It has been proposed, that the purpose of local translation is to define the site of nucleation of actin polymerization rather than affecting the rate of actin polymerization. This in turn may trigger various cellular pathways affecting the cytoskeletal network (Condeelis and Singer 2005). Our observation of upregulated local translation in response to Laminin211/221 signalling in *Smn-/-;SMN2* cells supports the idea that the purpose of local translation is not to provide the raw material for the actin polymerization but to act as a signalling cue.

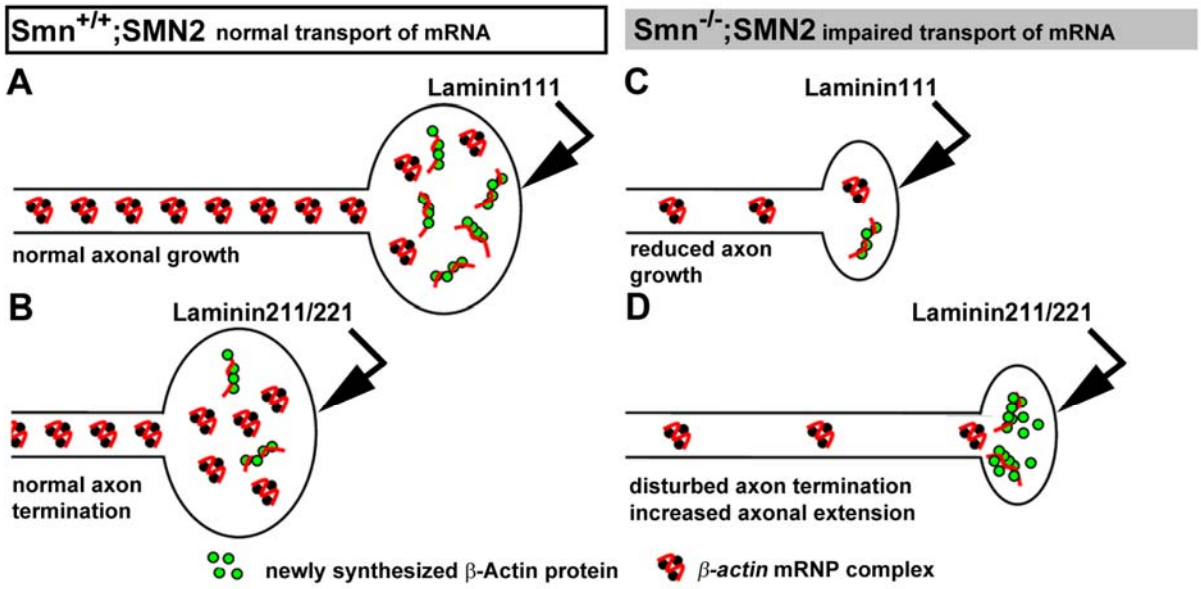

Fig 41 Model for the regulation of β-actin mRNA transport and local translation.

The axonal transport and accumulation of the β-actin mRNA depends on SMN levels but is independent of Laminin signalling. The translation of the β-actin mRNA is regulated by Laminin signalling. A) *Smn$^{+/+}$;SMN2* motoneurons cultured on Laminin111 show normal transport of β-actin reporter mRNA that is translated efficiently. The motoneurons show normal axonal extension. B) *Smn$^{+/+}$;SMN2* motoneurons when cultured on Laminin211/221 show normal transport of β-actin reporter mRNA which is locally translated with lower efficiency. The motoneurons show normal axon termination and therefore reduced axon length. C) *Smn$^{-/-}$;SMN2* motoneurons cultured on Laminin111 show reduced transport of β-actin reporter mRNA and reduced translational efficiency. They exhibit defective axon elongation as compared to *Smn$^{+/+}$;SMN2*. D) *Smn$^{-/-}$;SMN2* motoneurons cultured on Laminin211/221 show reduced transport of β-actin reporter mRNA but increased translational efficiency and increased axonal length and a defect in axon termination.

6.9 ES cell derived motoneurons

Primary cell cultures have been mainly used for dissecting out the pathways necessary for neuronal survival, differentiation and axon growth. Heterogeneity, difficulty in manipulating the genetic background and limited amounts of primary cells are some of the drawbacks of using primary neurons. Therefore, differentiation of embryonic stem cells into motoneurons serve as a valuable tool for the investigation of mechanisms that determine cell fate and play important future roles in applications ranging from regenerative medicine by transplantation of neurons for therapeutic purposes in neurodegenerative diseases, such as SMA and ALS, to drug screening efforts. Production and purification of physiologically active sonic hedgehog was established in order to differentiate ES cells into motoneurons. The N-terminal fragment of Sonic hedgehog is known to mediate all the physiological functions of Sonic hedgehog. The N-terminal fragment is palmitoylated, cleaved and secreted in the extracellular matrix where it forms a gradient of concentration. The enrichment of ES cell derived motoneurons can provide a homogenous population for all these studies. For directed differentiation of mouse embryonic stem cells into motoneurons, we compared three different stem cell lines E14Tg2A, J1, 159-2. All these ESC cell lines efficiently differentiated to motoneurons with similar efficacy. Typically, 65-70% of the cells obtained by the described technique are motoneurons. Previously published protocols without anti-$p75^{NTR}$ panning step usually end up with 20-35% of ESC-derived motoneurons within these cultures (Wichterle, Lieberam et al. 2002). After trypsinization, cell aggregates were dissociated and processed for panning. Only 10% of the cells remain attached to the panning plate after mild washing, similar to the washing step applied to purify primary motoneurons from mixed population of spinal neurons from embryonic mouse spinal cord (Wiese, Herrmann et al. 2010).

The anti-$p75^{NTR}$ panning step is useful for several reasons. It helps to enrich ES cell derived motoneurons, but it also eliminates cell aggregates which sometimes remain intact after trypsinization and trituration and stably attach on laminin-coated coverslips or culture dishes. Cultures contaminated by these aggregates form clusters with mixed neuronal populations. In cultures without the $p75^{NTR}$-antibody panning step, ES-derived motoneurons are present at relatively low percentage (about 20%). The $p75^{NTR}$-antibody panning step leads to enrichment of this population to nearly 90% of all DAPI positive cells that express $p75^{NTR}$. In addition, after $p75^{NTR}$-antibody panning at least 60% of all cells are $p75^{NTR}$ positive and express the motoneuron specific marker Islet 1/2, thus representing a fairly homogenous population of motoneurons.

Interestingly, such ES cell derived motoneurons also show some properties that differ from primary motoneurons that have been isolated from embryonic mouse spinal cord. ES cells

derived differentiated neurons develop long axons (1500 µm +/-50 µm) within 24 hours after plating. For comparison, axons of embryonic primary motoneurons of th mouse(Wiese, Herrmann et al. 2010) grow to a mean length of 700 µm (+/-50µm) within 7 days on the same laminin-111 substrate. Dendritic length is also increased in ES-cell derived motoneurons (50 µm +/-5 µm) when compared with primary mouse motoneurons at DIV 7 (30 µm +/-5 µm).

In the presence of the neurotrophic factors BDNF and CNTF, 80% of mouse primary motoneurons survive for at least 7 days in culture whereas survival of ES cell derived motoneurons obtained by this protocol is limited and decreases after 3 days in culture. The reasons for these differences are not known. However, enrichment by anti-$p75^{NTR}$ antibody mediated panning can be used for further biochemical analyses to characterize the reasons for these distinct properties.

The major advantage of this new technique is the possibility to generate high numbers of relatively homogenous populations of motoneurons for biochemical analyses that normally require high cell numbers, such as microarray based gene expression or DNA methylation analyses(Meissner, Mikkelsen et al. 2008; Guttman, Amit et al. 2009; Han, Do et al. 2009) or ChIP studies(Tollervey, Curk et al. 2011). Given that proteins such as TDP-43 and FUS that are altered in patients with motoneuron disease bind to RNA(Tollervey, Curk et al. 2011), such techniques can be used to perform ChIP experiments with enriched motoneurons from mouse and human ES cells in order to study specific alterations in motoneurons and to search for drugs that can interfere with such mechanisms.

7 Abbreviations

°C	Grad Celsius
µg	microgram
µl	microliter
A	amino acid alanine
A	ampere
ACSF	artificial cerebrospinal fluid
APS	ammoniumpersulfate
APV	2-Amino-5-phosphonovaleriansäure
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
bp	base pairs
cAMP	cyclic adenosine monophosphate
LTP	long-term potential
cm^2	square meter
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CO_2	carbon dioxide
DAG	diacylglycerole
DAPI	4´,6-diamidino-2-phenlyindol
ddNTP	didesoxyribonucleotide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotide
EGFP	enhanced green fluorescent protein
ER	endoplasmatical reticulum
ERK	extracellular-signal-regulated kinases
et al.	latin: and others
EtOH	ethanol
F	amino acid phenylalanine
FCS	fetal calf serum
FL	full-length
GABA	γ-aminobutyric acid
GFP	green fluorescence protein
GlutaMAX	L-alanyl-L-glutamine
GPCR	G-protein coupled receptor
HBSS	Hank´s Buffered Salt Solution
HCl	hydrogen chloride
K	amino acid lysine
KCl	potassium chloride
kDa	kilo Dalton
l	liter
M	molar
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute
ml	milliliter
mM	milimolar
mRNA	messenger ribonucleic acid
N	amino acid asparagine
NaCl	sodium chloride
NfH	Neurofilament heavy chain
ng	nanogram

NGF	nerve growth factor
nm	nanometer
NT	neurotrophin
OH	hydroxide
PAC-1 receptor	pituitary adenylate cyclase activating-polypeptide receptor
PBS	phosphate buffered saline
PKC	protein kinase C
PLCy	phospholipase C
proSAP	proline rich synapse associated protein
rpm	round per minute
SDS-PAGE	Sodium-Dodecylsulfate-Polyacrylamide-Gelelectrophorese
sec	second
Trk	tropomyosin-related kinase
TTX	tetrodotoxin
U	unit
UV	ultra violet
V	volt
W	watt
wt	wildtype
Y	amino acid tyrosine

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Affidavit

I hereby confirm that my thesis entitled Study of local protein synthesis in growth cones of embryonic mouse motor is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis. Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation “eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum

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9 Curriculum vitae

Academics

- **Ph.D. (Neurobiology)** Institute fur Klinisches Neurobilogie,Wuerzburg, Germany • September 2007 onwards
- **PG Certificate (Molecular Medical Microbiology)** Universtiy of East London, London, UK. 2003
- **M.Sc. (Microbiology)** Maharaja Sayajirao University of Baroda, Baroda, India • 2001
- **B.Sc. (Biochemistry)** St. Xavier's College Ahmedabad, India • 1999

Employment Details

UNIVERSITY OF WUERZBURG, GERMANY

Phd student (September 2007 onwards)

- Responsible for coursework completion, design and execution of laboratory experiments
- First project on Differentiation of embryonic stem cell differentiation into motor neurons was successfully completed and submitted for publication.
- Second project on "Regulation of Local translation of β-actin mRNA in the growth cone of mouse motor neurons to analyse the underlying mechanism of Spinal muscular atrophy" successfully completed with results submitted for publication

Faculty of Science Nirma University, Ahmedabad, India

Teaching Assistant (Octobar 2004 –July2009)

- Responsible for training and evaluation of masters students in microbiology and biotechnology (theory and practical)

Intas Pharmaceuticals R&D centre Biotech division Ahmedabad, India

Research Associate (July 2001-August 2002)

- Worked in analytical division on developing protein purification methods for enhanced production of anti cancer biotech drug suitable for industrial scale production.

Publications

- **Laminin induced local axonal translation of β-actin mRNA is impaired in SMN-deficient motoneurons**. Reena Rathod, Steven Havlicek, Nicolas Frank, Robert Blum & Michael Sendtner.Journal of Histochemistry and cellbiology.2012 (accepted)
- **Differentiation of embryonic stem cells into motor neurons.** Thomas Herrman, Rathod ReenaJ., Blum Robert, Drepper Carsten, Sendtner Micheal. (Manuscript in revision).
- **Neuronal translation, do you get the message? A review of mRNA-protein particles in neurons**. Ross Smith, Rathod Reena J., Shalini Rajkumar and Derek Kennedy. (Manuscript in preparation).

Symposia and Conferences Attended

- Presented a poster titled "Local protein synthesis in the growth cones of mouse motor neurons" at International Symposium of the Graduate School of Life Sciences, University of Wuerzburg 19th Oct - 20 Oct 2011 **"6th International Symposium organized by the students of the Graduate School of Life Sciences – BIOBANG"** Wurzburg, Germany.

- Presented a poster titled "Local protein synthesis in the growth cones of mouse motor neurons" at International Symposium of the SFB 487 of 28 July - 30 July 2011 **"Molecular Pharmacology of Receptors, Channels and Transporters"** Wurzburg, Germany.

- Presented a poster titled "Local protein synthesis in the growth cones of mouse motor neurons" at the **EMBO "SPATIAL"** symposium June ,2011, Engelberg, Switzerland

- Presented a poster titled "Local protein synthesis in the growth cones of mouse motor neurons" at the **German Cell biology Annual meeting** March 31-April2 ,2011, Bonn, Germany.

- Attended **National conference on Management of viral diseases in WHO regime** Jan 2002, at IVRS Banglore, India.

- Attended **International symposium on recent advances in molecular biology, allergy and immunology** by Maharaja Sayajirao university of Baroda and State university of New York at Buffalo in association with Indian Academy of allergy. October 2000, Baroda, India.

Transferable skills acquired

Writing publication

Poster presentation

Giving academic talks

Computer skills acquired

- Computer skills for scientific analysis, presentation and publication of the data. Well versed with imaging software such as ImageJ, NIS elements, LAS lite.
- Stastical data analysis software such as graphpad prism
- In silico clone designing software such as oligo6 and ApE.
- Use of bioinformatical tools such as UCSC genesorter, Ensemble genome browser, NCBI NIH portal, IMAGE and FANTOM clone data base.
- Word office, photoshop, endnote, etc

Languages known

English, Hindi, Gujarati, German

Personal Details

- Date of Birth : 18 Feb 1979
- Nationality : Indian
- Marital Status : Single

10 Publications

Laminin induced local axonal translation of β-actin mRNA is impaired in SMN-deficient motoneurons. Reena Rathod, Steven Havlicek, Nicolas Frank, Robert Blum & Michael Sendtner.Journal of Histochemistry and cellbiology.2012 (accepted)

11 Acknowledgements

मूकं करोति वाचालं पङ्गुं लङ्घयते गिरिम्

यत्कृपा तमहं वन्दे परमानन्द माधवम् ॥

With whose grace, a dumb can speak eloquently, with whose grace, a crippled can climb the mountains-Oh my Lord, my respectful obeisance and prostrations to thy Holy feet.

I thank my Lord –my Prabhuji who came to me through Prof. Sendtner with this opportunity to do my PhD and through this, discover myself a new.

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अज्ञानतिमिरान्धस्य ज्ञानाञ्जनशलाकया ।

चक्षुरुन्मीलितं येन तस्मै श्रीगुरवे नमः ॥

I was born in the darkest ignorance, and my spiritual master opened the eyes of me with the torch of knowledge. I offer him my respectful obeisances.

Ich wurde in finsterster Unwissenheit geboren, und mein spiritueller Meister oeffnete mir die Augen mit der Fackel des Wissens. Ich erweise ihm meine achtungsvollen Ehrerbietungen.

My lord has revealed himself as my spiritual master His Holiness Pramukh Swami Maharaj who opened my eyes and showed me my Lord everywhere and above all within me. All this work was done by my Lord himself present in me. I pray to them to choose me to be the instrument in their hands for betterment of all.