Characterization of the Functional Domains of a Novel Vertebrate Specific Presynaptic Protein - Mover

Dissertation
for the award of the degree
"Doctor rerum naturalium"
of the Georg-August-Universität Göttingen

within the doctoral program Sensory and Motor neuroscience
of the Göttingen Graduate School for Neurosciences, Biophysics, and Molecular Biosciences (GGNB)
of the Georg-August University School of Science (GAUSS)

submitted by
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Affidavit

I hereby declare that the PhD thesis entitled "Characterization of functional domains of a novel vertebrate specific presynaptic protein-Mover" has been written independently and with no other sources and aids than quoted.

Asha Kiran Akula

Göttingen, May 26th, 2015

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Abbreviations

HRP	Horse-radish peroxidase
TBS	Tris-buffer Saline
TBST	Tris-buffer Saline with Tween 20
RT	room temperature
PVDF	Polyvinylidene fluoride
PEI	Polyethylenimine (Sigma 482595)
ON	overnight
DMSO	Dimethyl sulfoxide
GFP	Green fluorescent protein
TAE	Tris-acetate buffer
PLL	Poly-L-lysine
MOPS	3-(N-morpholino) propansulfonic acid
MES	2-(N-morpholino) ethane sulfonic acid
CaMKII	Ca^{2+}-Calmodulin dependent protein kinase II
LB medium	Luria-Bertani medium.
DTT	Di thiothreitol
MgCl2	Magnesium chloride
CaCl2	Calcium chloride
EDTA	Ethylene diamine tetra acetic acid
PMSF	phenyl methane sulfonyl fluoride
SDS	Sodium dodecyl sulphate
HCl	hydrochloric acid
FBS	Fetal bovine serum
BSA	Bovine serum albumin
PFA	paraformaldehyde
SV	Synaptic vesicle
aa	amino acid
CaM	Calmodulin
Ca^{2+}	Calcium
Syt1	Synaptotagmin1
SyPhy	Synaptophysin
IP	Immunoprecipitation
Ms	Mouse
Gp	Guinea pig
Rb	Rabbit
Ch	Chicken

Acknowledgements

I would like to express my sincere gratitude to my supervisor Prof. Thomas Dresbach for giving me an opportunity to work with him. I want to thank him for all the support, encouragement and guidance throughout my PhD. I would like to express special appreciation for being available all the time for discussions and help offered during my PhD.

I would like to thank my thesis committee members Prof. Tobias Moser and Prof. Nils Brose for their time and discussions during thesis committee meetings. In addition, I appreciate Prof. Tobias Moser for being the second reviewer of my thesis.

I want to thank Prof. Christoph Viebahn for giving me an opportunity to be a part of the anatomy and embryology department. In addition, I thank all the members of the department for a nice and friendly atmosphere.

I extend my sincere thanks to Frau Irmgard Weiss and appreciate for all the assistance and help during the last four years of PhD.

I am thankful to all colleagues of my lab: Nina, Andonia, Julio, Freida, Tina and Moritz for the comments, discussions and a friendly working atmosphere all through the years of my PhD.

I would like to extend my special thanks to Eric, Olga and Prof. Blanche Schwappach from the department of biochemistry for their valuable support and help.

I would like to express my special thanks to the GGNB for their support during the last four years of my PhD and for providing me the bridging fund for three months during the last stage of my PhD.

I would like to thank all my friends who made my stay in Germany wonderful during the last four years of my PhD.

Last but not least, I thank my family-mother, father, sisters, brother-in-laws, Srikanth, Yamini, Phani and brothers for their enormous support, motivation and love not only during PhD but also throughout my life. In addition, I thank my grandfather who was always with me discussing about my career. Above all, I want to thank the almighty for everything.

1. Introduction

1.1. Synaptic transmission

Synaptic transmission is an essential process of communication for all neuronal processes in the brain. Human brain performs several functions such as conduction, computation, storage and modulation of electrical information. These functions are mediated by highly differentiated cells of the brain- neurons. Mammalian brain consists of billions of neurons organized into a sophisticated network of connectivity. The connectivity among neurons is maintained through the intercellular connections called synapses.

Synapses are asymmetric intercellular junctions that aid in the transmission of information either through chemical (neurotransmitter) or electrical signals. Each synapse comprises of two functional units: a presynaptic compartment consisting of large number of proteins involved in synaptic vesicle release machinery and a postsynaptic compartment that receives the transmitter and propagates the signal. Electrical synapses are characterized by 2-4nm area of apposition and chemical synapses by a distance of 20-40nm between pre- and post-synaptic membranes (Hormuzdi et al., 2004). The transmission of information occurs through gap junction channels by a passive flow of current between adjacent cells. Chemical synapses communicate through the release of neurotransmitter from presynaptic to postsynaptic neuron upon arrival of an action potential.

1.2. Chemical synapses

Signal transduction in the mammalian brain happens mainly through chemical synapses, where the synaptic transmission occurs mainly by Ca^{2+} triggered release of neurotransmitter. Upon arrival of an action potential in the presynaptic terminal, Ca^{2+} enters through the voltage gated calcium channels triggering the Ca^{2+} dependent exocytosis of synaptic vesicles and ultimately the release of neurotransmitter into the synaptic cleft. The released neurotransmitters from the synaptic cleft subsequently bind to the receptors present on the postsynaptic

membrane leading to the translation of chemical signal back into an electrical signal. Exocytosis of synaptic vesicles occurs at a specialized area of the plasma membrane in the presynaptic terminal called the active zone (AZ).

1.3. Synaptic vesicle cycle

The Ca^{2+}-triggered exocytotic release of neurotransmitter from synaptic vesicles includes a series of cyclic events in a process known as synaptic vesicle cycle. The synaptic vesicle cycle involves three phases : i) formation of synaptic vesicles and their organization into functional pools, during which neurotransmitter is loaded onto synaptic vesicles through neurotransmitter transporters ii) exocytosis, a process that involves docking of vesicles to the presynaptic plasma membrane and the docked vesicles undergo maturation, called priming. The primed vesicles fuse with the plasma membrane following the influx of calcium through voltage-gated calcium channels mediated by a calcium sensor iii) endocytosis or recycling of synaptic vesicles occurs following fusion, vesicles and vesicle associated proteins are recycled to sustain neurotransmitter release.

The synaptic vesicle serves as an organelle for neurotransmitter storage and release. To do this, synaptic vesicles rely on restricted set of membrane proteins (Fig 1.1). The synaptic vesicle biogenesis involves a series of events starting from synthesis of proteins in the cell body, followed by their sorting and their targeting to the membrane along the axon. The formation of vesicles through endocytosis and the formed vesicles undergo a maturation process that includes the active uptake of neurotransmitter from the cytoplasm (Bennett and Scheller, 1994).

Functionally, synaptic vesicles are distinguished into three types of pools basing on their differential ability to be recruited for fusion. The three pools are:

- Readily Releasable Pool (RRP)
- Recycling Pool
- Reserve Pool (RP)

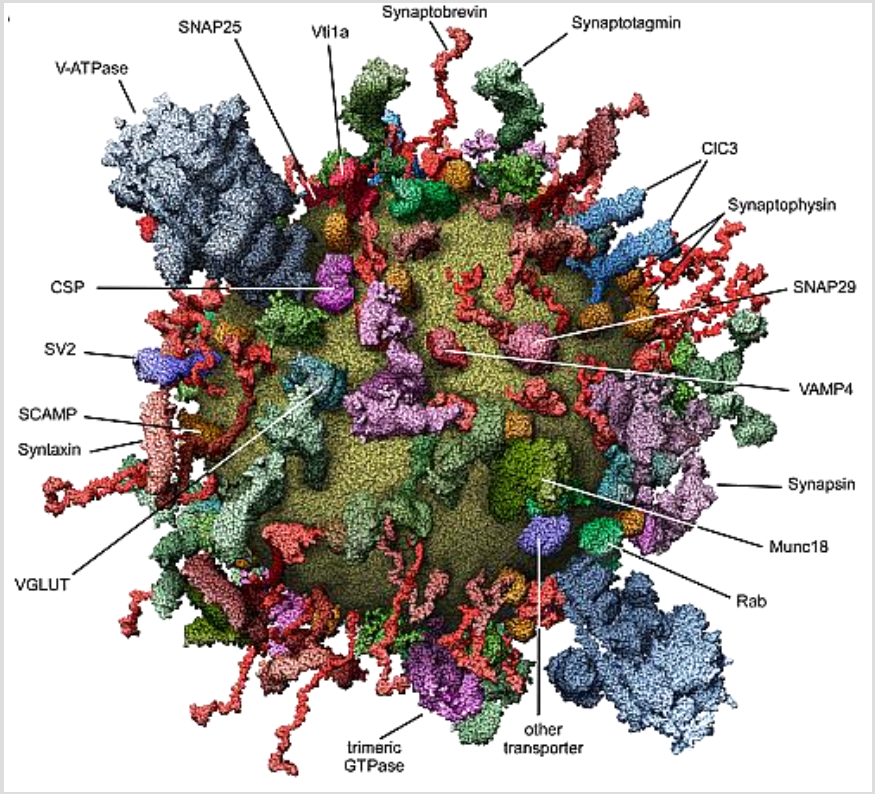


Fig. 1.1. Model of synaptic vesicle. 3-dimensional pictorial representation of synaptic vesicle representing various proteins. Fig adapted from Takamori et al., 2006.

According to Ting and Philips 2008, the RRP constitutes about 1-2%, the recycling pool 10-20% and the reserve pool of 80-90% when vesicle pools are expressed as percentages of the total synaptic vesicle cluster. RRP is the pool of vesicles that are available immediately on stimulation, and can be defined as those that are docked and primed at the active zone, rendering them fusion competent to be able to fuse upon arrival of an action potential (Schikorski et al., 2001, Rizzoli and Betz 2005). The second type of pool is the recycling pool, as the name implies, these vesicles are recycled repeatedly upon physiological stimulation. The recycling pool and readily releasable pool of vesicles are rapidly retrieved by endocytosis upon fusion with the presynaptic plasma membrane. Reserve pool is the third and the largest among the pools that do not contribute to neurotransmitter release.

Exocytosis is a tightly regulated process that involves multiple intermediate steps in the release of neurotransmitter from synaptic vesicles present in the presynaptic boutons. A large number of proteins have been implicated to play a role and

regulate various intermediate steps of the synaptic vesicle release machinery (SNAREs, Munc18s, SNARE proteins, SNAP25, Syntaxin, Synaptotagmin, Complexins, RIMs, and Munc13s). Exocytosis involves three intermediate steps-docking, priming and fusion.

Synaptic vesicles from the reserve pool are docked to the release sites of the presynaptic membrane in a process known as docking. Docking of synaptic vesicles at the active zone is mediated by Munc18-1, a member of SM family of proteins and Rab family of proteins (Voets et al., 2001a; Weimer et al., 2003). The latter have also been found to play a role in priming. Munc18-1 interacts with Syntaxin1 in its closed confirmation and disrupts the formation of a functional SNARE complex, important for vesicle fusion (Dulubova et al., 1999). Formation of a functional SNARE complex is mediated by the activation of syntaxin1 by Munc13 that disrupts the Munc18-1 and syntaxin1 interaction. Therefore, allowing Munc13 family of proteins to be essential priming components of the release machinery (Basu et al., 2005, Ma et al., 2011; Südhof 2012). Rab proteins are small GTPases that serve divergent functions from vesicular trafficking to the release of neurotransmitter. Rab proteins are involved in docking and priming through their interaction with RIM proteins (Rab3-interacting molecules). Members of the Rab protein family, Rab3 (Wang et al., 1997) and Rab27 (Fukuda et al., 2003) bind to a heterodimer of RIM and Munc13 (Dulubova et al., 2005) promoting an indirect role for Rab proteins in synaptic vesicle docking and priming (Südhof, 2012).

Docked vesicles are not yet fusion competent, hence they undergo a series of priming steps in order to be able to fuse rapidly in response to calcium influx. Several proteins are implicated to play a role in the priming process. Munc13, a mammalian homologue of Unc13 is an essential vesicle priming protein of active zones (Brose et al., 1995; Augustine et al., 1999). Fusion of a synaptic vesicle with its target membrane, following Ca^{2+} influx to release neurotransmitter into the synaptic cleft is a fundamental process essential to synaptic transmission. Many proteins are found to be involved in Ca^{2+}-triggered exocytosis of synaptic vesicles. In particular, three important proteins-soluble N-ethyl-maleimide-sensitive factor

attachment protein receptors (SNAREs), Synaptotagmin1 and Complexin have been found to be the core components of the release machinery.

SNARE proteins are the central components of membrane fusion machinery that share a 60 amino acid coiled-coil domain called SNARE motif (Weimbs et al., 1997) and SNARE proteins form extremely stable complexes when bound to each other through their SNARE-motifs. SNAREs can be classified into two types basing on their localization: v-SNAREs associated with vesicles and t-SNAREs, localized on the target membrane (Rothman 1994).

One of the critical steps of exocytosis is mediated by a SNARE complex, formed by two t-SNAREs-Synataxin1a and SNAP-25 (N-ethylmaleimide sensitive factor attachment protein-25), and a v-SNARE-Synaptobrevin/VAMP2 (Sutton et al., 1998). These three proteins of SNARE complex form a parallel four-helix bundle aligning vesicle and plasma membrane in a zippering fashion (Hanson et al., 1997). Munc13 promotes SNARE complex assembly by disrupting the interaction of the Munc18-Synataxin complex that is known to inhibit SNARE complex formation. In addition to SNARE proteins, Ca^{2+}-triggered neurotransmitter release is mediated by Synaptotagmin1, a Ca^{2+} sensor protein present on synaptic vesicles (Brose et al., 1992). It is believed that calcium bound to the C2-domains of Synaptotagmin1 mediate buckling of the active zone membrane with SNARE complex. This brings membranes together in a zippering fashion allowing the completion of the fusion process (Martens et al., 2007; Ting and Phillips, 2008).

Vesicle fusion and neurotransmitter release at the plasma membrane occurs by two modes. Neurotransmitter release from synaptic vesicles occurs either through complete fusion releasing all of its components into the synaptic cleft in a process known as full-fusion or full-collapse model. The other way in which the neurotransmitter release is via a small opening of a fusion pore in a process known as Kiss-and-run mode of fusion or flicker-fusion. The unaltered synaptic vesicles from kiss-and-run fusion are available for future use. The question of how kiss-and-

run fusion influences neurotransmitter release at CNS synapses is still under debate and is the topic of research yet to be resolved (Smith et al., 2008).

Endocytosis is an essential mechanism for the retrieval of synaptic vesicles to sustain synaptic transmission by refilling the vesicle pool and facilitating continuous release. During intense synaptic activity, vesicle release can reach maximum leading to the depletion of vesicles; hence vesicles are retrieved and recycled via endocytosis to maintain the process of neurotransmission. Depending on the synapse in the brain, the process of vesicle retrieval and recycling can occur by three modes i) Clathrin-mediated endocytosis ii) kiss-and-run, and iii) activity-dependent bulk endocytosis (ADBE) (Nyugen et al., 2014). Depending on the synaptic activity, different synapses in the brain adopt a different mode of retrieval. For example, kiss-and-run mode of endocytosis varies between small and large nerve terminals with an estimation of 80% in small nerve terminals like hippocampal boutons and around 3% at the Calyx of Held, although controversies still remain (Smith et al., 2008).

1.4. Active zone

Neurotransmitter release occurs at highly specialized area on the presynaptic plasma membrane called the active zone (AZ). The term active zone was first used by Couteaux and Pecot-Dechavassine in 1970.The regulated neurotransmitter release at the active zone involves a series of membrane trafficking events and the synaptic vesicle cycle. Each AZ is defined by the presence of an electron dense membrane specialization comprised of a meshwork of proteins called the cytomatrix of the active zone (CAZ, Dresbach et al., 2001; Schoch and Gundelfinger, 2006).

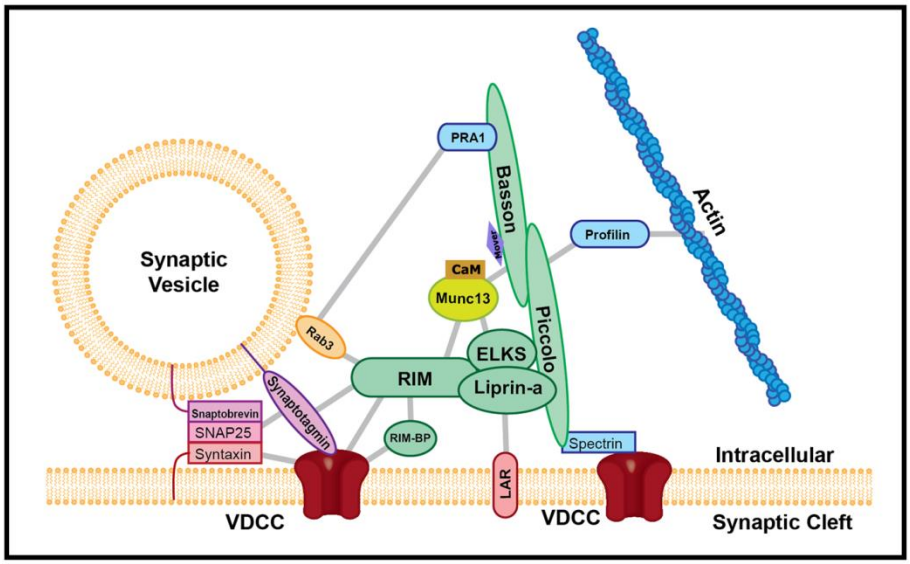


Fig.1.2. Synaptic vesicle protein machinery implicated in the neurotransmitter release process. Fig adapted from Mittelstaedt et al., 2010.

Five major multi-domain proteins-RIMs, Munc13s, CAST/ELKS proteins, Piccolo/Aczonin and Bassoon have been found at the CAZ. Bassoon and Piccolo/Aczonin are two large protein families that have been identified as vertebrate specific whereas the RIMs, Munc13s and CAST/ELKS proteins are found to be evolutionarily conserved (Gundelfinger and Fejtova 2012). The CAZ proteins form a supramolecular complex and regulate key steps of neurotransmitter release such as organization of synaptic vesicles into distinct pools (Rizzoli and Betz 2005), docking and priming of synaptic vesicles, recruitment of calcium channels to the release sites and tethering of vesicles and calcium channels to cell-adhesion molecules. (Südhof 2012)

RIMs or Rab3 interacting molecules are a family of multi-domain proteins (Wang et al., 1997 and Schoch et al., 2010) and the central elements of CAZ. RIMs mediate presynaptic functions and plasticity through their interaction with all other active zone proteins and presynaptic components (Wang et al., 2009;Schoch et al., 2010).RIM proteins are involved in two principal functions :i) docking and priming of synaptic vesicles and ii) recruitment and tethering of Ca^{2+} channels to the active zone. RIM binds to a homodimer of Munc13 thus promoting synaptic vesicle priming (Augustine et al., 1999). Knock-out studies from RIM1α resulted in severe

impairment of synaptic vesicle priming (Andrews-Zwilling et al., 2006). This impairment was rescued by the expression of monomeric Munc13 (Deng et al., 2011).

Earlier, an indirect interaction of RIM proteins to calcium channels was observed by binding to RIM-binding proteins (RIM-BPs) (Wang et al., 2000) that in turn bind to L-,N-, P/Q-type Ca^{2+} channels (Hibino et al., 2002). Later, a direct interaction of RIM with N- and P/Q-type Ca^{2+}channels was identified that is essential for recruiting ca2+ channels to active zones. Conditional knock-out mice lacking all RIM isoforms containing a PDZ domain exhibit a selective loss of Ca^{2+} channels at the active zones and are rescued by a RIM fragment with PDZ domain. (Kaesar et al., 2011).

Munc13-, a mammalian homologue of Unc13 is an essential vesicle priming protein of active zones (Brose et al., 1995; Augustin et al., 1999). Munc13 proteins promote synaptic vesicle priming by activating syntaxin1, a member of the SNARE complex essential for fusion (Ma et al., 2011, Südhof, 2012). Munc13-1 binds to Ca^{2+}-Calmodulin and mediates presynaptic short-term plasticity (Junge et al., 2004; Lipstein et al., 2013).Munc13 modulates neurotransmitter release by forming an interaction web with the active zone components Piccolo, Bassoon, CASTs and RIMs (Wang et al., 2009). Recent electron tomography studies on hippocampal organotypic cultures indicate that Munc13s not only participate in the priming process but also aid in the recruitment of synaptic vesicles and docking along with CAPS proteins and neuronal SNARES with the exception of Synaptotagmin or Complexin (Imig et al., 2014) .

Bassoon (tom Dieck et al., 1998) and Piccolo (Cases-Langhoff et al., 1996 are the two largest vertebrate specific proteins of the CAZ that are highly homologous and share ten so-called Piccolo-Bassoon homology domains (PBH). Most of the studies involving Piccolo deficient synapses do not show any major impairment in synaptic transmission, in contrast synapses lacking Bassoon or both Piccolo and Bassoon involve in regulating several stages of synaptic transmission. Moreover, they exhibit different functions at different synapses from small hippocampal synapses to large Calyx of Held synapses. Due to their large size of 420kDa (Bassoon) and 530kDa

(Piccolo), most of the studies were done using partial knock-out or knock-down strategies using RNA interference.

In addition to Piccolo-Bassoon homology domains, Piccolo consists of two C2 domains (different from others) and one of the C2 domain binds to Ca^{2+}, suggesting an indirect role for Piccolo in the process of Ca^{2+}-mediated exocytosis which is yet to be resolved (Gerber et al., 2001, Südhof, 2012). Hair cell ribbon synapses lacking Bassoon show a reduction in the number of calcium channels, reduced neurotransmitter release and an impairment in vesicle replenishment thus suggesting a role of Bassoon in organizing Ca^{2+} channels and vesicles to release sites at ribbons and promoting vesicle replenishment (Frank et al., 2010).Disruption of Bassoon at end bulbs of Held synapses resulted in a reduction of the readily releasable pool size and in the rate of vesicle replenishment. In addition, increase in vesicular release probability and postsynaptic densities suggesting a role of Bassoon in homoeostatic plasticity and promoting vesicle replenishment (Mendoza et al., 2013). Piccolo and Bassoon have various functions like maintaining synaptic vesicle clustering and synapse integrity via ubiquitination (Waites et al., 2013; Mukherjee et al., 2010).

Bassoon and Piccolo are vertebrate-specific proteins that are possibly adding vertebrate-specific features to the presynaptic nerve terminals. Exogenous expression of full-length and deletion constructs of Bassoon did retain their presynaptic targeting capacity in cultured hippocampal neurons (Dresbach et al., 2003). The C-terminal region of Bassoon with a stretch of glutamine residues also showed presynaptic targeting in cultured hippocampal neurons. This led to the idea that the presynaptic targeting of C-terminal region of Bassoon (aa3263-3938) could be due to its binding to other yet unknown proteins. In the process of identifying interacting partners of Bassoon, Mover, a novel also vertebrate specific protein, was identified in yeast 2-hybrid assay using the C-terminal region of Bassoon as bait (Kremer et al., 2007).

1.5. Presynaptic targeting of proteins

Little is known about the targeting of proteins to the presynaptic membrane. The proteins synthesized in the cell body are targeted to various components of synaptic vesicles and participate in various phases of the synaptic vesicle cycle either alone or by interacting with other proteins. The proteins associated with the synaptic vesicle cycle are recycled during endocytosis suggesting the need for sorting mechanisms that generate highly organized synaptic structures at the presynaptic plasma membrane. Each protein of the synaptic vesicle may undergo selective sorting consisting of several signals - mediating its targeting to the TGN, the plasma membrane and endosomes. Having several signals mediating the targeting of these proteins, makes understanding the mechanisms underlying their sorting more complicated and difficult to characterize. Furthermore, no common targeting information, sequence or motif is known to be involved in the sorting of proteins. Moreover, the targeting signals involved in polarized trafficking of SV proteins are more complicated to understand. The targeting of proteins to synaptic vesicles along the axon may rely on protein-protein, protein-lipid interactions and post-translational modifications
(Bonanomi et al., 2006).

1.6. Modulation of neurotransmitter release

Synaptic plasticity, the central and key element of information processing between neurons in the brain is characterized by the activity-dependent changes in strength of synaptic connections that can be long-term or short-term. Synaptic plasticity can be divided into pre- and post-synaptic plasticity. Considerable progress has been made in elucidating the mechanisms of post-synaptic plasticity. The complex synaptic vesicle release machinery at the presynaptic terminal makes it difficult to understand the molecular and cellular mechanisms underlying presynaptic plasticity.

One possible approach to studying presynaptic plasticity is to determine the mechanisms in neurotransmitter release. Neurotransmitter release is modulated through release probability of readily releasable pool of vesicles or though regulated

exocytosis. The latter, in turn is modulated through second second-messenger mediated signaling pathways resulting in phosphorylation of proteins by protein kinases, involved in the exocytotic release machinery (Leenders and Sheng, 2005). Protein phosphorylation plays an important role both in pre-and post-synaptic plasticity. Several proteins that are involved in multiple steps of synaptic vesicle cycle are also regulated by phosphorylation and the best studied and characterized example is Synapsin. Phosphorylation of Synapsin results in its dissociation from SVs in the reserve pool and mobilizing SVs to the active zone for exocytosis (Hosaka et al., 1999). Phosphorylation of proteins is mediated by different protein kinases such as ca^{2+}-Calmodulin dependent protein kinase II (CaMKII), protein kinase A (PKA) and protein kinase C (PKC).

Ca^{2+}-triggered neurotransmitter release occurs via exocytosis of synaptic vesicles with an influx of Ca^{2+}, upon arrival of an action potential. The

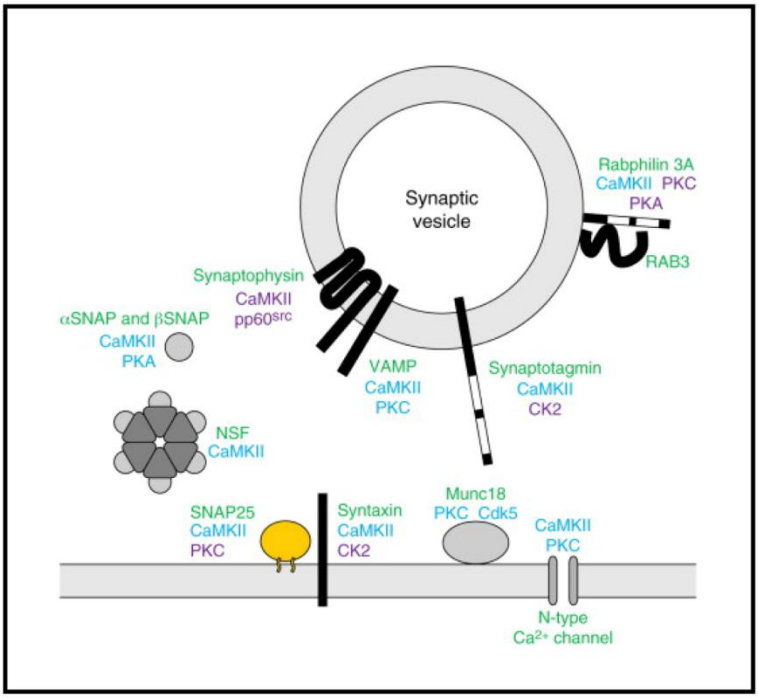


Fig 1.3. Phosphorylation of proteins implicated in exocytosis. Phosphorylation of synaptic vesicle proteins, proteins of the SNARE complex, calcium channels by CaMKII or PKC or PKA alone or in combination. Adapted from Turner et al., 1999.

release of neurotransmitter from vesicles occurs with a certain probability known as release probability. The release probability can be defined as the probability with which a number of vesicles are released immediately in response to an action potential (Leenders and Sheng, 2005). The release probability depends on several factors such as the size of the readily releasable pool,Ca^{2+}-sensitivity of release, vesicular release and the responsiveness of the release machinery to Ca^{2+}influx(Subramanian, 2011).

1.7. Ca^{2+} and Calmodulin in the brain

Calcium ions play essential roles in many cellular processes including transcription control, neurotransmitter release, muscle contraction and cell survival. Calcium ions form a gradient across the membrane being at higher concentrations extracellular and with low intracellular concentrations. Many proteins are involved in Ca^{2+}-mediated signaling process altering their activity in response to the availability of free Ca^{2+}, but the interaction of proteins with Ca^{2+} is indirect and modulated by Ca^{2+}-binding proteins. One such protein is Calmodulin (CaM), highly conserved and best studied among the E-F hand family of Ca^{2+}- sensing proteins that has been implicated to participate in many cellular functions.

Calmodulin (CaM) is an E-F hand family member of Ca^{2+}- sensing proteins that comprises of four E–F hands. Calmodulin consists of an N-terminal globular domain connected by a short linker to a C-terminal domain. Both N- and C-terminal domains each, have a pair of E-F hands accommodating four Ca^{2+} ions. The two domains of CaM adopt different conformations in the presence or absence of Ca^{2+}. The N-terminal domain of apo-CaM adopts a closed conformation in the absence of Ca^{2+}while the C-terminal adopts a semi-open conformation exposing hydrophobic methionine residues to interact with target proteins. Binding of Ca^{2+} changes both domains of CaM to produce an open conformation leading to structural rearrangements exposing hydrophobic residues accessible to target proteins (Chin and Means, 2000).The target proteins can be divided into two groups of calcium-dependent and calcium-independent binding proteins.

Most of the Ca^{2+}-dependent CaM interacting proteins - are neuronal proteins. Nevertheless the Ca^{2+}sensor essential for neurotransmitter release is Synaptotagmin (Brose et al., 1992). However, Calmodulin- aids in regulating the recruitment of synaptic vesicles through the activation of Ca^{2+}- Calmodulin dependent protein kinases (CaMKs), ultimately leading to the phosphorylation of vesicle proteins. Phosphorylation of proteins is an important process that modulates presynaptic changes and plasticity.

1.8. Aims of this study

Mover is a novel vertebrate-specific protein of 266 amino acids (apparent molecular weight 30KDa) (Kremer et al., 2007. Mover has also been identified in SV proteomic analysis as SVAP-30 (synaptic vesicle associated protein-30) (Burre et al., 2006; Böyken et al., 2013) Evident from subcellular fractionation experiments showing the association of Mover with synaptic vesicles (Kremer et al., 2007; Ahmed et al., 2013). The Mover gene shares 49% amino acid homology with the Tprg gene that is expressed in skin and also is hence also known asTprgl1 (Tprglike-1) (Antonini et al., 2008).

Over expressed recombinant Mover in neuronal and non-neuronal cells, forms aggregates due to its partial solubility. There are no predicted homology domains to study except for a predicted HSac2domain (aa53-163) and a Calmodulin binding site (aa206-218) identified through database analysis. However, the C-terminus of Mover is highly conserved in all vertebrates, while its N-terminus is less well conserved. Mass spectrometry studies of presynaptic proteins detected a threonine phosphorylation site at amino acid 13 (Munton et al., 2007). Data base analysis suggests additional predicted phosphorylation sites at aa14 (Serine), aa64 (Threonine), aa 257 (Tyrosine) and aa221 (Serine).

Mover is a presynaptic protein that exhibits differential expression among synapses in the brain, raising the possibility that Mover may account for the presynaptic functional heterogeneity (Kremer et al., 2007). This is also evident from the differential expression of Mover at a subset of end bulbs of Held, in the auditory brainstem (unpublished data).

In order to understand the role and function of Mover, it is essential to identify the functional domains of Mover. The aims of the current study were to: 1) generate a Mover Knock-out mouse line and 2) characterize the functional domains of Mover and their role in

1) Subcellular localization
2) Dimerization/Oligomerization
3) Binding interactions with Munc13, Calmodulin and Bassoon
4) Synaptic vesicle recycling.

2. Materials and Methods

2.1. Materials

2.1.2. Chemicals for biochemistry

Reagents	Company	Catalogue number
Calmodulin Human	Enzo	BML-SE325-0001
CaMKII	Enzo	BML-SE470
CaMKII	England Biolabs	P6060L
Gelcode Blue protein stain	Thermo Scientific	24594
Cheluminate HRP femto detect plus	Applichem	A7879,0200
Western Bright Chemilumiscence Substrate Sirius	BioZym	541021
Glutathione Sepharose	GE HealthCare	17-5132-01
Protein A/G agarose	Pierce	PI-20423
L-Gluthione Reduced	Sigma	G4251-109
Nonidet P-40	Applichem	A1694,0250
Thrombin	Sigma	T6884-100UN
Lysozyme from Chicken	Sigma	62970-1G-F
Protease Inhibitor cocktail tablets EDTA-free	Roche	04693132001
MES SDS Running buffer	Life Technologies	NP0002
MOPS SDS Running buffer	Life Technologies	NP0001
Gel Blot Paper	Omni Lab	GB003
Ficoll	Applichem	A2252,0100
Novex 4-12% Bis-Tris Gels	Life Technologies	NP0335BOX
Immunoblot PVDF membrane	Bio-Rad	162-0177

2.1.3. Buffers

10X Laemli buffer

Tris	29g
Glycine	144g
SDS	10g

Western Blot Buffer

Dis.H2O	760ml
Methanol	200ml
25X Transfer Buffer	40ml

Make the day before

1X TBST

Tris HCl	0.02M
NaCl	0.137M
Tween 20	0.1%

Blocking reagent 4%

4 gm of milk powder in 100ml of TBST
Concentration can vary from 1% to 5%.
Instead of milk powder 10% FCS can also be used.

Sample Buffer

10% SDS	20ml
DTT	1.542g
1M Tris (PH: 6.8)	6ml
Glycerin	10ml
Bromophenol Blue	5mg

Make it to 50ml.

Transfer Buffer 25X/500ml

Tris	18.2g
Glycine	90g

2.1.4. Reagents for cell culture

Solution	Company	Catalogue no
Dulbecco's Modified Eagle's Medium	Invitrogen,	41966-029
Neurobasal Medium	Invitrogen,	21103-049
Hanks buffered salt solution	Invitrogen,	14170-088
Fetal Calf Serum (FCS)		
OPTIMEM	Invitrogen,	31985-047
Trypsin-EDTA 0.05%	Invitrogen	25300
Trypsin 2.5%	Invitrogen	15090-046
Pen strep		15140-122
B27 supplement		17504-044
L-Glutamine 200mM		25030-024
PEI	Sigma	482595
poly L-lysine	Sigma	P4832
Lipofectamine 2000	Invitrogen	
PEI for transfection	Sigma	
1X Dulbecco's PBS	Invitrogen	14190-094
1X Distilled water	Invitrogen	15230-089

2.1.5. Drugs

Chemical	Company	Catalogue no
Tetrodotoxin	Tocris Bioscience	1069
4-AP	Sigma	275875-1G
Forskolin	Sigma	F6886
MG-132	Sigma	M7449-200UL

2.1.6. Reagents for molecular biology

Reagent	Company	Catalogue no
CIP	England Biolabs	M02905
Restriction endonucleases	England Biolabs	----------
Gene ruler 1Kb DNA Ladder	Thermo scientific	SM0311
Gene ruler 100bp DNA ladder	Thermo scientific	SM0241
Pageruler Prestained protein Ladder	Thermo scientific	26616
DreamTaq Green PCR Master Mix (2x)	Thermo scientific	K0161
Midori Green	Nippon Genetics	MG04

2.1.7. Kits

Kit	Company	Catalogue no
Plasmid Endo-free Maxi Kit	Qiagen	12362
PCR and Gel Purification Kit	Promega	A9281
BCA assay kit	Thermo scientific	23227
Spectra Por Dialysis membranes	Omni Lab	132650
Spin-X-UF Protein Concentrator	Corning	431487
Nexttec DNA extraction Kit	Biozym	391025N

Equipment	Company
SDS-PAGE	Bio-Rad
Glass-Teflon potter	Omni Lab
Sterile hood for cell culture work	Thermo Electron
	Zeiss
Metamorph analysis software	Visitron Systems,
Inverted Fluorescence Microscope	
Centrifuges	Eppendorf
Ultracentrifuges	Beckman Coulter,
KS-15 control bacterial shaker device	

2.1.8. Cell lines and bacterial strains

Eukaryotic cell lines

HEK 293T: Human embryonic kidney tumor cell line, additionally expressing "SV40 large T-Antigen", capable for DNA-replication of episomal plasmids

E.coli XL1 Blue

Rosetta Electro competent cells

2.1.9. List of primary antibodies

Antibody	Company		Western	ICC
Rb Mover	Synaptic Systems	248003	1:2000	1:1000
Rb Synapsin 1/2	Synaptic Systems	106002		1:2000
Ms MAP2	Synaptic Systems	188011		
Gp MAP2	Synaptic Systems			1:1000
Ch MAP2	Biosensis	C-1382-50		1:6000
Ms Synaptophysin	Sigma	S5768	1:2000	1:1500
Rb GFP	Synaptic Systems	132002	1:2000	1:1000

Ms Bassoon	Enzo	SAP7F407		1:1000
Ms Myc	Calbiochem	OP10		
Ms GFP	Thermoscientific	MA5-15256		
Ch GFP	Abcam	13970		1:2000
Rb β-3-tubulin	Synaptic Systems	302302	1:2500	
Rb VAMP2	Synaptic Systems	104202		1:1000
Gp VGLUT1	Synaptic Systems	131004		1:1000
Gp VGAT	Synaptic Systems	135304		1:1000
Ms GFAP	Synaptic Systems	173011		1:1000
Ms Calmodulin	Merck Millipore	05-173		1:2000
Ms CaMKII	Abcam	22609		1:1000
CaMKII-P	Merck Millipore	101004		1:1000
Gp Synaptophysin	Synaptic Systems	101004		1:1000
Ms Synaptotagmin	Synaptic Systems	105311		1:100

2.1.10. Secondary antibodies

Immunofluorescence

Secondary antibodies coupled to alexa flour 488, Cy3 and 647 at a concentration of 1:1000 were used to stain neurons and HEK cells appropriately and as required.

Western blots

Anti-Mouse and anti-Rabbit Horse-radish peroxidase at a concentration of 1:10000 was used to probe western blots

2.1.11. List of Plasmid DNA's

pEGFPC-1	available in the lab
pEGFPC-1-Helix Long (53-266)	cloned personally
pEGFPC-1-Helix Short (53-253)	Genscript
pEGFPC-1-HSac	cloned personally
pEGFPC-1-DE2	cloned personally
FL-Mover- pEGFPN-1	cloned personally
52-266- pEGFPN-1	cloned personally
91-266-pEGFPN-1	cloned personally
52-253- pEGFPN-1	Genscript
53-163- pEGFPN-1	Genscript
ΔExon2 (93-151)- pEGFPN-1	Genscript
pEGFPC-1-90	available in the lab
pEGFPC-1-180	available in the lab
pEGFPC-91-180	available in the lab
pEGFPC-181-266	available in the lab
pEGFPC-91-266	available in the lab
T13D- pEGFPN-1	Genscript
T13A- pEGFPN-1	Genscript
T13A-T64A- pEGFPN-1	Genscript
T13A-Y257F- pEGFPN-1	Genscript
T64A- pEGFPN-1	Genscript
Y257- pEGFPN-1	Genscript
yEFP-Synapsin	Genscript

Mover-myc	available in the lab
GFP-Mover (Ku)	available in the lab
GFP-Mover	available in the lab
GFP-VAMP	available in the lab
Synaptophysin mOrange	Available in the lab
Synaptophysin-mOrange-pHluorin	Provided by Dr. Camin Dean
EGFP-Munc-13	available in the lab
Complexin-1, 2, 3 and 4	provided by Dr. Kerstin Reim
F206R-mGFP	Genscript
GFP-F206R	Genscript
GFP-4-Mutation	Genscript
GST-Calmodulin	available in the lab
GST-Mover	available in the lab
GST	available in the lab
GST-F206R	Genscript
GST-4-mutations	Genscript
GST-HL-F206R	Genscript
GFP-HL-F206R	Genscript

2.2. Experimental methods

2.2.1. Neuronal cultures

Coverslips-

Cover slips were sterilized by autoclaving at 180°C for 2 hours. These cover slips were distributed into 24-well plate, rinsed once with 1X DPBS and coated with PEI (rat cultures) and PLL (mouse cultures) for 2 hours at 37°C in the incubator. PEI was removed by three washes with 1X DPBS and stored in the incubator.

Preparation of primary hippocampal cultures

1x Dulbecco's PBS (Gibco)
1x Hank's Buffered Salt Solution (Gibco)
1x trypsin solution (Sigma)

Plating Medium:
1x Dulbecoo's Modified Eagle Medium (DMEM), Invitrogen
10% (v/v) Fetal Calf Serum (FCS)
1% (v/v) Penicillin/Streptomycin
2 mM Glutamine

Growth Medium:
1x Neurobasal Medium,
B27 supplement
0.5 mM Glutamine
1% (v/v) Penicilline/Streptomycin

Hippocampi were dissected from E19 rat embryonic brains and trypsinized for 20 mins at 37°C with 0.25% trypsin. After trypsinization, the hippocampi were washed three times with HBSS and triturated through 20G and 25G needles for three times each, in order to dissociate the neurons. The dissociated neuronal suspension was then passed through the filter to remove the residual tissue. The cells were counted using trypan blue exclusion method to distinguish the viable and non-viable cells.

The cell suspension and trypan blue were mixed at 1:1 ratio and the resultant mixture was put on the Haemocytometer to count the cells. Viable cells do not take up dye and appear as white spots. The cells were diluted with plating medium to have a low-density culture of 50.000 cells/well and plated on to the PEI coated cover slips in a 24-well plate. All the cells were maintained at 37°C with 5% CO2. The plating medium was replaced with growth medium the following day.

Primary cortical neurons

Cortices from the hippocampal preparation of E19 embryos were collected and chopped into small pieces with a scissor after removing meninges. The same protocol is followed as described above for hippocampal cultures. The dissociated cells were plated at 1 million cells per well of a 6-well plate coated with PEI. Drug treatment experiments were done using 1 million cells per well of a 6/well plate.

Mover knock-out mouse hippocampal cultures

P0 litter from heterozygous Mover mice were used for cultures after genotyping. The same protocol is followed for both hippocampal and cortical mouse neuronal cultures as described for rat embryonic cultures. Mouse hippocampal neurons were plated at 50,000 cells/ well and cortical neurons at 80,000 cells/well of a 24-well plate.

2.2.1.2. Transfection

Calcium phosphate transfection

Primary cultured hippocampal neurons were transfected using calcium phosphate transfection method (for long-term expression) on 3DIV. First the DNA-CaCl2 mixture was made accordingly:

DNA-CaCl2 mixture for 3 Coverslips

DNA-3µg
CaCl2-5.6µl
Make up to 45µl with Gibco distilled water

Then add 45µl of transfection buffer slowly and drop wise with gentle mixing. Store the mixture for 20-30 mins at RT. During this incubation, the medium on the neurons was exchanged with pre-warmed Opti-MEM and placed back into the incubator at 37°C and 5% CO2. Removed growth medium is stored in a petri-dish and placed into the incubator for later use. Add 30µl of the transfection mixture per well of a 24-well plate and the neurons were placed back into the incubator for 75 mins. Neurons were washed by exchanging the medium with 1ml of pre-warmed neurobasal once, followed by 750µl twice. Finally the medium was exchanged with stored growth medium.

Lipofectamine transfection

Primary cultured hippocampal neurons were transfected using lipofectamine transfection method (for short-term expression). Firstly Opti-MEM and growth medium were pre-warmed at37°C in the water bath.

DNA-lipofectamine mixture for 3 coverslips

Epi 1. 75µl of pre-warmed Opti-MEM+3µl of lipofectamine reagent were added and mixed well
Epi 2. 75µl of pre-warmed Opti-MEM + 3µg of DNA were added and mixed well
Mix both 1 and 2 and incubate for 30 mins at RT.

The cultured medium from the neurons is exchanged with pre-warmed growth medium. The cultured medium is stored in a petri-dish and placed it in incubator for later use. Add 50µl of the transfection mixture per well of a 24-well plate and the neurons were placed back into the incubator for 75 mins. Neurons were washed by exchanging the medium with 1ml of pre-warmed neurobasal once, followed by 750µl twice. Finally the medium was exchanged with stored growth medium.

2.2.1.3. Immunocytochemistry

Primary antibody buffer
10% 10X PBS
10% FCS
5% Sucrose
2% BSA
0.3% Triton X-100

Secondary antibody buffer
10% 10X PBS

5% Sucrose
2% BSA
0.3% Triton X-100

Mounting medium
Mowiol with DABCO
2.4g Mowiol
6g Glycerol

Mix by vortexing. 6 ml of H_2O was added and mixed well, incubated for 2 hrs at RT. 12ml of 0.2M Tris pH 8.5 was added and heated at 50°C for 10 mins. Centrifuge at 5000g for 15 mins. A final concentration of 2.5% DABCO was added, aliquot and stored at -20°C.

Eukaryotic cells and primary hippocampal neurons (14DIV or 7DIV) are fixed with 4% PFA in PBS for 20mins at RT. Cells were washed three times with 1X PBS for 5 mins each. Cells were permeabilized and blocked for 30 mins-1 hour using primary antibody buffer. After blocking, respective antibodies diluted in primary antibody buffer were added and incubated overnight at 4°C. Following day, the cells were washed three times of 10 mins each with 1X PBS and incubated with secondary antibodies coupled to Alexa 488, Alexa Cy3 or Alexa 647 diluted at 1:1000 in secondary antibody buffer for 30mins-1 hour at RT in the dark. Further washes were done with 1X PBS for three times of 10 mins each and the cover slips were mounted using mounting medium containing Mowiol. Mounted cover slips were air-dried in the dark and used for examination.

2.2.1.4. Synaptotagmin 1 antibody uptake assay

10X Depolarization buffer

640mM NaCl
700mM KCl
20mM $CaCl_2$
10mM $MgCl_2$
200mM HEPES pH 7.4
300mM Glucose

Synaptotagmin 1 is an integral membrane protein of synaptic vesicles that serves as a calcium sensor in the process of vesicular trafficking and exocytosis, thus linking calcium influx during depolarization to neurotransmitter release. Upon Ca^{2+} binding, Synaptotagmin triggers exocytosis of synaptic vesicles. Antibodies raised against luminal domain of Synaptotagmin are used to label recycling synaptic vesicles in live cultured neurons. Cultured rat hippocampal neurons were co-transfected on 3DIV with either with mGFP, Mover-mGFP or 52-266-mGFP along with Synaptophysin mOrange. Following expression, Synaptotagmin antibody uptake was performed on 14DIV by adding 200µl of the depolarization buffer containing Synaptotagmin antibody at a dilution of 1:100, incubated at RT for 90secs. Neurons were washed with pre-warmed neurobasal medium for two times and third time for 5 mins in the incubator. Following the third was in the incubator for 5 mins; neurons were fixed with 4% PFA, stained with anti GFP and incubated overnight at 4°C. Following day, the cells were washed with 1X PBS for three times of 10 mins each. Secondary antibodies coupled to alexa flour 488 against rabbit GFP and alexa 647 for mouse Synaptotagmin.

2.2.1.5. Drug treatment and protein extraction

Hypotonic buffer

10 mm Tris-HCl
pH 7.4

Homogenization buffer

320mM sucrose
1mM EDTA
10mM Tris-HCl
pH 7.4

Fourteen DIV and twenty one DIV hippocampal neurons growing in 6-well plates at a density of 100,000cells/well were treated with 50 µm forskolin for 8 h, 50 µm MG132 for 8 h, 1 µm TTX for 48 h, 4-AP-50µM for 30 h or in control conditions. Neurons were washed twice with PBS and 1 ml of hypotonic buffer was added per well for 5 min. Cells were harvested in 0.5 ml of homogenization buffer by scraping and passed through a 25-gauge needle 10 times. A final concentration of 150 mm NaCl was added to the lysate. Cell lysates were centrifuged at 4000 rpm for 10 min to pellet nuclei and cellular debris. The supernatant was collected and the protein concentration determined by Nano Drop. 100 micrograms of total protein per lane was resolved by SDS-PAGE and analyzed by immunoblotting for Mover (30 kDa band) and β-3-tubulin as a control (55kDa band).

2.2.2. Maintenance of HEK293T cell lines

1x Dulbecco's PBS (Gibco)
1x trypsin-EDTA solution (Sigma)

Growth Medium:
1x Dulbecoo's Modified Eagle Medium (DMEM), Invitrogen
10% (v/v) Fetal Calf Serum (FCS)
1% (v/v) Penicillin/Streptomycin

HEK293T cells were grown in T100 flasks at 37°C with 5% CO2. Cells were washed once with 1X DPBS and trypsinized with 2ml of 1X trypsin-EDTA solution for 1-2 mins on the bench in the sterile hood. Detached cells were collected by adding 10 ml of pre-warmed growth medium and sedimented at 1000rpm for 5mins.Cell pellet

was resuspended by adding 10ml of pre-warmed growth medium to have a single cell suspension.

Freezing and thawing of HEK293T cells

Freezing Medium

1x Dulbecoo's Modified Eagle Medium (DMEM), Invitrogen with
20% FBS and
10% DMSO

HEK293T cells were grown in T75 flasks at 37°C with 5% CO2. Cells were trypsinized as described and cell pellet was resuspended in 500µl of growth medium. Resuspended cell suspension and the freezing medium were added at 1:1 ratio, transferred into 2ml cryovials and transferred incubated at -80 °C overnight in isopropanol-surrounded plastic tanks to assure a slow decrease in temperature. The following day, cryovials were transferred into a liquid nitrogen tank for long-term storage.

Thawing of HEK293T Cells

Cells frozen in liquid nitrogen were quickly thawed at 37 °C in a water bath. Cells were then transferred into a 15ml falcon tube with 10 ml fresh growth medium and sedimented at 1000rpm for 5mins to remover residual DMSO. Sedimented cells were then resuspended in 10ml of growth medium and plated onto the 10cm dishes or T75 flask as required. Cells were further maintained and cultured as described.

Transfection of HEK293T cells

HEK293T cells plated on 10cm cell culture dishes were used for transfected with PEI transfection method.

Transfection mixture for one 10cm dish

22.5µg of DNA

67.5µl of PEI

make it to 1ml with pre-warmed OptiMEM medium

Transfection mixture was mixed gently and incubated for 20-30 mins at RT.1ml of the transfection mixture was added to the dish and incubated at 37°C from 30 mins-2hrs in the incubator. Cells were washed by exchanging the medium with 10 ml of pre-warmed growth medium.

2.2.3. Biochemistry

2.2.3.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

10X Running buffer/1 litre

Tris	29g
Glycine	144g
SDS	10g

Proteins were separated using SDS-polyacrylamide gel electrophoresis where in SDS is an anionic detergent that imparts a negative charge to the linearized proteins. The amount of SDS bound is proportional to the molecular weight of proteins and migrate towards anode. The smaller the percentage of acrylamide the larger the size of the proteins resolved. The different percentages of gels used to separate proteins are tabulated below.

Resolving gel				Stacking gel	
	8%	10%	12%		5%
H_2O	2.31ml	1.98ml	1.65ml	H_2O	3.4 ml
Acrylamide	1.34 ml	1.67 ml	2 ml	Acrylamide	850µl
1.5M Tris pH 8.8	1.27 ml	1.27 ml	1.27 ml	1.0M Tris pH 6.8	625µl
10% SDS	50µl	50µl	50µl	10% SDS	50 µl
10% APS	34µl	34µl	34µl	10% APS	50 µl
TEMED	3.4µl	3.4µl	3.4µl	TEMED	10 µl

Western blotting

Proteins separated through polyacrylamide gel electrophoresis are transferred on to either PVDF or nitrocellulose membrane for detection through antibodies.

Western blot transfer

Transfer buffer

Tris buffered saline 25X	40 ml
Methanol	200 ml
make it to 1 liter with water	

Tris buffered saline 25X

Tris	18.2g
Glycine	90g

Semi-dry transfer was performed for all the experiments. For semi-dry transfer, the gel and activated PVDF membrane were sandwiched between two blotting pads on each side. Transfer was performed at 64mA for 1one hour at RT.

Western blot detection

1X TBST

Tris HCl	0.02M
NaCl	0.137M
Tween 20	0.1%

After transfer, membranes were blocked with 4% milk in TBS (for pre-cast Bis-Tris gels) or 10% FBS in TBS (for poured gels) for an hour at RT to avoid unspecific binding of antibodies. After blocking, the membranes were probed with respective antibodies diluted in blocking buffer overnight. Following day, the membranes were washed three times with 1X TBST for 10 mins each. The membrane is incubated with secondary antibody conjugated to Horse-radish peroxidase at a dilution of 1:10000 in 1X TBST for 1 hour at RT (for poured gels) or 4% milk in 1X TBST at 4°C for 1 hour (for pre-cast gels). The membrane was washed three times of 10 mins each with 1X TBST at RT. For the detection of proteins, CheLuminate-HRP FemtoDetect Plus was used, that utilizes the reaction of luminol and Hydrogen

peroxide catalyzed by HRP. The membrane was incubated in CheLuminate-HRP Femto detects solution for 5 mins in the dark and exposed to hyper film ECL (BioZym) for the visualization of proteins.

Stripping and reprobing the western blot

Stripping buffer-100 ml

SDS 10%	20ml
Tris HCl pH 6.8, 0.5M	12.5ml
Ultra-pure water	67.5ml

Add 0.8 ml ß-mercaptoethanol under the fume hood.

Add the buffer to a small plastic box which has a tight lid. Use a volume that would cover the membrane. Add the membrane and incubate at 50°C for up to 20 minutes with some agitation. Rinse the membrane under running water tap for 2-3 times. Traces of ß-mercaptoethanol will damage the antibodies. Wash extensively for 5 minutes in TBST for twice and then go for blocking.

2.2.3.2. Antibody characterization

Several versions of Mover antibodies were present in the lab. To detect the best working antibodies, two approaches were used-one is western blotting and the other immunofluorescence, Cell lysates expressing GFP-Mover, Mover-mGFP and TPRG-mGFP were separated by SDS-PAGE and transferred onto PVDF membranes. The separated proteins were immunoblotted with the several versions of Mover antibodies. Using immunofluorescence, 14 DIV rat embryonic cortical and hippocampal neurons were fixed and stained with Mover antibodies to detect endogenous mover, and HEK293T cells over-expressing GFP-Mover, Mover-mGFP and TPRG-mGFP were fixed and stained with Mover antibodies to detect the specificity of Mover antibodies. All the antibodies were used at different dilutions starting from 1:500-1:3000.

2.2.3.3. Brain homogenates

Homogenization buffer

0.32 M sucrose
1 mM NaHCO3

To make brain homogenates from rats, wild type and knock-out mice, one cortex was transferred to glass Teflon homogenizer and homogenized using 1.5 ml of homogenization buffer with freshly added protease inhibitor cocktail and Benzonase (sigma). Homogenization is performed by giving 10 strokes at 1200rpm on ice. The protein concentration was determined by BCA assay.

2.2.3.4. Determination of Protein concentration by BCA assay

Protein concentrations were determined using BCA (Bicinchoninic acid) assay kit (Pierce). Under alkaline conditions, Bicinchoninic acid protein assay relies on the formation of a Cu^{2+} protein complex followed by reduction of the Cu^{2+} to Cu^{1+} (purple-blue complex). The amount of reduction is proportional to the amount of protein present. The assay was performed using the manufacturer's protocol.

2.3.4.5. Synaptosomal preparation

Sucrose buffer

320mM sucrose
5mM HEPES
Protease inhibitors (PMSF, Final concentration 200mM/Pepstatin (Final concentration 1µg/ml)

4%, 6% and 13% ficoll were made in sucrose buffer.

Prepare the non-continuous ficoll gradient in SW41 tube, from bottom to top: 4ml 13%, 1ml 9%and 4ml 6% (w/v) ficoll. The 9% and 6% ficoll has to be added very gently to avoid mixture of them. At the end you have to be able to see two interface

bands between different concentrations. One gradient per rat brain is used. Decapitate rats (5-6 weeks old) and remove cortex into ice cold sucrose buffer and homogenize in 30 ml (for 2 rat brains) sucrose buffer at 900rpm for 9 strokes. Centrifuge at 5000 rpm for 2 min at 4°C, in fixed angle SS34 rotor to pellet the cell debris. Decant supernatants in to new SS34 tube (**S1**) and re-centrifuge at 11,000rpm for 12 min at 4°C. Resuspend pellet (leaving the dark brown part, **P2**) in 6ml (3 ml for each gradient) sucrose buffer and collect them in 15 mL Falcon tube. Load 3 ml of resuspended pellet onto a Ficoll gradient with pre-cut 1 ml pipette tips. Centrifuge at 22,500 rpm, 35 min and 4°C in SW41 rotor. Isolate bands at the interfaces between 13% and 9% Ficoll. Dilute the bands with 30 ml sucrose buffer containing pepstatin/PMSF in SS34 tubes. Centrifuge for 12 min in SS34 at 11 000rpm, 4°C to wash the traces of ficoll. Resuspend pellet in 5 ml sucrose buffer containing pepstatin/PMSF (2.5 ml per cortex).

2.2.3.6. Binding interactions

Cell Lysis and Co-Immunoprecipitation

Lysis Buffer

50 mM Tris-HCl pH 8.0
150 mM NaCl
2 mM EDTA
0.5% NP40
EDTA free complete protease inhibitor cocktail (Roche)

Cells expressing the protein/s of interest were lysed using appropriate volume of lysis buffer with freshly added protease inhibitor cocktail. The cells were scraped, collected into eppendorf tubes and incubated on ice for 20-30 mins. After incubation, the cell lysate was centrifuged at 4°C for 10 mins and 12000rpm to remove the cell debris. The supernatant is transferred into fresh eppendorf tubes and used as required.

The cell lysate (reaction volume of 400µl) was incubated with 4µl of anti-Myc antibody and incubated at 4°C for 6-8 hours on the shaker. 30µl of protein G

Sepharose beads were equilibrated by adding 1ml of lysis buffer, centrifuged at 3000rpm for 1 min at 4°C for three times. Equilibrated beads were then added to the cell lysates with pre-incubated antibody and incubated for 1 hour to overnight at 4°C on the shaker. The sepharose beads with the bound proteins and antibody were washed three times with 1 ml of lysis buffer at 3000rpm for 1 min each. 30µl of 2X sample buffer is added to the samples and boiled at 95°C for 5 mins. The samples were analyzed by SDS PAGE and western blotting.

Immunoprecipitation of Mover with Calmodulin

Lysis Buffer

50 mM Tris-HCl pH 8.0
150 mM NaCl
4 mM CaCl2
1% NP40
EDTA free complete protease inhibitor cocktail (Roche)

Wash Buffer

50 mM Tris-HCl pH 8.0
150 mM NaCl
4 mM CaCl2
0.1% NP40

4-12% Bis-Tris pre-cast gels (Invitrogen)

20X MES SDS running buffer

50 mM MES
0 mM Tris Base
0.1% SDS
1 mM EDTA
pH 7.3

HEK 293T cells grown on 10cm dish were transfected using PEI with GFP-Mover. Cells were lysed 48 hours post transfection. The cell lysate (reaction volume of 400µl) was incubated with 20µg of recombinant human Calmodulin (Enzo) in the presence of 4mM Ca^{2+} over night at 4°C. 4µl of anti-GFP antibody was added and incubated at 4°C for 6-8 hours on the shaker. 30µl of protein G Sepharose beads

were equilibrated by adding 1ml of lysis buffer, centrifuged at 3000rpm for 1 min at 4°C for three times. Equilibrated beads were then added to the cell lysates with pre-incubated antibody and incubated for 4 hours at 4°C on the shaker. The sepharose beads with the bound proteins and antibody were washed three times with 1 ml of wash buffer in the presence of Ca^{2+}; centrifuged at 3000rpm for 1 min each. 30µl of 2X sample buffer is added to the samples and boiled at 95°C for 5 mins. The samples were analyzed by SDS PAGE and western blotting. After separation, the proteins were blotted on to PVDF membrane (0.2µm) and fixed with 0.2% glutaraldehyde. PVDF membrane with fixed proteins was blocked using 4% milk solution in TBS and probed with anti-GFP and anti-Calmodulin antibodies.

Interaction of Mover on Munc13 and Calmodulin complex

Lysis Buffer

50 mM Tris-HCl pH 8.0
150 mM NaCl
2 mM CaCl2
1% NP40
EDTA free complete protease inhibitor cocktail (Roche)

Wash Buffer

50 mM Tris-HCl pH 8.0
150 mM NaCl
2 mM CaCl2
0.1% NP40

4-12% Bis-Tris pre-cast gels (Invitrogen)

20X MOPS SDS running buffer

50 mM MOPS
50 mM Tris Base
0.1% SDS
1 mM EDTA pH 7.7

HEK 293T cells grown on 10cm dish were transfected using PEI with Munc13-EGFP. Cells were lysed 48 hours post transfection. The cell lysate (reaction volume of 600µl) was incubated with 30µg of recombinant human Calmodulin (Enzo) in the presence of 2mM Ca^{2+} over night at 4°C. Mover-Myc expressing HEK 293T cells

were lysed as described above and 50µl was added to the Munc13-Calmodulin complex, incubated overnight at 4°C. 5µl of anti-GFP antibody was added and incubated at 4°C for 6-8 hours on the shaker. 30µl of protein G Sepharose beads were equilibrated by adding 1ml of lysis buffer, centrifuged at 3000rpm for 1 min at 4°C for three times. Equilibrated beads were then added to the cell lysates with pre-incubated antibody and incubated for 4 hours at 4°C on the shaker. The sepharose beads with the bound proteins and antibody were washed three times with 1 ml of wash buffer in the presence of Ca^{2+}; centrifuged at 3000rpm for 1 min each. 30µl of 2X sample buffer is added to the samples and boiled at 55°C for 10 mins. The samples were analyzed by SDS PAGE using 4-12% Bis-Tris gel and 1X MOPS running buffer from Invitrogen. After separation, the proteins were blotted on to PVDF membrane (0.2µm) and fixed with 0.2% glutaraldehyde. PVDF membrane with fixed proteins was blocked using 4% milk solution in TBS and probed with anti-GFP, anti-Mover and anti-Calmodulin antibodies.

2.2.3.7. Expression and purification of GST-Mover

Transformation of chemo-competent E. coli

LB-medium
Chemo-competent XL1-BLUE, Rosetta
Bacterial agar plates

Chemo-competent cells were thawed on ice for 15 mins. 0.5-1µg of DNA was added to a 100µl cell aliquot and incubated for 30 Min on ice. Cells were heat shocked for 45s at 42 °C and immediately cooled on ice for 1 min. 200µl LB medium was added and cells were incubated for 45-60 Min at 37 °C on a horizontal shaker. The bacterial-DNA mixture was plated on bacterial agar plates and incubated over night at 37 °C.

Transformation of electro competent E.coli

Electro-competent cells were thawed on ice for 15 mins. 0.5-1µg of DNA was added to a 40µl cell aliquot and transferred into ice-cold cuvette. Cells were treated by electric shock using a micropulser (Bio-Rad) and 1ml 0f SOC medium was added, and incubated for 45-60 min at 37 °C on a horizontal shaker. 200µl of bacterial-DNA mixture was plated on agar plates with appropriate antibiotic and incubated over night at 37 °C.

Liquid cultures of bacteria

Small scale liquid cultures

A single colony was inoculated into 3 ml LB medium supplemented with the appropriate antibiotic and incubated over night at 37 °C and 250rpm in a shaker. The bacterial culture was used for small scale DNA preparation (Mini Prep).

Large scale liquid cultures

For large scale DNA preparation (Maxi Prep) and protein purification, a single colony was inoculated into 2-3 ml of LB medium supplemented with appropriate antibiotic to prepare a starter culture and incubated for 6-8 hours at 37°C and 250rpm in a shaker. Starter culture was inoculated into 200 ml LB-medium supplemented with the appropriate antibiotic, and incubated over night at 37 °C and 250rpm.

Purification of GST-Mover

LB medium

Rosetta 2 BL21 competent cells

IPTG

Lysis buffer

50mM Tris-HCl
10mM NaCl
10mM EDTA
1.5% TritonX-100
pH 7.5
Protease inhibitors

Elution Buffer

20mM HEPES

150mM potassium acetate
10mM Glutathione
5mM Magnesium acetate
1mM EDTA
1mM DTT
pH 9.5

Transform Rosetta 2 BL21 cells with GST-Mover using electroporation transformation protocol as descried earlier. A single colony was inoculated into 5ml of LB medium, incubated over night at 37°C and 220rpm.Next day, inoculate the overnight culture into 500ml LB medium and shake at 37°C until an OD600nm of 0.5 is reached. Add 1mM IPTG and shake the culture at 20°C and 150rpm for 24h. Harvest the bacterial culture by centrifugation at 4°C and 4000rpm for 25 mins. Resuspend the bacterial pellet in 20ml of lysis buffer with freshly added protease inhibitors. Cells were disrupted by sonicating three times on ice for 15 sec with intervals. The cell lysate was centrifuged at 12000rpm for 70 mins. 1 ml of glutathione sepharose beads were equilibrated by adding 1ml of lysis buffer, centrifuged at 4000rpm for 5 min at 4°C for three times. Equilibrated beads were then added to the cell lysate and incubated overnight at 4°C on the shaker. The sepharose beads with the bound protein was washed three times with 5 ml of lysis buffer and centrifuged at 4000rpm for 5 min each. The glutathione sepharose-bound protein was eluted by adding 1-5 ml of elution buffer at RT for 30 mins-1 hr on a shaker and centrifuged at 4000rpm for 5 mins. The eluted protein is dialyzed against 1X PBS over night at 4°C using a dialysis membrane of MW cut off 6-8 kDa (Omni lab). The dialyzed protein is concentrated using a spin-X-UF concentrator (Corning) by centrifuging at 5000 rpm for 10 mins. The protein estimation is done using Nanodrop.

2.2.3.8. CaMKII phosphorylation assay

Reaction buffer

20 mM Tris-HCl (pH 7.5),
10 mM MgCl2,
0.5 mM DTT, 0.1 mM EDTA,
2.4 µM Calmodulin,

2 mM CaCl2,
100 µM ATP

To observe the effect of Mover on the phosphorylation of CaMKII (Ca^{2+}- Calmodulin dependent protein kinase) - a target of Calmodulin, an in vitro phosphorylation assay of CaMKII was performed with varied concentrations of Mover (from 1, 2, 5 and 10 molecules) and GST as a control. But the concentrations of CaMKII and Calmodulin were kept constant. The reaction was executed in the reaction buffer with 25 ng of recombinant CaMKII with 1, 5 and 10 molecules of purified GST-Mover to one molecule of recombinant human Calmodulin (Enzo), incubating the reaction at 30 °C for 30 min in the presence of 2mM Ca^{2+}. The proteins were concentrated using 50% TCA and suspended in 2 X SDS-PAGE sample buffers and incubated at 55 °C for 5 mins. The proteins were resolved by SDS-PAGE were transferred to PVDF membranes. Resolved proteins were detected by immunoblotting with respective antibodies- anti- Mover, anti-CaM and anti-CaMKII. Two CaMKII antibodies were used- one that detects phosphorylated CaMKII and the other dephosphorylated form of CaMKII.

2.2.4. Molecular biology

2.2.4.1. Cloning

cDNA's of Mover deletion constructs were synthesized by Genscript and provided in PUC vector. The cDNA's were digested with the appropriate restriction endonucleases (Fermentas Fast Digest and New England Biolabs) and the reaction incubated at 37°C from 30 mins-6 hrs depending on the requirement. The general digestion reaction was set up accordingly

Fast digest green buffer or NEB 10X	2µl
Restriction enzymes	1µl
DNA	10µg
Water	make it to 20µl

The cleaved products of DNA were analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis

The digested DNA samples were analyzed by agarose gel electrophoresis. Depending on the size of the cleaved products, an appropriate percentage of agarose is chosen as the separation is based on the concentration of the agarose. The smaller the percentage of agarose the larger the size of the DNA fragments resolved. Appropriate percentage of agarose is weighed and boiled with 1X TAE buffer in a microwave oven until a clear transparent solution is reached. Then 4-5µl of Ethidium bromide or Midori green is added to agarose that helps in visualizing the DNA fragments on the gel upon exposure to ultraviolet light. The required fragments of DNA are cut using a scalpel and transferred to eppendorf tubes for purification. The purification of DNA from agarose gel is done using a purification kit from Promega following manufacturer's protocol.

Ligation

Digested DNA was ligated into the appropriate linearized plasmid DNA by using T4-DNA ligase (NEB). T4-DNA ligase is an enzyme that catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA. The plasmid DNA and the insert DNA were used in the ratio of 1:2 to 1:5 respectively. Ligation reaction was done as follows:

Linearized vector	25ng
10X T4 DNA-ligase ligation buffer	1µl
insert DNA	100ng
T4-DNA Ligase	0.5µl
Nuclease-free water	to 10µl

The ligation reactions were incubated at 16°C overnight and heat inactivated the enzyme at 65°C for 10 mins. 1-5µl of the ligation mixture was used for transformation.

2.2.4.2. Mini and Maxi preparation of DNA

A Single colony was inoculated into 3 ml (mini) or 200 ml (maxi) LB medium with appropriate antibiotic and plasmid DNA was isolated using Qiagen endofree Mini prep or Maxi prep DNA isolation kit following manufacturer’s protocol.

2.2.4.3. Determination of DNA concentrations using spectrophotometer

Concentrations of DNA were measured using a spectrophotometer (Eppendorf). DNA was diluted to 1:20 in water for measurement. The purity of DNA was verified by looking at the absorbance ratio at 260nm/280nm that is supposed to be 1.8.

2.3. Generation of Mover knock-out mouse line

Conditional targeting vector harboring Exons 1 to 3 of Mover flanked by lox sites was generated by Polygene. The targeting vector was electroporated into 129Ola ES-cells. After screening, four ES cell clones harboring the conditional targeting vector of Mover were provided by Polygene. All the documentation provided by Polygene is maintained in the lab.

2.3.1. ES Cell culture and DNA extraction

ES cells were maintained on inactivated MEFs. First, MEFs were taken into culture with MEF medium. The confluent MEFs were inactivated using Mitomycin C for the maintenance of ES cells. Mover ES cell clones were taken into culture with inactivated MEFs. The confluent ES cells were split and plated onto a 6-well plate to extract DNA for genotyping.

MEFs into culture

MEF medium

500 ml	KO-DMEM	(GIBCO 10829-018)
95 ml	FCS	(HyClone SH30070-03)
6 ml	MEM	(GIBCO 11140-035)
6 ml	Glutamine	(GIBCO 25030-024)
6 ml	2-Mercaptoethanol	(SIGMA M7522) diluted! (7.2 µl 14 M - 99% - in 10 ml PBS, filter sterilize; PAA H15-002)
3ml	Pen/Strep	(GIBCO 15140-122)

T75 flasks precoated with 0.1% gelatin for 2 hrs at RT.

Wash cells once with 15 ml PBS. Add 2 ml of 0.05% Trypsin-EDTA (GIBCO 25300-054) and incubate for 5 min at 37°C. Stop digestion with 7 ml medium, triturate cells carefully (9 ml) and add 3 ml of cell suspension to each precoated T75 flask containing 12ml medium. Move T75 flask with MEFs to distribute the cells.

Inactivation of MEFs

MEFs were inactivated using Mitomycin C (SIGMA M0503-2MG) as follows: dissolve 2 mg Mitomycin C in 4 ml PBS (50x stock) and add 300 µl 50x Mitomycin C (final concentration: 1x) incubate for 2 hrs at 37°C and 5% CO_2. Wash cells twice with PBS and add MEF medium (15 ml), incubate at least for 2 hrs and then split inactivated MEFs using the protocol used for splitting MEFs and plate on to 6-well plates.

Maintenance of ES cells on inactivated MEFs

ES cell medium

500 ml	KO-DMEM	(GIBCO 10829-018)
95 ml	FCS	(HyClone SH30070-03)
6 ml	MEM	(GIBCO 11140-035)
6 ml	Glutamine	(GIBCO 25030-024)
6 ml	2-Mercaptoethanol	(SIGMA M7522) diluted! (7,2 µl 14 M - 99% - in 10 ml PBS, filter sterilize; PAA H15-002)
3 ml	Pen/Strep	(GIBCO 15140-122)
65 µl	LIF (ESGRO Millipore ESG 1107)	

Exchanged the MEF medium from inactivated MEFs with ES cell medium and four Mover ES cell clones were taken into culture.ES cells were confluent and have been split at 1:3 ratio as follows: wash the cells with 5 ml PBS. Add 1 ml 0.25% trypsin (GIBCO 25200-056) and incubate for 8 min at 37°C. Stop digestion by adding 5 ml ES cell medium triturate the cells carefully and distribute into the wells. Following day prepare ES cells for DNA preparation by trypsin digestion.

DNA preparation from ES cells

ES cells from the 6 well plate without MEFs were used for the extraction of DNA. Following protocol was followed: wash the cells with 5 ml PBS. Add 1 ml 0.25%trypsin and incubate for 8 min at 37°C. Stop digestion by adding 5 ml ES medium and triturate the cells carefully. Collect cell suspension and centrifuge at 800 rpm for 5 min. Resuspend cell pellet in 1 ml of ES cell medium and make two aliquots in E-cups. Centrifuge at 800 rpm for 5 min and DNA was extracted using the DNeasy® Blood & Tissue Kit from QIAGEN.

2.3.2. Microinjection, Mouse breeding and Genotype analysis

The amplified ES cell clones (2B11 and 1F10) were injected into blastocysts of C57/BL6 mice to generate chimeric mice. Genotyping was done to identify the chimerism by using PCR protocol I and II. The obtained chimeric mice were bred with Rosa Flip mice to remove the Frt-flanked neo expression cassette and verified by using PCR protocol III. At this stage the mice have a Mover gene that is flanked by loxp sites, hence called as 'flox mice'. These mice were genotyped for the presence of lox allele using the PCR protocol I. In order to obtain heterozygous Mover mice, the flox mice were bred with heterozygous Cre mice and the obtained progeny was verified for the Cre positive heterozygous Mover mice using the PCR protocol IV and finally the heterozygous mice were inbred to generate global Mover knock-out mice. Global Mover knock-out mice were verified by genotyping using a multiplex PCR protocol VI and V.

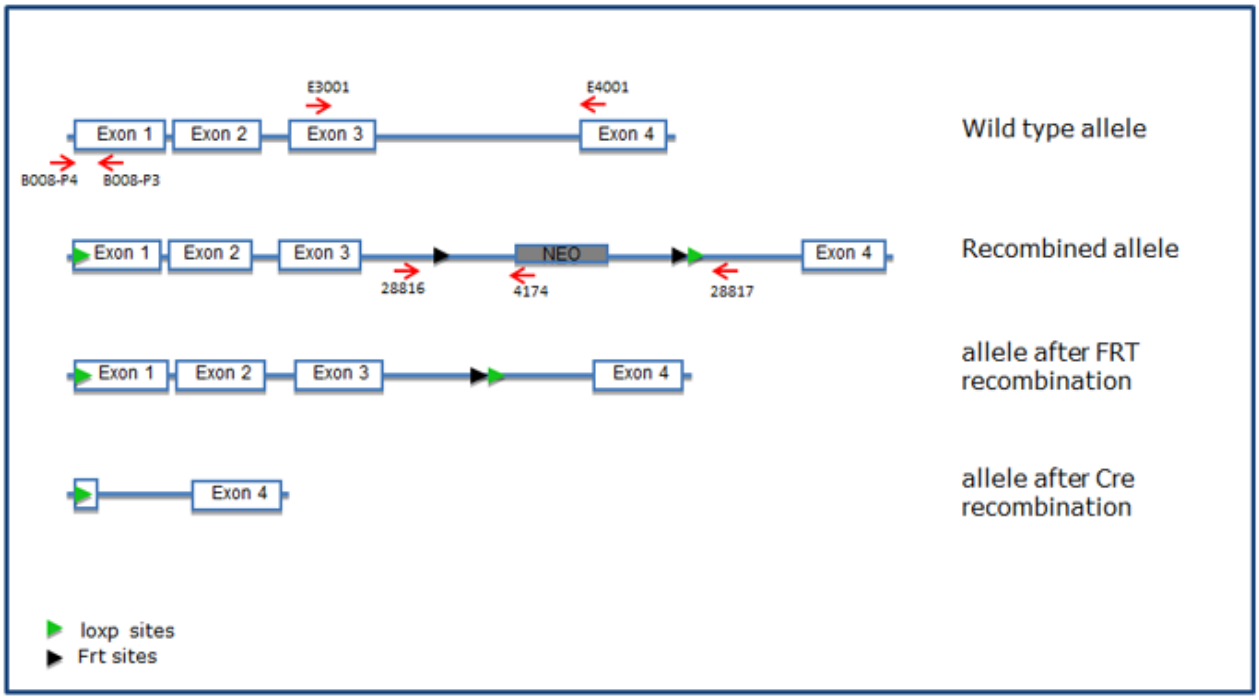


Fig.2.1. Schematic overview of the generation of Mover knock-out mice. Wild type and modified Mover gene with 5' and 3' loxp sites (in green triangles) and neo cassette flanked by Frt sites (in black triangles). Forward and reverse for genotyping are labeled.

PCR protocol I

To identify 5' lox sites			**PCR conditions**		
DMSO	1µl		95°C	03:00	
DNA	1µl		95°C	00:30	
B008-P3	1µl	5pmol/µl	55°C	00:30	
B008-P4	1µl	5pmol/µl	69.2°C	00:30	goto 2 for 40X
H2O	8.5µl		69.2°C	01:00	
2X MM	12.5µl		4°C	pause	
B008-P3GTAGGGTCGTGGACGTTCAG					
B008-P4CCAATCACAAGGCGAACGAG					
Expected bands-			**Wt allele**	**234bp**	
			Lox allele	**267bp**	

PCR protocol II

To identify 3’ lox sites			PCR conditions		
DMSO	1µl		95°C	03:00	
DNA	1µl		95°C	00:30	
28816	1µl	5pmol/µl	57°C	00:30	
28817	1µl	5pmol/µl	69.2°C	00:30	go to 2 for 40X
H2O	8.5µl		69.2°C	01:00	
2X MM	12.5µl		4°C	pause	
28816 GGATCCAGATTTAAGGCCTTG					
28817 CGACTCACTAGTGGGCAGATC					
Expected bands-			**Lox**	**213bp**	

PCR protocol III

To identify the presence and absence of neo			PCR conditions		
DMSO	1µl		98°C	03:00	
DNA	1µl		98°C	00:30	
28816	1µl	5pmol/µl	55°C	00:30	
4174	1µl	5pmol/µl	72°C	00:30	go to 2 for 35X
H2O	8.5µl		72°C	10:00	
2X MM	12.5µl		10°C	pause	
28816 GGATCCAGATTTAAGGCCTTG					
4174 CGCATCGCCTTCTATCGCCTTCTT					
Expected bands-			**Neo**	**619bp**	
			Without neo	**no band**	

PCR protocol IV

To identify the presence of Cre			PCR conditions		
DMSO	1µl		95°C	03:00	
DNA	1µl		95°C	00:30	
24365	1µl	5pmol/µl	61°C	00:30	
24366	1µl	5pmol/µl	72°C	00:30	go to 2 for 35X
H2O	8.5µl		72°C	07:00	
2X MM	12.5µl		4°C	pause	
24365GCG GTC TGG CAG TAA AAA CTA TC					
24366 GTG AAA CAG CAT TGC TGT CAC TT					
Expected bands-			**Cre positive**	**100bp**	
			Cre negative	**no band**	

PCR protocol V

To identify the presence of Wt and lox alleles			PCR conditions		
DMSO	1µl		95°C	03:00	
DNA	1µl		95°C	00:30	
B008-P3	1µl	5pmol/µl	55°C	00:30	
	1µl	5pmol/µl	69.2°C	00:30	go to 2 for 40X
H2O	8.5µl		69.2°C	01:00	
2X MM	12.5µl		4°C	pause	
B008-P3GTAGGGTCGTGGACGTTCAG					
B008-P4CCAATCACAAGGCGAACGAG					
Expected bands-			**Wt allele**	**234bp**	
			Lox allele	**267bp**	

Multiplex PCR protocol VI

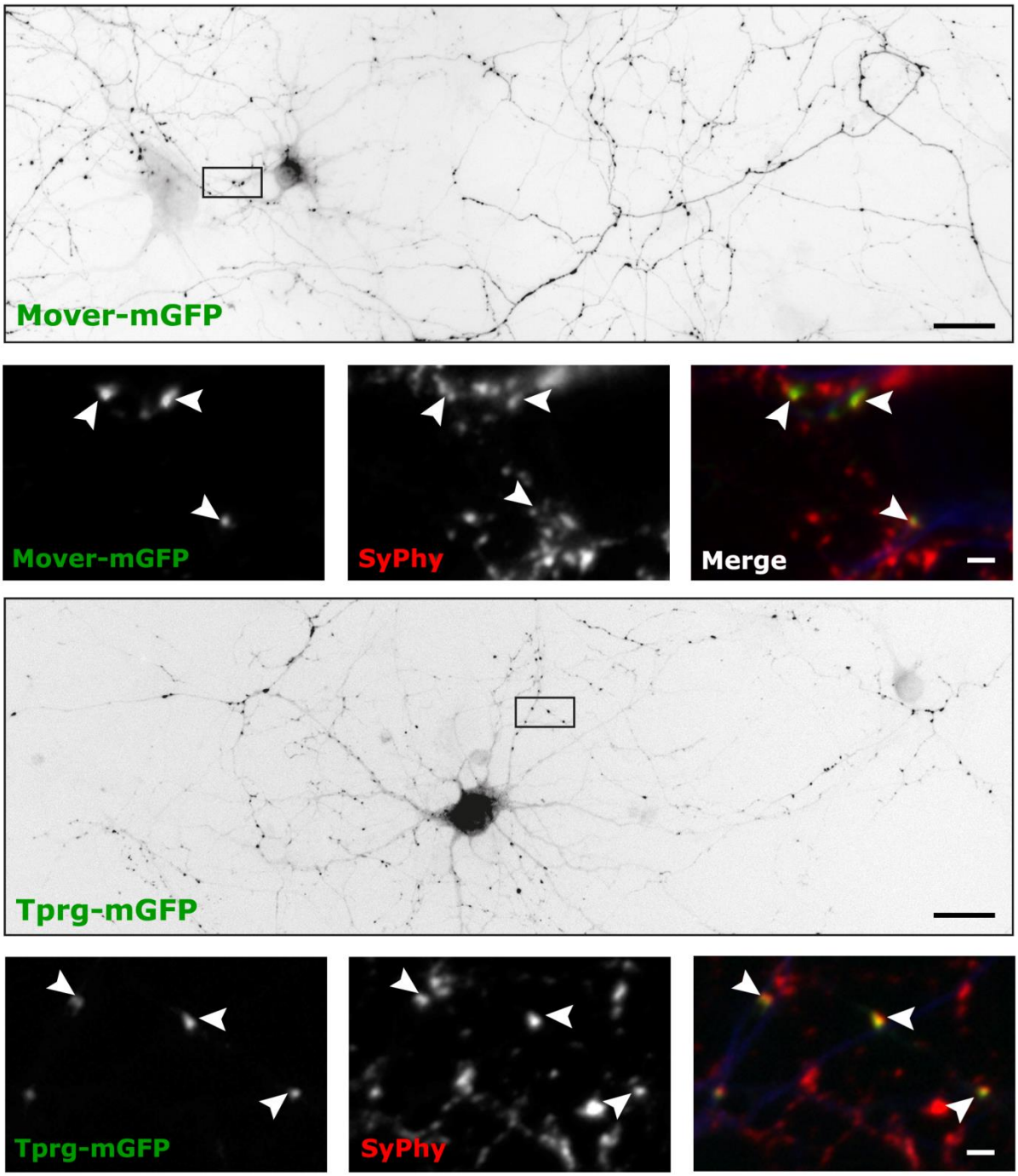
To identify the knock-out allele			PCR conditions		
DMSO	1µl		98°C	03:00	
DNA	1µl		98°C	00:30	
B008-P4	1µl	5pmol/µl	55°C	00:30	
E-4001	1µl	5pmol/µl	72°C	00:30	go to 2 for 35X
E-3001	1µl		72°C	10:00	
H2O	7.5µl		10°C	pause	
2X MM	12.5µl				
B008-P4 CCAATCACAAGGCGAACGAG					
E-3001 CATTCAGTGGGACAAGCAGA					
E-4001 CAAGGCTCTCCTGATCCAAG					
Expected bands-			**Wt**	867bp	
			Knock-out	697bp	
			flox	1106bp	
			heterozygous	867bp & 697bp	

3. Results

3.1. Tprg, a homologue of Mover

Mover shares homology with Tprg (Transformation-related protein 63regulated), a gene predominantly expressed in skin, and Mover is also known as Tprgl or Tprg1-like (*Antonini et al., 2008)*. Mover/Tprgl/Tprg1-like protein shares 49% amino acid identity with Tprg protein. Exogenous expression of Mover-mGFP produced a punctate pattern in 14DIV cultured hippocampal neurons. Unexpectedly, the expression of Tprg-mGFP also showed punctate fluorescence pattern in cultured neurons. Both recombinant Mover and recombinant Tprg punctae co-localize with endogenous Synaptophysin indicating their accumulation at synapses (Figure 3.1.1; A). Although, no expression of Tprg was reported in the brain, the recombinant version of Tprg was still targeted to presynaptic terminals. Because Mover has a strong tendency to dimerize Mover may also heterodimerize with TPRG, and this may affect presynaptic targeting of both proteins. I tested heterodimerization by a co-immunoprecipitation assay from Hek293T cell lysates co-expressing Mover-myc along with recombinant GFP-Mover or recombinant Tprg-mGFP. The proteins were immunoprecipitated with ant-myc antibody and analyzed by SDS-PAGE and western blotting. The western blot is probed for anti-GFP antibody that detected the GFP-tagged proteins of Mover and Tprg. Recombinant Mover and recombinant Tprg failed to heterodimerize *in vitro* (Fig.3.1.2; A). Failure to heterodimerize with Mover indicates that Mover and Tprg are two different proteins that may have different roles to play. I also used expression of recombinant Mover-mGFP and TPRG-mGFP in Hek293T cells to test the specificity of antibodies against these two proteins. Lysates from HEK cells expressing GFP-Mover, Mover-mGFP and Tprg-mGFP were analyzed by SDS-PAGE and immunoblotted against anti-GFP, anti-Mover and anti-Tprg antibodies. The antibodies showed specificity for respective recombinant proteins whereas anti-GFP antibody detected all the three recombinant proteins (Fig.3.1.2; B).

A.



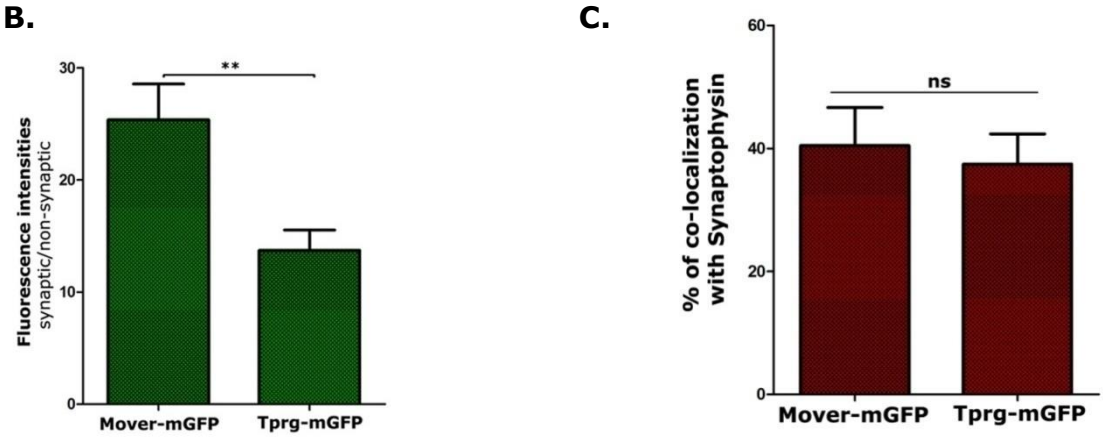
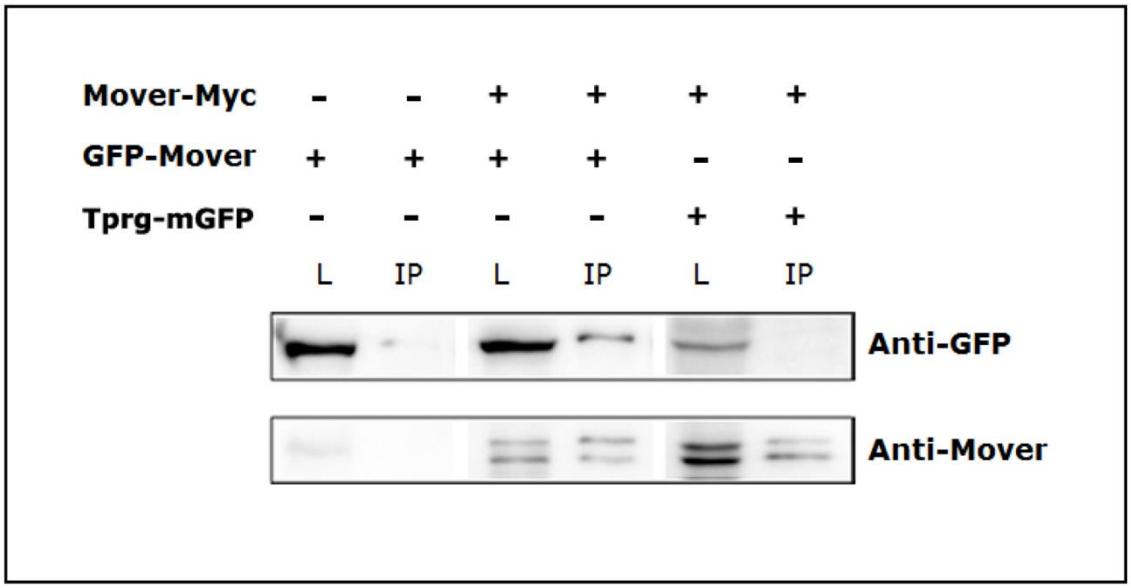


Fig.3.1.1. Mover-mGFP and Tprg-mGFP accumulate at presynaptic sites. Expression of C-terminal mGFP-tagged Mover and Tprg showing a punctate fluorescence pattern in 14DIV cultured hippocampal neurons. Inlay is shown as higher magnification images (left to right) of the construct (green), endogenous Synaptophysin (red) and merge (along with MAP2). Scale bar: 10µm for grey panel and 2µm for higher magnification images (A).Bar graph representing the enrichment of Mover-mGFP and Tprg-mGFP at presynaptic sites. Fluorescence intensities ratio were measured for Mover-mGFP and Tprg-mGFP synaptic puncta to the intervening axonal regions. Enrichment of Mover-mGFP at presynaptic sites is statistically significant from Tprg-mGFP.t-test. P≤ 0.005 (**). N=2 (B). Bar graph illustrates the percentage of co-localization of Mover-mGFP and Tprg-mGFP puncta with endogenous Synaptophysin. No statistical significance is observed in the co-localization of Mover-mGFP and Tprg-mGFP puncta with Synaptophysin (C).

Enrichment of Mover-mGFP and Tprg-mGFP at presynaptic sites was determined by measuring fluorescence intensities of synaptic puncta along the axon to the intervening axonal regions (synaptic versus non-synaptic). Presynaptic targeting capacity of Mover-mGFP showed a two fold increase to that of Tprg-mGFP (Fig.3.1.1; B). No statistical difference was observed in the percentage of co-localization with Synaptophysin between Mover-mGFP and Tprg-mGFP puncta (Fig.3.1.1; C).

A.



B.

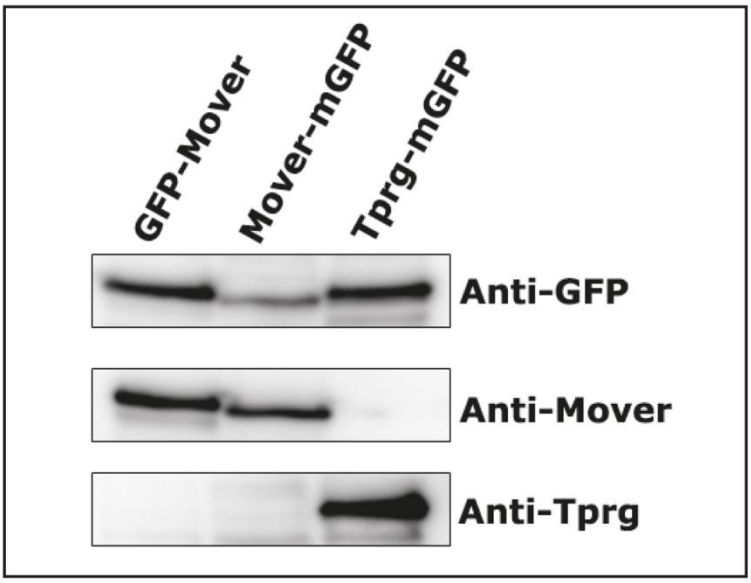


Fig.3.1.2. Tprg does not heterodimerize with Mover *in vitro*. GFP-Mover was immunoprecipitated with co-expressed Mover-myc from HEK293T cell lysates. Tprg-mGFP does not IP with Mover-myc. Protein complexes were immunoprecipitated using sepharose coupled antibodies against myc epitope .L= Total lysate, IP= immunoprecipitate.(A).Specificity of Mover and Tprg antibodies.HEK293T cells lysates expressing GFP-Mover, Mover-mGFP and Tprg-mGFP were analyzed by SDS-PAGE and western blotting. The blot was probed with anti-GFP, anti-Mover and anti-Tprg antibodies (B).

3.2. Presynaptic targeting of Mover

Mover has been identified as a Bassoon binding partner in yeast-2-hybrid assay, using C-terminal region of Bassoon as a bait (Kremer et al, 2007).It has been identified as a peripheral membrane protein associated with synaptic vesicles (Ahmed et al, 2013). Structurally and functionally nothing is known about Mover. Mover has no homology domains except for a HSac2 domain spanning aa53-163 and a predicted Calmodulin binding site between aa206-218. HSac2 domain belongs to family of inositol phosphatases (Minagawa et al., 2001) and functions as inositol phosphate-5 polyphosphatase. Inositol phosphatases are involved in several functions such as synaptic vesicle recycling and actin polymerization. Data base analysis suggests that the Mover gene is alternatively spliced producing two isoforms, a long isoform harboring exons 1-4 and a short isoform lacking exon2 (aaΔ93-151). In the current study, I aimed to characterize the functional domains of Mover mediating cellular properties such as presynaptic targeting, self-interaction and potentially affecting synaptic vesicle recycling. Expression of recombinant N-terminal GFP-tagged full length Mover generated aggregates in cultured hippocampal neurons (Appendix Fig.7.1), thus making it difficult to study. Therefore, full length constructs with C-terminal tags and several deletion mutants of Mover fused with either N-terminal GFP or C-terminal mGFP were generated to identify the domains required for presynaptic targeting and self-interaction of Mover. Fig.3.2. shows the list of all deletion mutants used in the current study.

In general, deletions were introduced to generate constructs encoding

- aa52-266 and aa91-266
- aa 52-253 and aa 53-163 (the latter encoding the HSac2 homology domain)
- a construct lacking aa 93-151 (deleting the region encoded by Exon2)

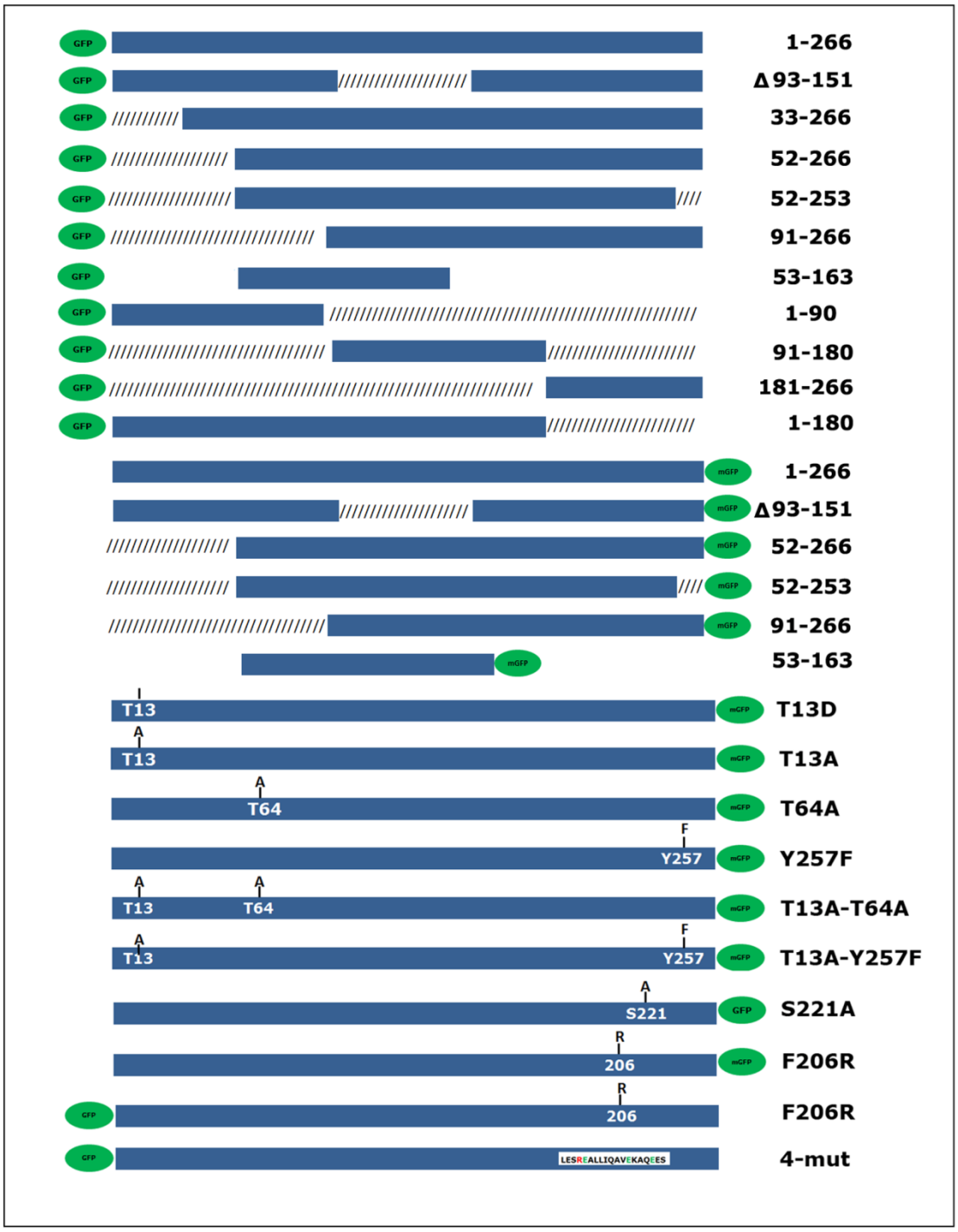


Fig. 3.2. List of Mover deletion mutants used in the current study. Schematic representation of full length and the deletion mutants of Mover fused with either N-terminal GFP or C-terminal mGFP used in the current study. Deletion constructs also include the predicted HSac2 (aa53-163) and a construct with a point mutation in the predicted Calmodulin binding site (aa206-218. F206R). In addition mutations at predicted phosphorylation sites threonine 13, 64 and tyrosine 257. Mover amino acid sequence is represented in blue boxes and the crosshatched boxes indicate deleted regions. Point mutations are indicated with amino acid number. The deletion mutants were labeled with corresponding amino acid deletions and are labeled adjacent to the amino acid sequence.

3.2.1. Localization of GFP-VAMP (Vesicle Associated Membrane Protein) to presynaptic sites.

VAMP or Synaptobrevin is a synaptic vesicle associated membrane protein that is part of the SNARE complex. The SNARE complex mediates fusion of synaptic vesicles with the plasma membrane. I used recombinant VAMP fused with N-terminal GFP as a typical example of presynaptic targeting in cultured hippocampal neurons. Expression of GFP-VAMP produced punctate fluorescence along the axon with minimal signal detected between intervening axonal regions (Fig 3.2.1; A). These puncta co-localize with endogenous Synaptophysin indicating the accumulation of GFP-VAMP at presynaptic sites.

A.

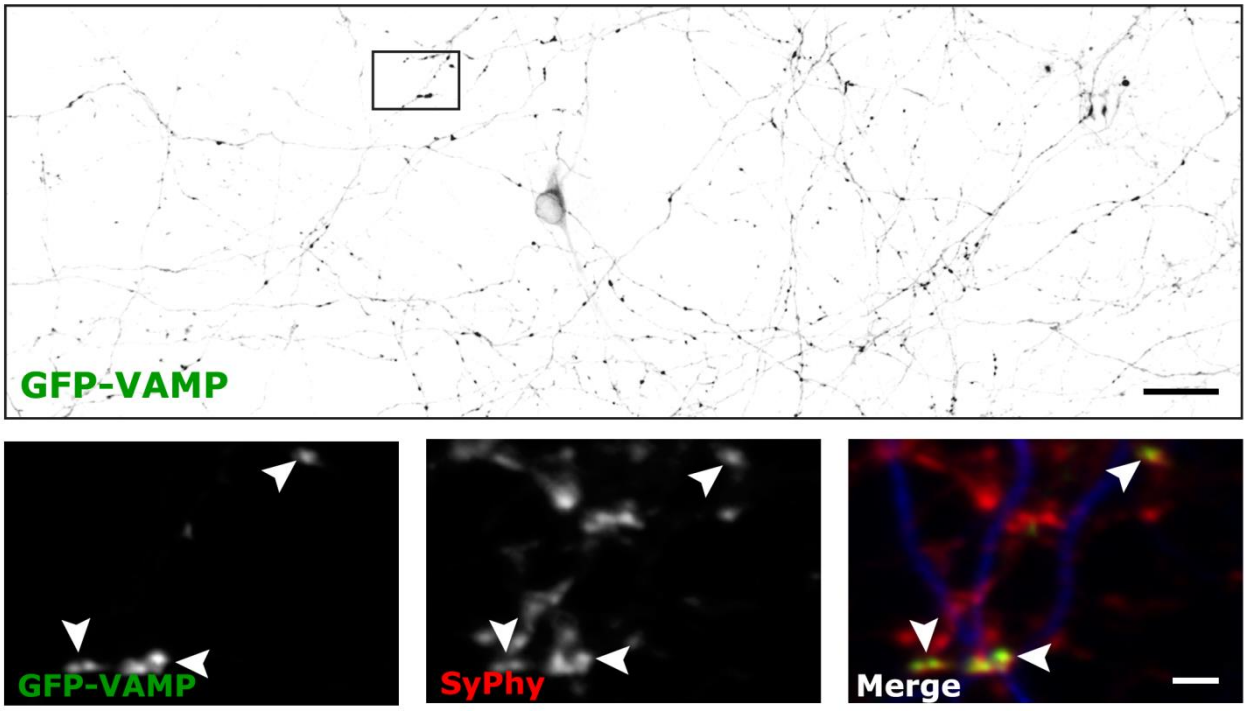
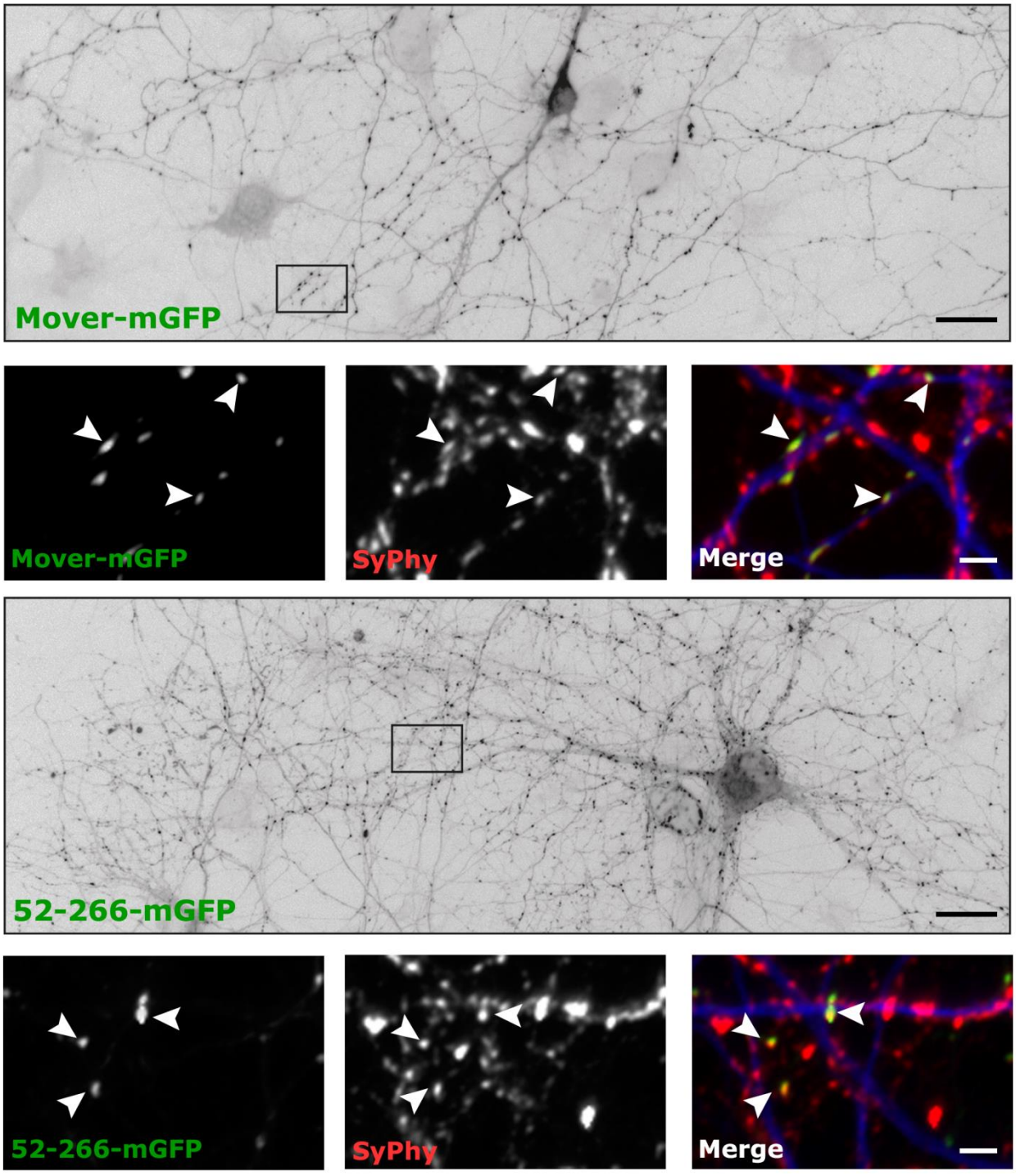


Fig.3.2.1. Localization of GFP-VAMP to presynaptic sites. Localization of GFP-tagged VAMP in 14DIV cultured hippocampal neurons and immunostained for endogenous Synaptophysin (SyPhy) (in red), a presynaptic marker and MAP2 (in blue), a dendritic marker. Higher magnification images of the inlay are shown in individual channels (from left to right) - over-expressed protein in green, Synaptophysin in red and a merge along with MAP2. GFP-VAMP puncta positive for Synaptophysin are shown with arrow heads. Scale bar: 10µm for white panel and 2µm for magnified images (A).

3.2.2. Localization of Mover deletion mutants in cultured hippocampal neurons.

The regions or amino acid sequences necessary for the presynaptic targeting of Mover were unknown. Expression of mGFP tagged full length recombinant Mover (Mover-mGFP) and the N-terminal deletion mutant of aa51 (52-266-mGFP) in cultured neurons produced punctate fluorescence along the axon. These puncta co-localize with endogenous Synaptophysin indicating the localization of Mover at presynaptic sites (Fig.3.2.2.1; A). In order to compare the presynaptic targeting of full length Mover-mGFP and 52-266-mGFP, fluorescence intensities of synaptic puncta and intervening axonal regions (non-synaptic regions) were measured using Metamorph image analysis software. Full length Mover-mGFP and 52-266-mGFP showed no significant difference in their targeting capacity to presynaptic terminals (Fig.3.2.2.1; B). No significant difference was observed in the percentage of co-localized Mover-mGFP puncta and 52-266-mGFP puncta with Synaptophysin (Fig 3.2.2.1; C).Co-localization analysis with Synaptophysin was performed using Puncta Analyzer program written by Bary Wark (Ippolito and Eroglu, 2010). Expression of several mGFP tagged deletion mutants each showed a diffuse distribution along the axon with weaker expression. These mutants included a construct encoding the predicted HSac2 domain (aa53-163), a construct lacking the part encoded by exon2, a construct lacking the N-terminal 91 amino acids, and a construct lacking both the N-terminal 51 and the C-terminal 13 amino acids (aa52-253). This indicates that N-terminal, C-terminal and central deletions are each required for targeting (Fig 3.2.2.2; and Fig. 3.2.2.3.) only the N-terminal 51 amino acids are not necessary for the presynaptic targeting of Mover. In particular, regions between aa52-90, aa253-266, aa53-163 (HSac2) and aa93-151 (exon2) are necessary for the targeting of Mover to presynaptic sites.

A.


Mover-mGFP
Mover-mGFP
SyPhy
Merge
52-266-mGFP
52-266-mGFP
SyPhy
Merge

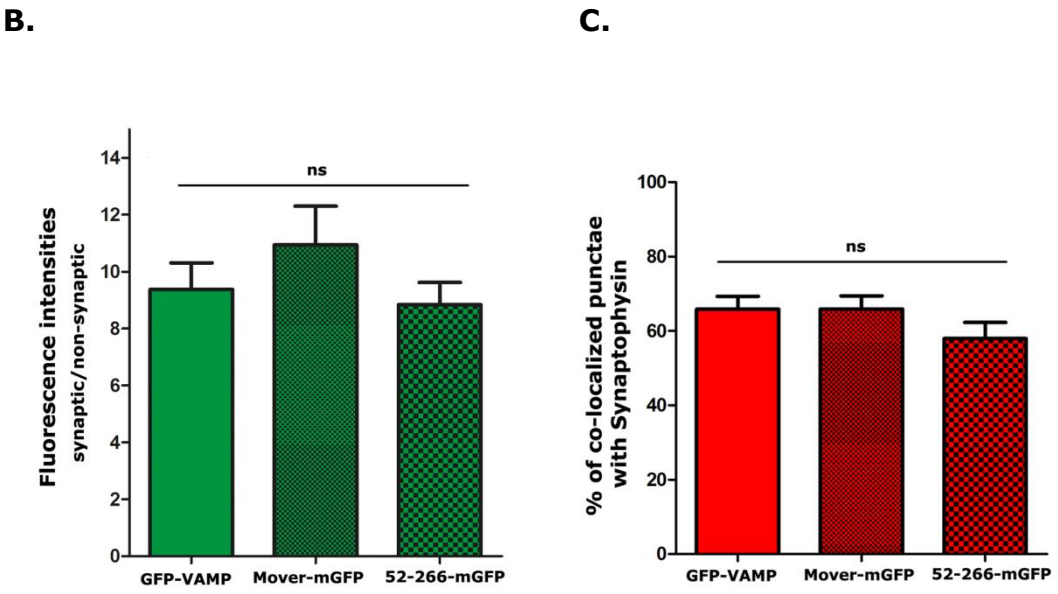


Fig.3.2.2.1. The deletion mutant 52-266-mGFP targets to presynaptic terminals. Expression of mGFP-tagged full length Mover and the deletion mutant lacking N-terminal aa51 showing a punctate fluorescence pattern in14DIV cultured hippocampal neurons. Mover-mGFP and 52-266-mGFP puncta co-localize with Synaptophysin (SyPhy). Inlay is shown as higher magnification images (left to right) of the construct (green), endogenous Synaptophysin (red) and merge image (along with MAP2 in blue). Scale bar: 10µm for grey panel and 2µm for magnified images (A) Presynaptic targeting of full length Mover and 52-266 deletion mutant of Mover. Average fluorescence intensities of the synaptic puncta were measured for full length Mover-mGFP, deletion mutant of aa51 (52-266-mGFP) and GFP-VAMP. No statistical difference in the targeting of Mover-mGFP and 52-266 deletion mutants at the presynaptic sites was observed (B). Co-localization analysis of full length and 52-266 deletion mutant of Mover with Synaptophysin in comparison to GFP-VAMP(C). No significant difference either in their targeting or co-localization with Synaptophysin was observed between full length and deletion mutant (52-266) of Mover compared to GFP-VAMP. One way ANOVA with Bonferroni's posthoc test. Values represented as mean±SEM.

Deletion mutants with N-terminal GFP tag did not produce any difference in the targeting of Mover when compared to the mutants with C-terminally tagged mGFP. Similar to the targeting of deletion mutants with C-terminal mGFP tag, expression of deletion mutants with N-terminal GFP tag - aa91-266, aa52-253, HSac2 domain (53-163), short isoform (excluding the expn 2 region spanning aa93-151) displayed diffuse labeling along the exon except for the GFP-52-266 (Appendix Fig. 7.1 and 7.2).

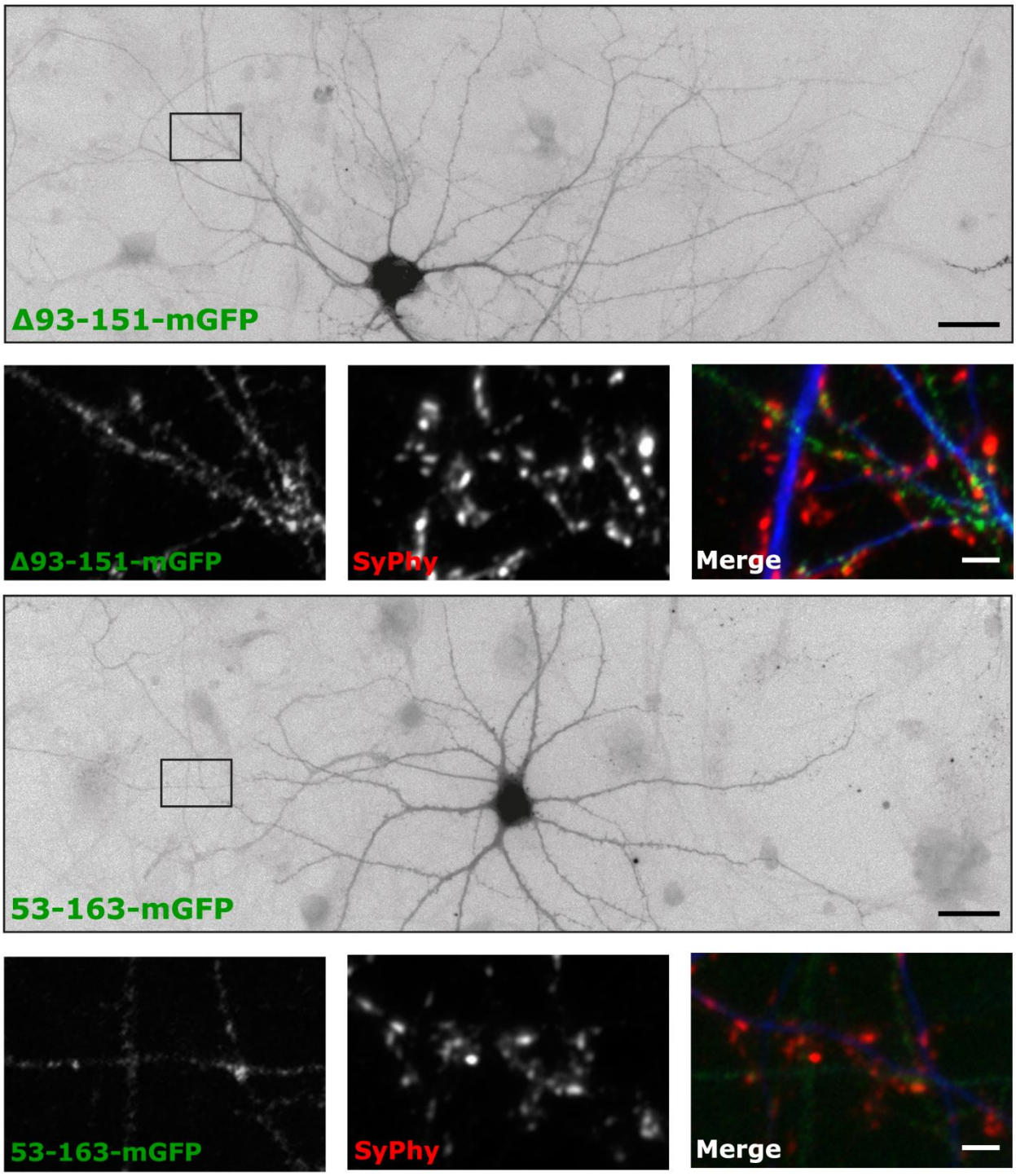


Fig.3.2.2.2. Δ93-151-mGFP and 53-163-mGFP do not target to presynaptic sites. Localization of mGFP-tagged deletion mutant lacking exon 2 region (Δ93-151-mGFP) and the other encoding the predicted HSac2 domain (53-163-mGFP) show diffuse labeling along the axon. Co-stained with Synaptophysin (SyPhy) and MAP2. Higher magnification images of the inlay are shown in individual channels (from left to right) - over-expressed protein in green, Synaptophysin in red and a merge along with MAP2 (in blue). Scale bar: 10µm for grey panel and 2µm for small panels (A).

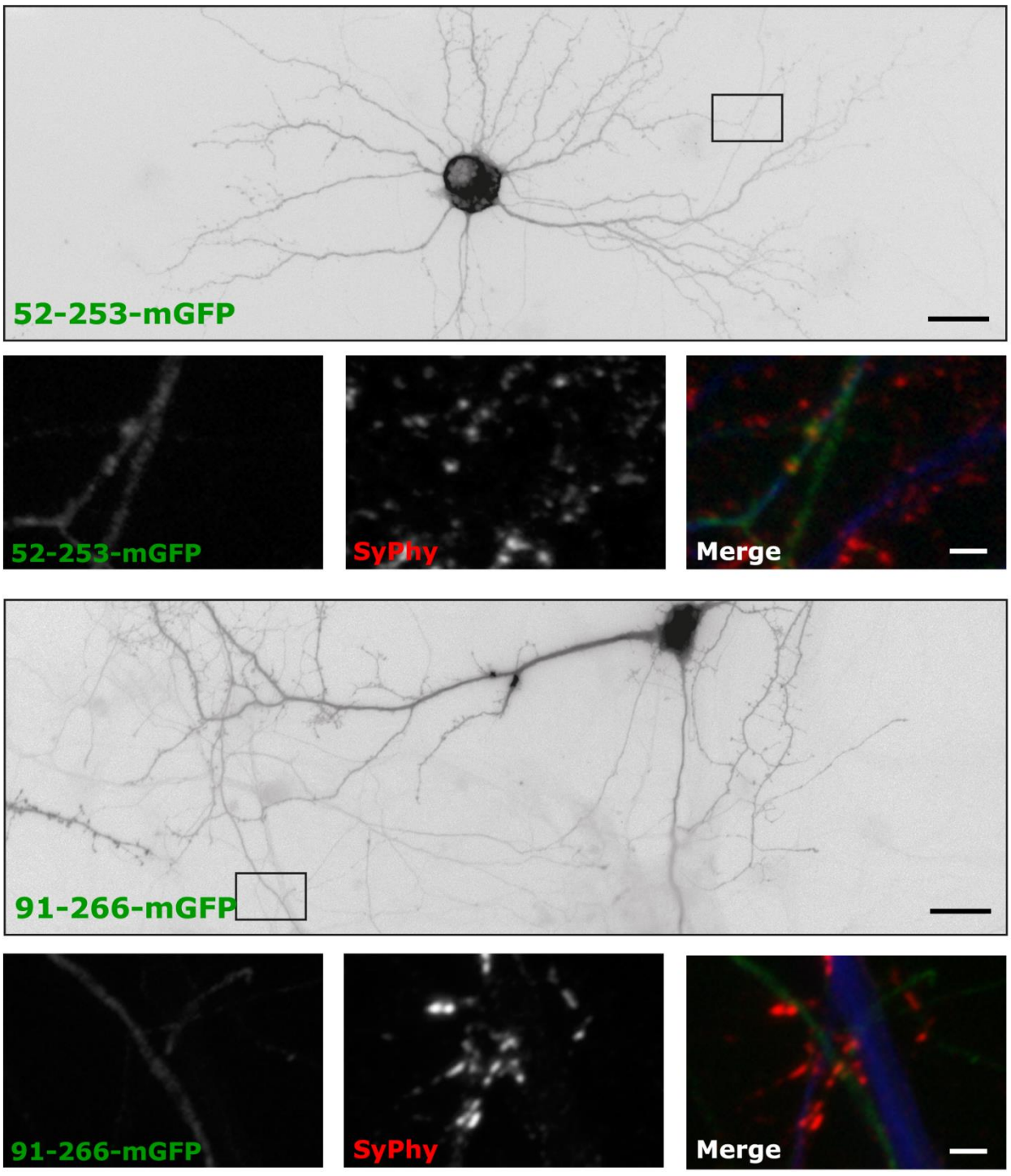


Fig.3.2.2.3. Diffuse distribution of 52-253-mGFP and 91-266-mGFP deletion mutants of Mover. Neurons expressing 52-253 and 91-266 with a C-terminal mGFP tag were diffusely distributed along the axon in 14DIV cultured hippocampal neurons. Co-stained for Synaptophysin (SyPhy, in red) and MAP2 (in blue).Higher magnification images of the inlay are shown in individual channels (from left to right) - over-expressed protein in green, Synaptophysin in red and a merge along with MAP2 in blue. Scale bar: 10µm for grey panel and 2µm for small black panels (A).

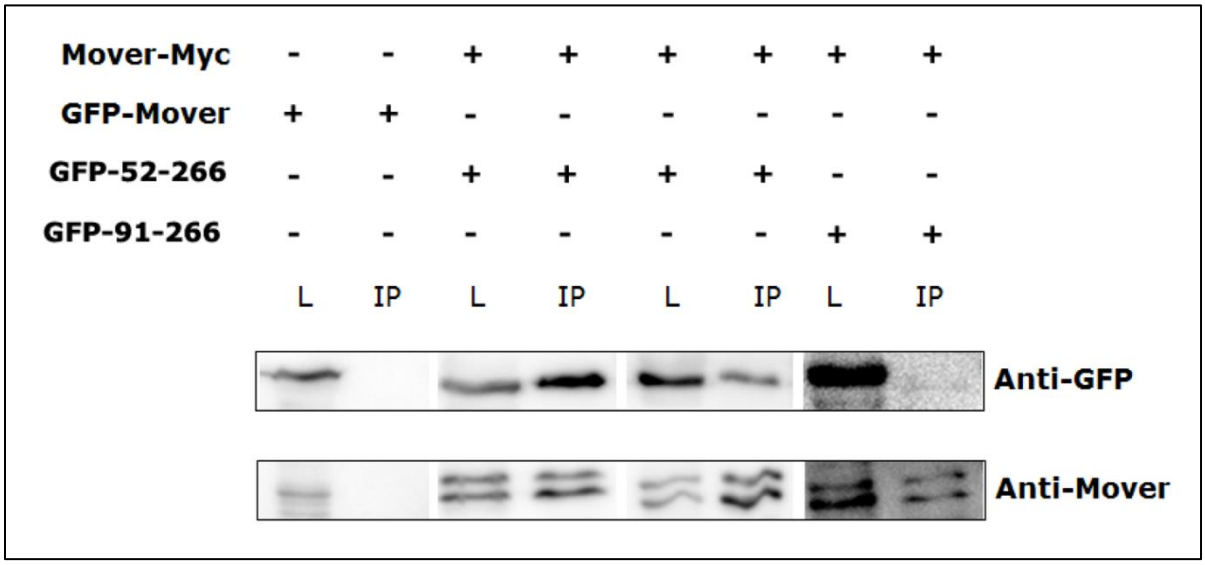
3.3. Self-interaction of Mover

Yeast 2-hybrid assay suggests that full-length Mover undergoes self-interaction (Ahmed et al., 2013). The amino acid sequences or regions necessary for Mover to undergo self-interaction are unknown. The yeast 2-hybrid data that full length Mover undergoes self-interaction was further corroborated with a co-immunoprecipitation assay. Hek293T cell lysates co-expressing GFP-Mover and Mover-myc were immunoprecipitated using sepharose or agarose coupled antibodies against myc tag. Immunoprecipitated complexes were analyzed by SDS-PAGE and western blotting.

The deletion mutants used for identifying the regions required for the presynaptic targeting of Mover were also used to test for their role in self-interaction. N-terminal GFP-tagged deletion mutants of Mover encoding aa52-266, aa 91-266, aa52-253, the HSac2 domain (53-163), or lacking the central exon2 region (lacking 93-151) were co-expressed with Mover-myc in Hek293T cells. The cell lysates with the respective GFP-deletion mutant along with Mover-myc were immunoprecipitated using sepharose coupled antibodies against myc tag. All the deletion mutants failed to dimerize *in vitro* except for GFP-52-266. Thus, co-immunoprecipitation experiments with GFP-deletion mutants identified the regions between aa52-90, aa253-266, aa53-163 (HSac2) and aa 93-151 (exon2) to be necessary for Mover to undergo self-interaction *in vitro* (Fig. 3.3; A and B).

Interestingly, the regions or amino acid sequences required for self-interaction of Mover were also found to be necessary for the presynaptic targeting of Mover. All the deletion mutants that impaired presynaptic targeting of Mover also failed to dimerize *in vitro,* suggesting dimerization /oligomerization of Mover as a prerequisite for the targeting of Mover to presynaptic terminals.

A.



B.

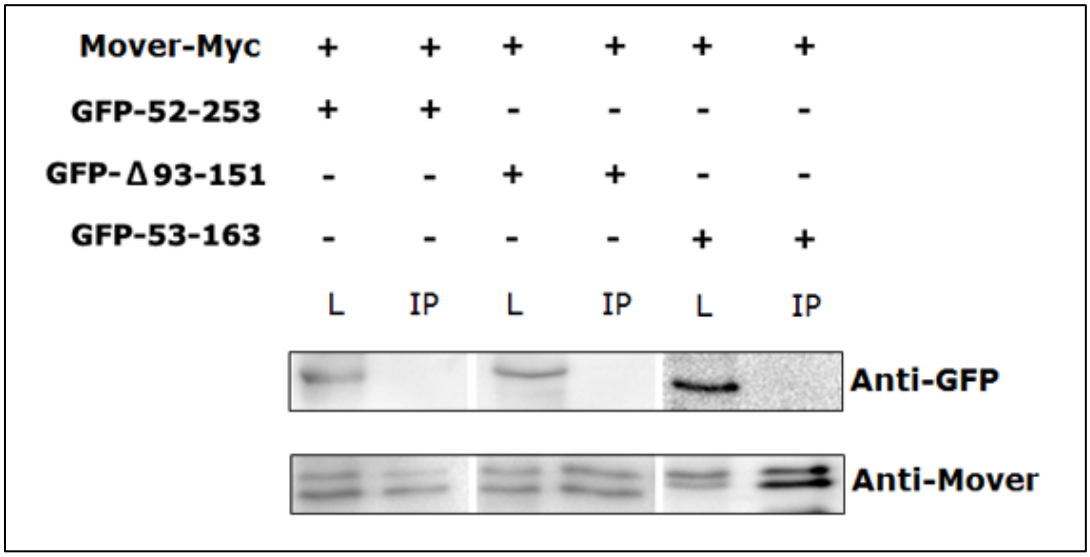


Fig.3.3. Deletion mutants- GFP-52-253, GFP-53-163, GFP- Δ93-151 and GFP-91-266 fail to dimerize *in vitro*. GFP-Mover and GFP-52-266 were pull-down with Mover-myc co-expressed in HEK293T cells using an anti-myc antibody. Mover-myc. Protein complexes were immunoprecipitated using sepharose beads coupled antibodies against myc epitope (A).GFP-52-253, GFP-Δ93-151, GFP-53-163 and GFP-91-266 were not immunoprecipitated upon pull-down with anti-myc antibody. Protein complexes were immunoprecipitated using sepharose coupled antibodies against myc epitope. Immunoprecipitated pellets were analyzed by SDS-PAGE and detected through western blotting by probing against anti-GFP and anti-Mover (A). L= Total lysate, IP= immunoprecipitate.'+' and '-' indicate the presence or absence of the construct in the assay.

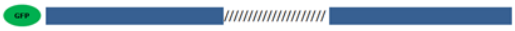
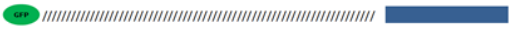
Deletion Construct	Amino acid	Presynaptic targeting	Self-interaction
	1-266	x	x
	Δ93-151	-	-
	33-266	x	x
	52-266	x	x
	52-253	-	-
	91-266	-	-
	53-163	-	-
	1-90	-	-
	91-180	-	-
	1-180	-	-
	181-266	-	-

Table 1. Illustrating the deletion mutants and the regions required for presynaptic targeting and self-interaction of Mover

3.4. Phosphorylation of Mover

3.4.1. Presynaptic targeting of Mover with mutations at predicted phosphorylation sites

Neurotransmitter release is modulated through second-messenger mediated signaling pathways resulting in phosphorylation of proteins by protein kinases and phosphatases (Leenders and Sheng, 2005). Protein phosphorylation plays an important role in pre-and post-synaptic plasticity. Most of the proteins involved in multiple steps of exocytotic release machinery are found to be regulated by phosphorylation. Phosphorylation of proteins has been shown to be necessary for various forms of synaptic plasticity (Turner et al., 1999).

Mass spectrometry analysis of presynaptic proteins detected a threonine phosphorylation site at amino acid 13 of Mover. Bioinformatical analysis also suggested additional predicted phosphorylation sites at aa14 (Serine), aa64 (Threonine) and aa 257 (Tyrosine) (Fig. 3.4.1.1; A).

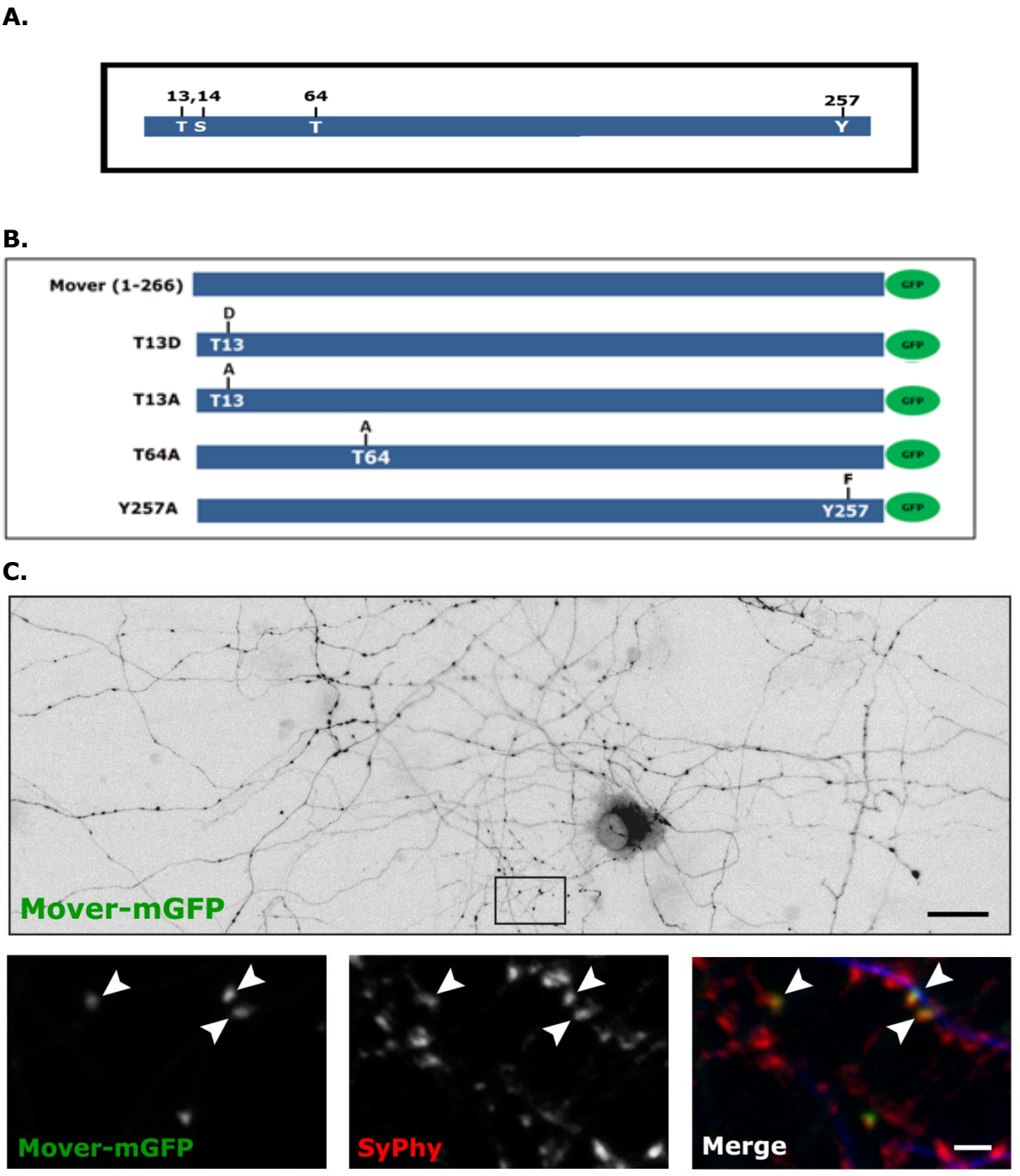


Fig. 3.4.1.1. Targeting of Mover-mGFP to presynaptic terminals. Mover protein with predicted phosphorylation sites (A) Single point mutations introduced at phosphorylation sites threonine 13 and 64 and at tyrosine 257 (B). Expression of recombinant full length Mover tagged with C-terminal monomeric GFP (mGFP) showing a punctate pattern in 14DIV cultured hippocampal neurons. Higher magnification images of the inlay are shown in individual channels (from left to right) - over-expressed protein in green, Synaptophysin in red and a merge along with MAP2 in blue. Mover puncta co-localizing with Synaptophysin are pointed with arrow heads. Scale bar: 10µm for grey panel and 5µm for small black panels (C).

A

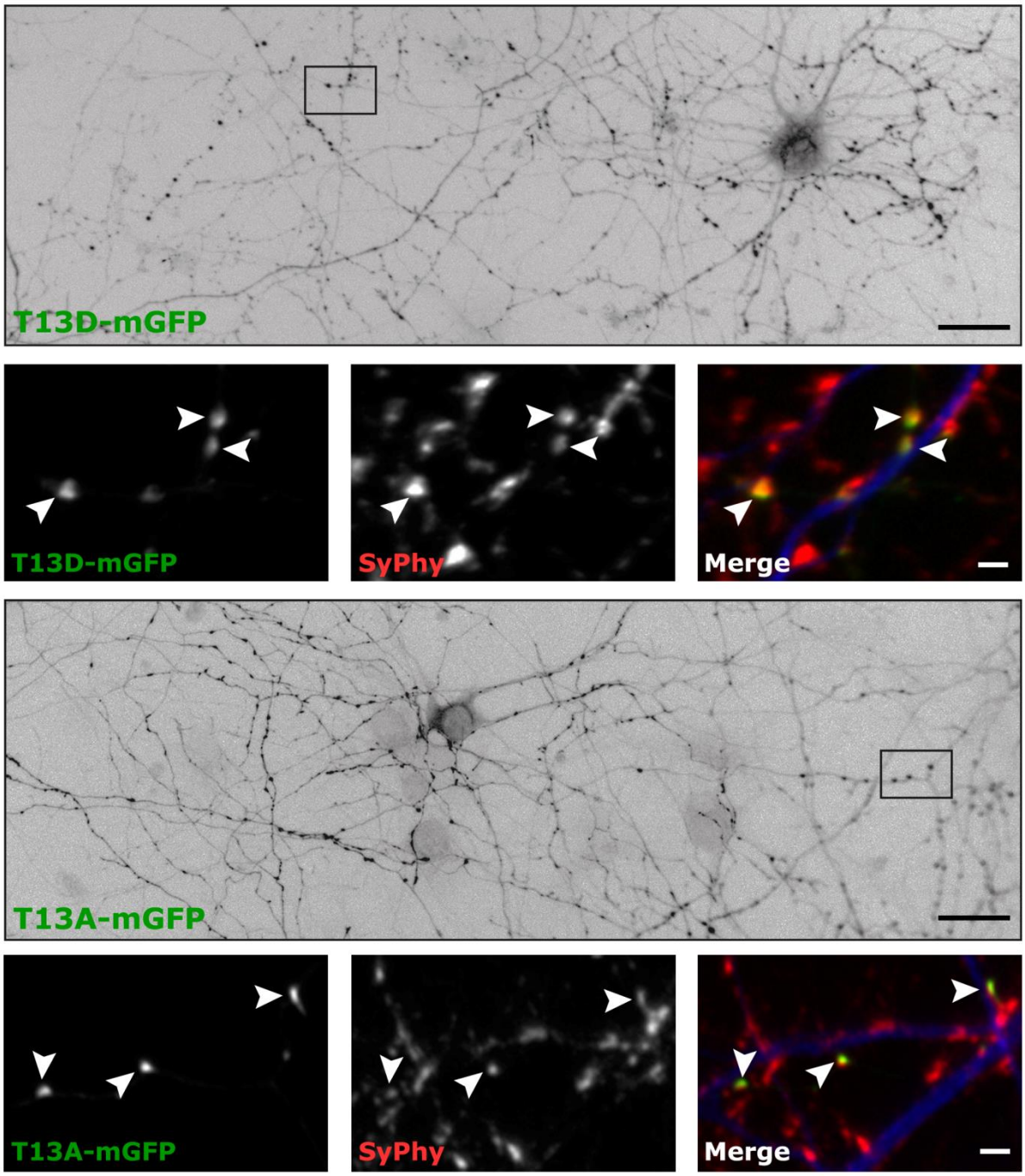


Fig.3.4.1.2. Point mutations at phosphorylation site-threonine 13 do not affect presynaptic targeting of Mover. Expression of C-terminally tagged full length Mover-mGFP with a point mutation at threonine 13 to aspartate (T13D-mGFP) or alanine (T13A-mGFP)in 14DIV cultured hippocampal neurons display a punctate pattern. Puncta co-localizing with synaptophysin are indicated with arrow heads. Higher magnification images of the inlay are shown in individual channels (from left to right) - over-expressed protein in green, synaptophysin in red and a merge along with MAP2 in blue. Scale bar: 10µm for grey panel and 2µm for small black panels (A).

Here I expressed constructs mutated at predicted phosphorylation sites rendering them non-phosphorylatable by changing threonine to alanine, and tyrosine to phenylalanine, or phosphomimetic by changing threonine to aspartate. mGFP tagged versions of phospho-mutants T13A-mGFP, T64A-mGFP, Y257F-mGFP and full length Mover-mGFP were immunostained with anti-GFP, anti-Synaptophysin and anti-MAP2 after their expression for 14DIV in cultured neurons. Expression of full length mGFP tagged Mover (Fig.3.4.1.1; C), single phospho-mutant at T13A and a phospho mimetic mutation at T13D in cultured hippocampal neurons produced discrete punctae along the axon that co-localize with endogenous Synaptophysin (Fig.3.4.1.2; A) Thus, phosphorylation mutations at site threonine 64 and tyrosine 257 do not impair presynaptic targeting in 14DIV cultured neurons (Fig.3.4.1.3.; A).

Single point mutations at phosphorylation sites threonine 13, 64 and tyrosine 257 had no effect on the presynaptic targeting of Mover. I next tested whether double mutations at threonine 13 and threonine 64 (T13A-T64A) as well as threonine 13 with tyrosine 257 (T13A-Y257F) had any effect on the presynaptic targeting of Mover. Both constructs produced a punctate staining pattern and co-localized with Synaptophysin puncta, suggesting that presynaptic targeting was not impaired (Fig.3.4.1.4; A)

In order to analyze presynaptic targeting quantitatively, the fluorescence intensities of the synaptic punctae (puncta that co-localize with Synaptophysin) were measured using Metamorph analysis software. No significant difference in the presynaptic targeting capacity of single or double phospho-mutants of Mover was observed compared to full length Mover (Fig.3.4.1.4; B and C). Similarly, no significant difference in the percentage of co-localization with Synaptophysin has been observed between full length Mover and either the single or double phospho-mutants (Fig.3.4.1.4; D and E).

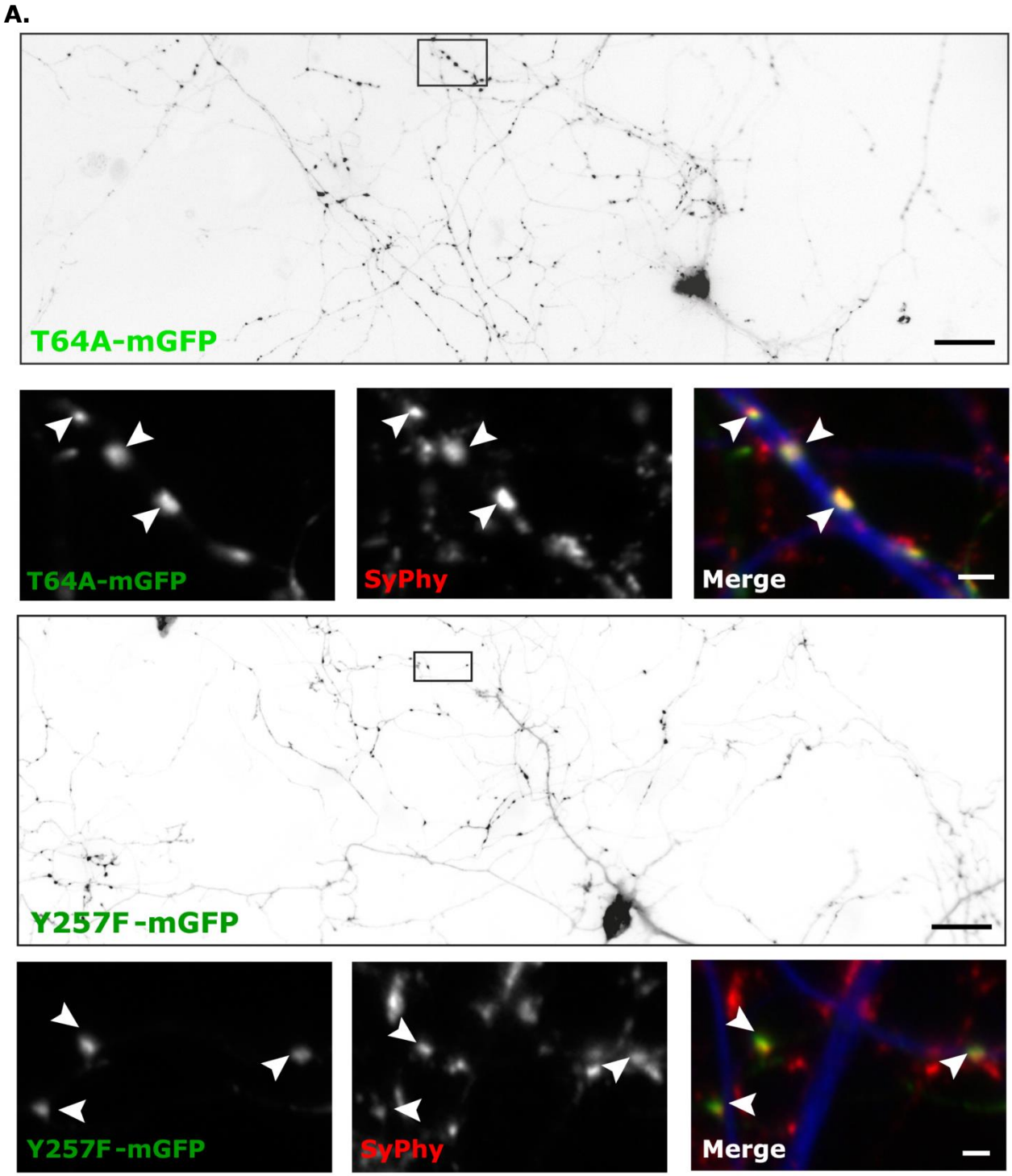
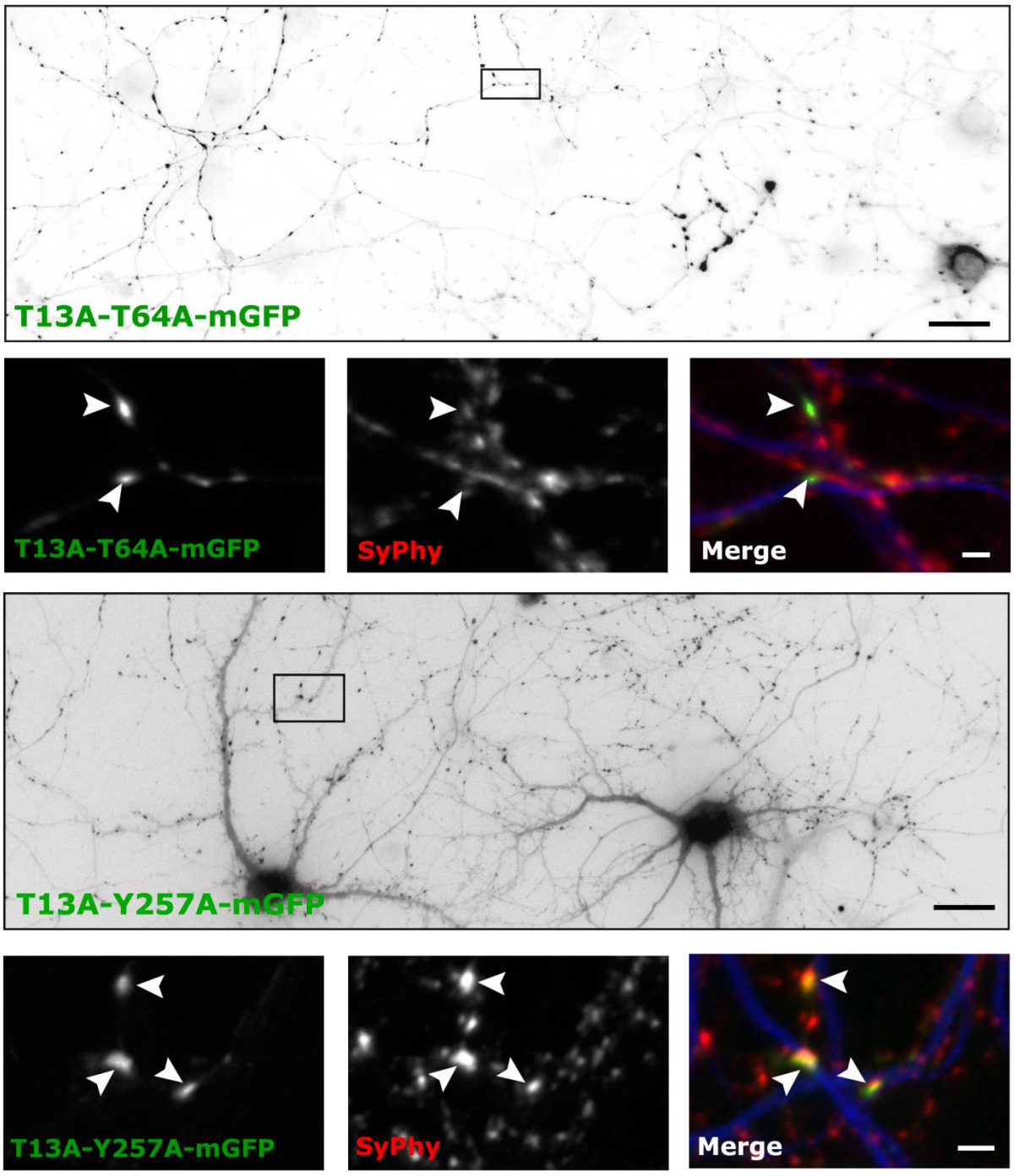


Fig.3.4.1.3. Point mutations at phosphorylation site threonine 64 and tyrosine Y257 do not affect presynaptic targeting of Mover. Expression of C-terminally tagged full length Mover- mGFP with a point mutation at threonine 64 to alanine (T64A-mGFP) and at tyrosine 257 to phenylalanine (Y257F-mGFP) 14DIV cultured hippocampal neurons display a punctate pattern. Expression of mGFP-tagged double mutated Mover at T64 (T64A), and Y257 (Y257F) in 14DIV cultured hippocampal neurons displaying a punctate pattern. Synaptic puncta are indicated with arrow heads. Higher magnification images of the inlay are shown in individual channels (from left to right) - Over-expressed protein in green, Synaptophysin in red and a merge along with MAP2 in blue. Scale bar: 10µm for grey panel and 2µm for small black panels (A).

A.


T13A-T64A-mGFP
T13A-T64A-mGFP
SyPhy
Merge
T13A-Y257A-mGFP
T13A-Y257A-mGFP
SyPhy
Merge

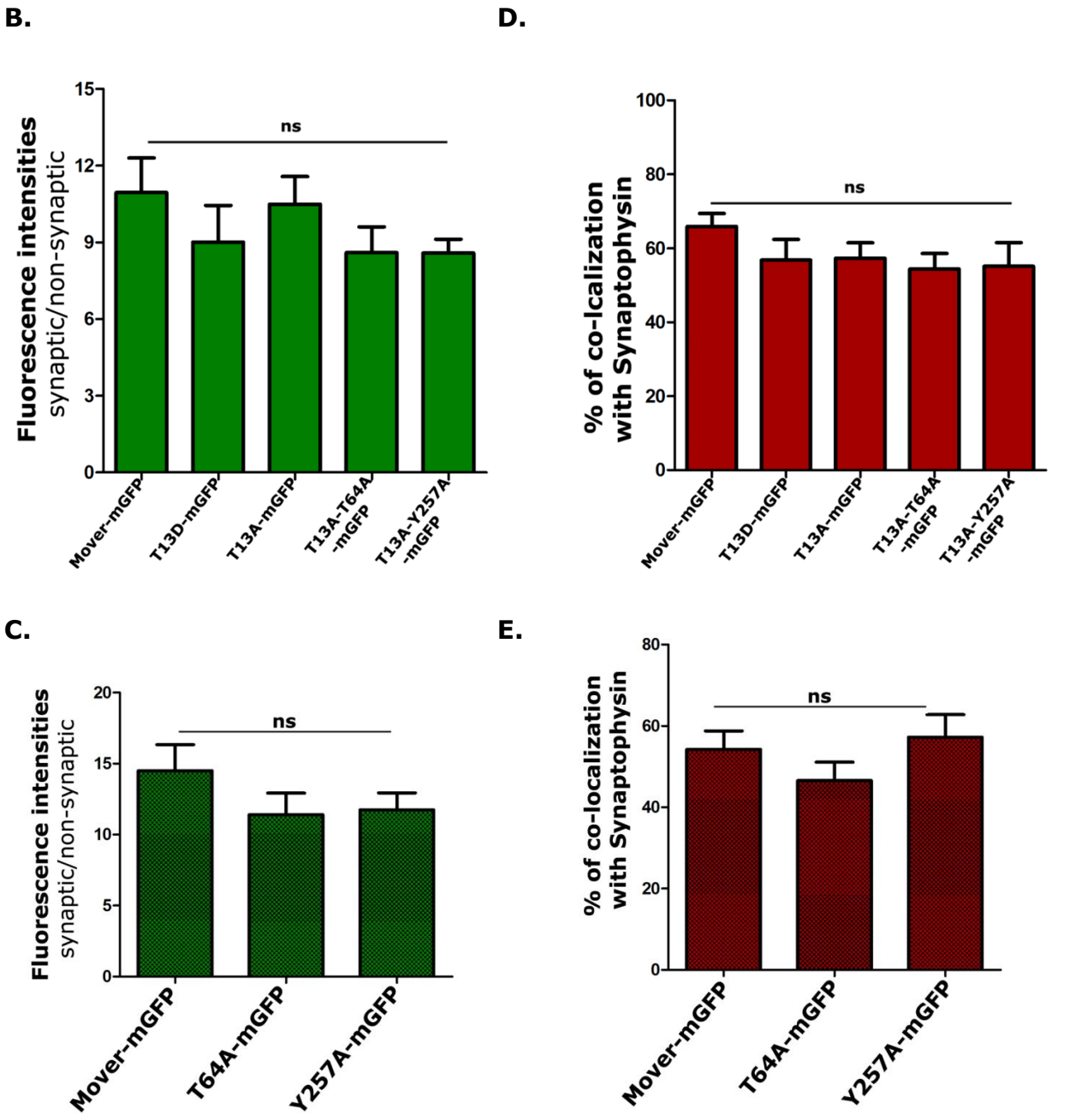


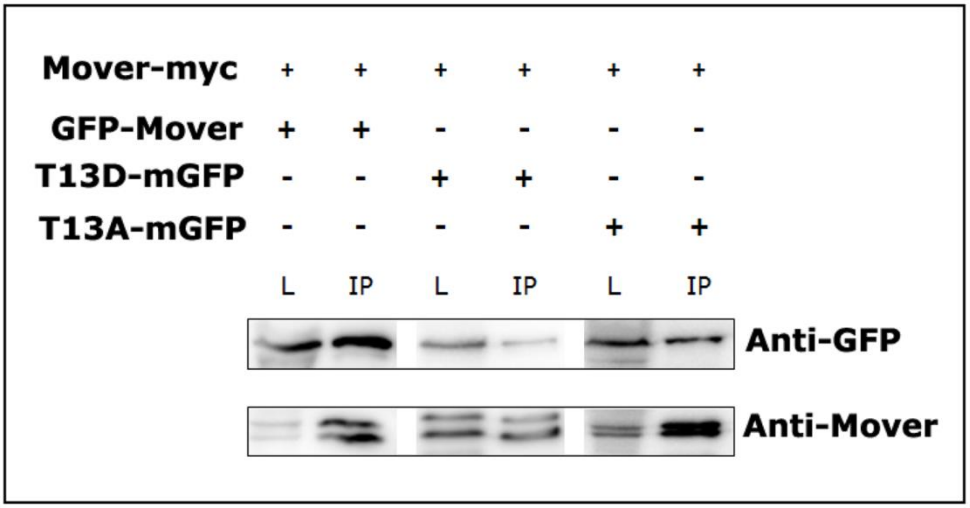
Fig.3.4.1.4. Double point mutations at threonine 13 along with T64 and Y257 do not affect presynaptic targeting of Mover. Expression of C-terminally tagged full length Mover- mGFP with a point mutations at threonine 13 and 64 to alanine (T13A-64A-mGFP) and; at threonine 13 to alanine and tyrosine 257 to phenylalanine (T13A-Y257F-mGFP) 14DIV cultured hippocampal neurons display a punctate pattern. Higher magnification images of the inlay are shown in individual channels (from left to right) - Over-expressed protein in green, Synaptophysin in red and a merge along with MAP2 in blue. Scale bar: 10µm for grey panel and 2µm for small black panels (A). Bar graph representing the presynaptic targeting capacity of single and double phospho-mutants compared to Mover-mGFP (B & C**).** No difference in the presynaptic targeting of Mover-mGFP and any of the phospho-mutants either single or double (T13D, T13A, T64A, Y257A, T13-T64A and T13A-Y257A) was observed. Co-localization analysis and phospho-mutants of Mover with synaptophysin in comparison to Mover-mGFP (D& E). One way ANOVA with Bonferroni's posthoc test. Values represented as mean±SEM.

The presynaptic targeting of phospho-mutants of Mover could be due to formation of dimers by the over-expressed Mover or with endogenous Mover in cultured rat neurons. In order to exclude the effect of dimerization/self-interaction on the phosphorylation of Mover, a Mover knock-out mouse line has been generated. Expression of phospho-mutants in Mover knock-out mice cultures would not be confounded by binding of these mutant variants to an intact endogenous protein.

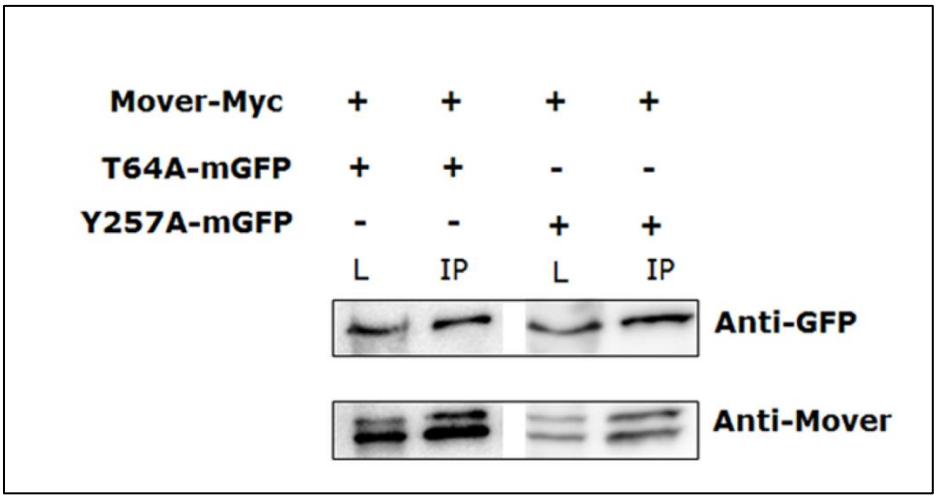
3.4.2. Self-interaction of phospho-mutants of Mover

From the targeting and self-interaction experiments, it had become clear that the regions or sites identified for Mover to undergo self-interaction were also necessary for presynaptic targeting of Mover. Hence, I tested whether the phospho-mutants dimerize with full length Mover *in vitro*. Hek293T cell lysates co-expressing either of the mGFP-tagged phospho mutants (T13A, T64A, Y257F, T13A-T64A and T13A-Y257F) and Mover-myc were tested for co-immunoprecipitation with a mouse monoclonal anti-myc antibody. Protein complexes were immunoprecipitated with sepharose beads against monoclonal antibody and analyzed by SDS-PAGE and western blotting. Phospho-mimetic and Mover mutants with either single or double mutations at phosphorylation sites and a phospho-mimetic mutation at threonine 13 did co-immunoprecipitate with Mover-myc, indicating that they are capable of interacting with full-length Mover (Fig.3.4.2. A, B and C).

A.



B.



C.

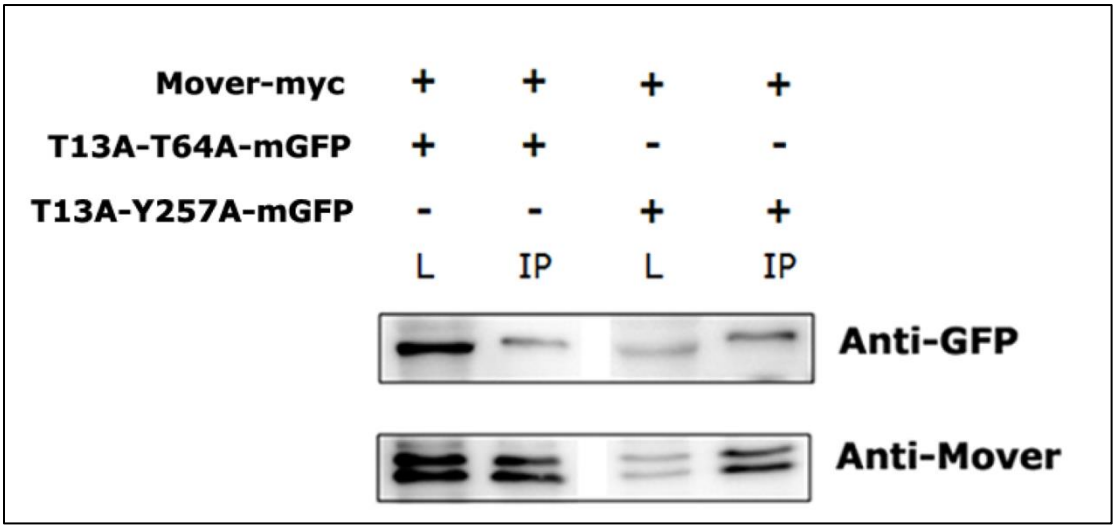
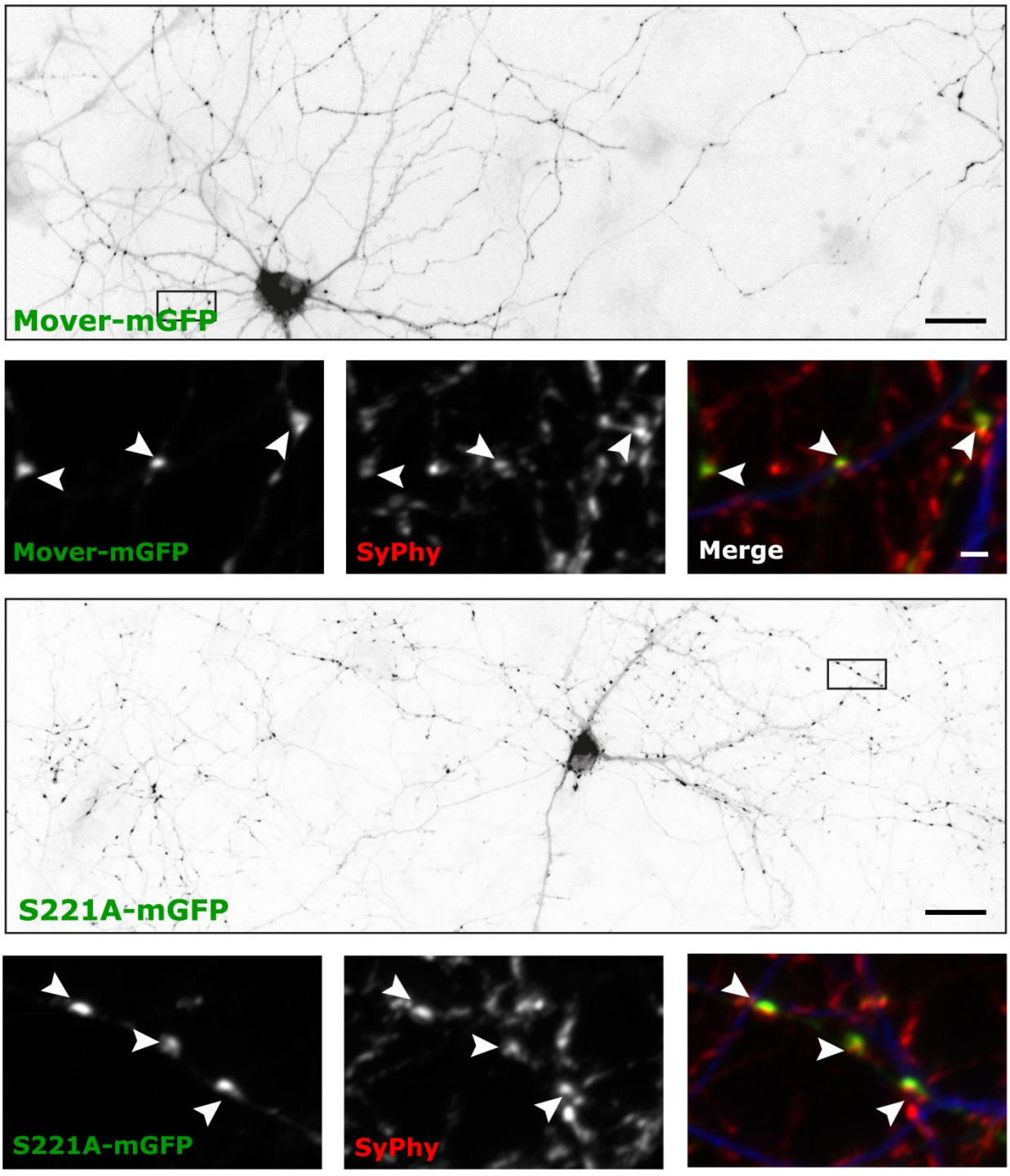


Fig.3.4.2. Mutations at phosphorylation sites of Mover had no effect on the self-interaction of Mover. GFP-Mover does immunoprecipitate upon IP with anti- myc antibody from co-expressed HEK293T cell lysates. Co-expressed phospho-mutants of Mover-T13A, T64A, Y257F, T13A-T64A and T13-Y257A (L) with Mover-myc from HEK293T cell lysates were immunoprecipitated with sepharose beads coupled against anti-myc antibody and analyzed by SDS-PAGE and western blotting. Protein complexes on the western blot were detected by probing against anti-GFP and anti-Mover. Phospho-mimetic mutation at threonine 13 self-interacts with Mover-myc from Hek293T cell lysates. (A, B and C). L=total lysate, IP=immunoprecipitate.

3.4.3. Analysis of ERK phosphorylation site in Mover

MAPK/ERK's (Mitogen Activated Protein Kinases/Extracellular signal Regulated Kinases) are protein kinases that are involved in intracellular signaling pathways. Mover is predicted to have an ERK phosphorylation site at aa221.

A.



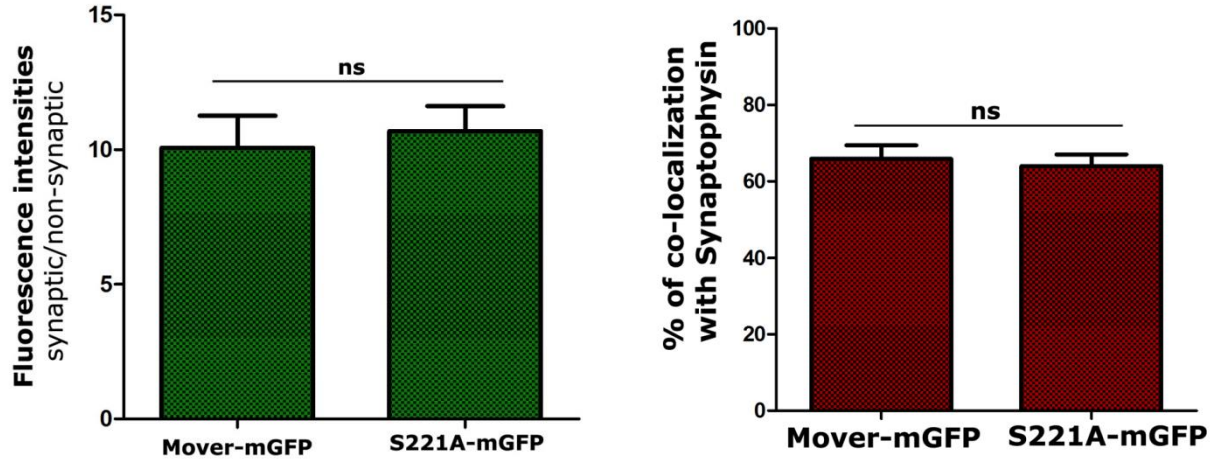


Fig.3.4.3. Mutation at the ERK phosphorylation site aa221 had no effect on the targeting of Mover to presynaptic sites Expression of C-terminally tagged full length Mover- mGFP with a point mutation at serine 221 to alanine (S221A-mGFP) in 14DIV cultured hippocampal neurons displayed a punctate fluorescence pattern along the axon. Puncta co-localizing with endogenous Synaptophysin are indicated with arrow heads. Higher magnification images of the inlay are shown in individual channels. Scale bar: 10µm for grey panel and 2µm for small black panels (A). Bar graph representing the fluorescence intensities of synaptic puncta produced by Mover-mGFP and S221A-mGFP. No difference in the targeting of Mover-mGFP and S221A-mGFP mutant of Mover at the presynaptic sites was observed (B). Co-localization analysis representing the percentage of co-localized Mover or S221A puncta with Synaptophysin (C). No statistical difference either in their targeting or co-localization with Synaptophysin was observed between full length and S221A of Mover. t- test. Values represented as mean±SEM.

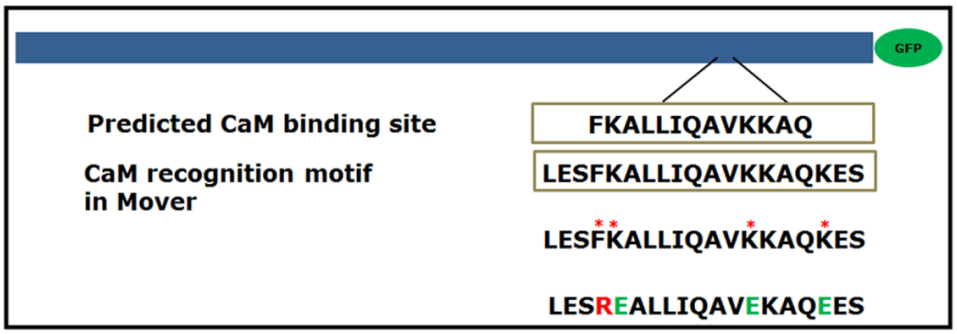
I tested to see whether a mutation introduced into this site would have any effect in the presynaptic targeting of Mover. mGFP-tagged Mover with a point mutation at phosphorylation site 221 from serine to alanine was used for their expression in 14DIV cultured neurons with full length Mover-mGFP as a positive control. Upon expression of S221A in cultured neurons, S221A displayed a punctate fluorescence pattern without any effect on the targeting of Mover to presynaptic sites (Fig.3.4.3; A). No difference in the targeting of S221A-mGFP at presynaptic sites (Fig 3.4.3; B) and percentage of co-localization with Synaptophysin was observed when compared to Mover-mGFP (Fig 3.4.3; C).

3.5. Interaction of Mover with Calmodulin

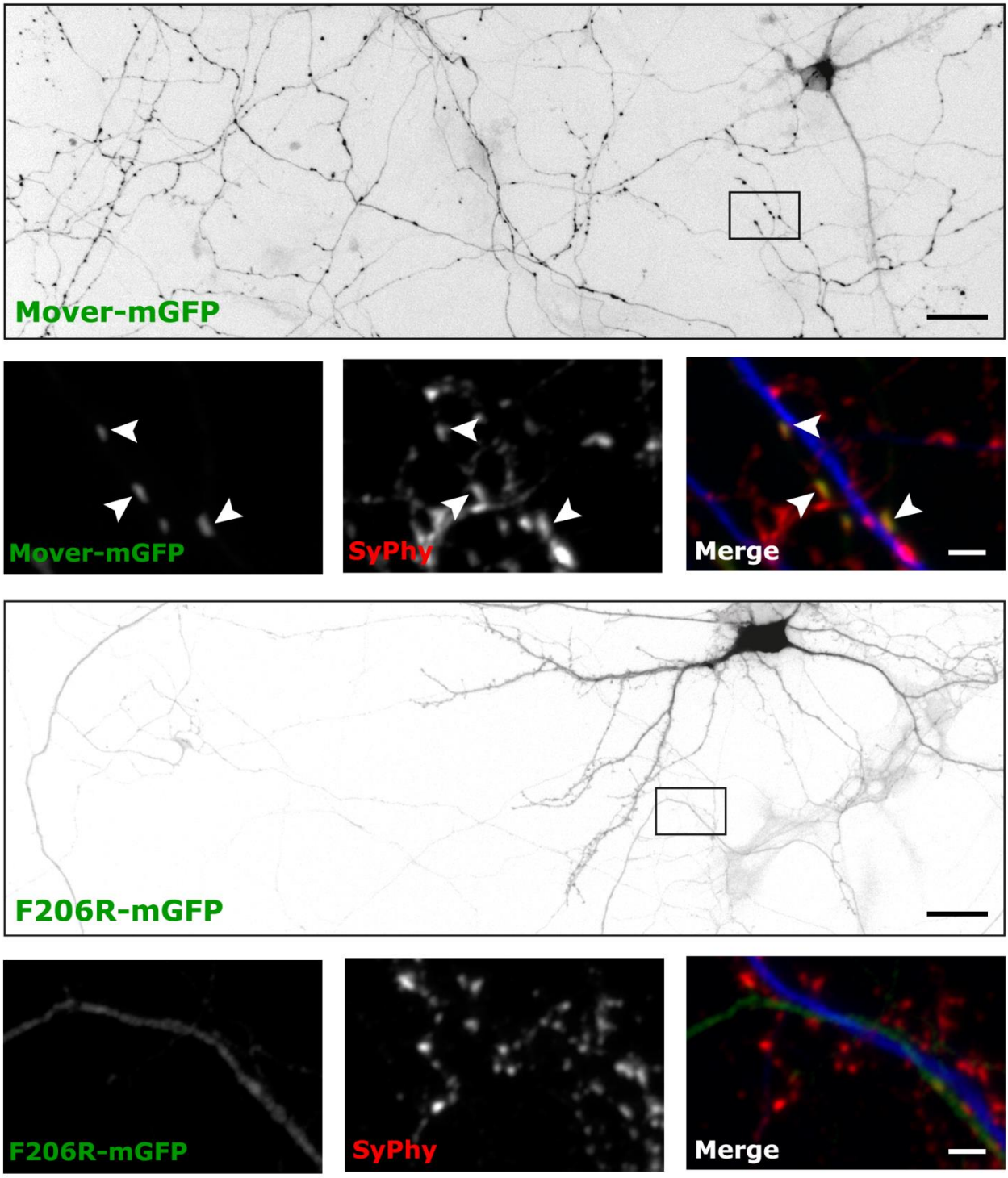
3.5.1. A point mutation at aa206 impairs presynaptic targeting and self-interaction of Mover

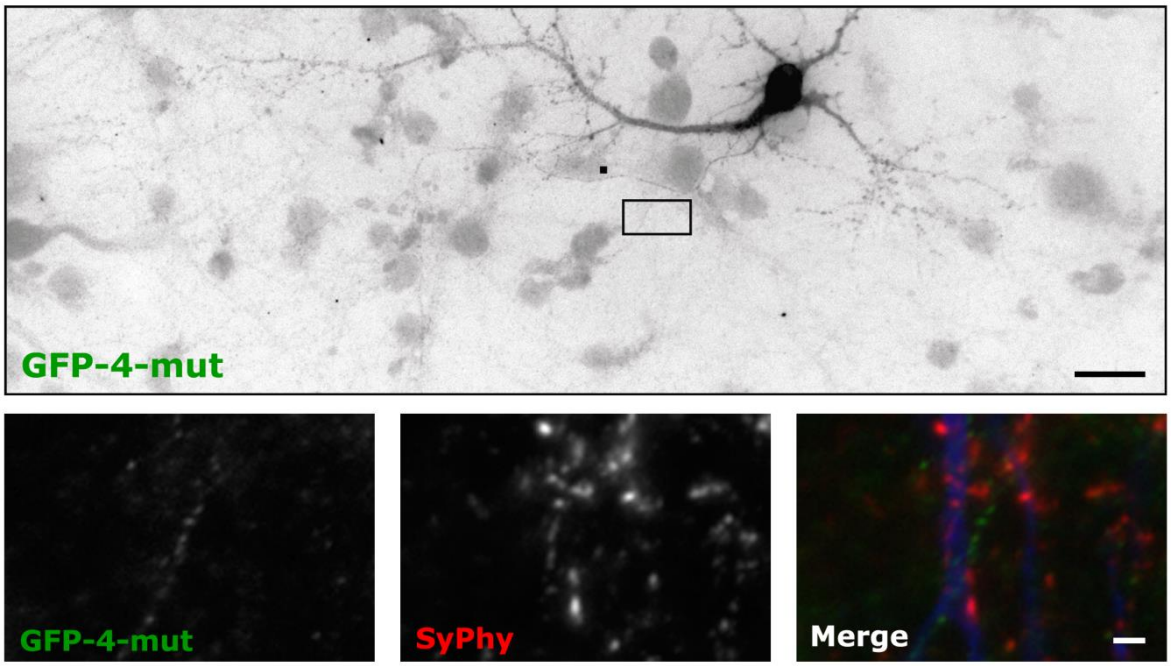
Calmodulin Target Database suggests that Mover has a predicted Calmodulin binding site of aa 206-218. The interaction of Calmodulin occurs through Calmodulin recognition motifs on target proteins and can be Ca^{2+} independent (such recognition motifs often include isoleucine and glutamine and thus called IQ motifs) or Ca^{2+} dependent. Canonical Ca^{2+}dependent motifs are characterized by positively charged amphipathic helices predominated by hydrophobic residues at certain positions, i.e. at positions 1,5 and 10, or at positions 1,8 and 14 of the motif (Junge et al., 2004). Amino acids of 206-218 contain both an IQ motif and a predicted amphipathic helix with a positive charge (Fig. 3.5.1; A). In addition, there is data supporting that Mover binds to Calmodulin in the presence of calcium with pull-down assays using GST-fusion proteins and synaptosomal extract from rat brains (Saheeb Ahmed, ENI, Goettingen, unpublished). Interaction of Calmodulin requires the presence of hydrophobic residues on the target protein that could serve as an anchor for binding. Comparing Mover to the CaM-binding protein Munc13-1 (Junge et al., 2004, Lipstein et al., 2013) reveals that Phenylalanine 206 qualifies as one such potential anchor. To test the role of the predicted CaM binding motif of Mover two full-length constructs were generated, one carrying a single point mutation (F206R), and one carrying the F206 mutation plus three additional mutations exchanging basic residues to acidic residues (Fig. 3.5.1; A). Four point mutations were introduced into the CaM recognition motif of Mover at phenylalanine to arginine at aa206 and lysine to glutamate at aa207, 215 and 219 (F206R, K207E, K215E and K219E) (Lipstein et al., 2012).

A.



B.


Mover-mGFP
Mover-mGFP
SyPhy
Merge
F206R-mGFP
F206R-mGFP
SyPhy
Merge



C.

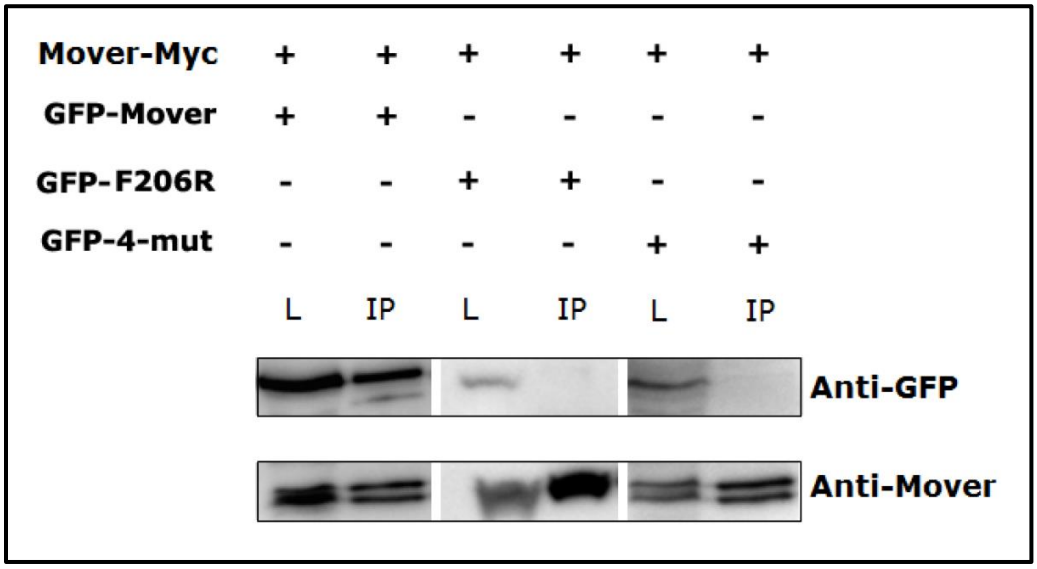


Fig.3.5.1. A point mutation at aa206 impairs presynaptic targeting and self-interaction of Mover. Amino acid sequence representing predicted Calmodulin binding site with the introduced point mutations. Amino acids with point mutations are denoted by '*' in red color and the exchanged amino acids in red (F) and green (E) (A).Expression of mGFP-tagged full length Mover, F206R mutant and a Mover mutant harboring four mutations in the predicted Calmodulin binding site (GFP-4-mut) displaying diffuse distribution along the axon in DIV14 cultured hippocampal neurons. Inlay shown as higher magnification images (left to right) of the construct (green), endogenous Synaptophysin (red) and merge image (along with MAP2). Scale bar: 10µm for grey panel and 2µm for magnified images (B).GFP-Mover does immunoprecipitate upon IP with anti- myc antibody from co-expressed HEK293T cells lysates. Co-expressed GFP-F206R and GFP-4-Mut (four exchanged amino acids in the CaM recognition motif of Mover) with Mover-myc from HEK293T cell lysates were immunoprecipitated, analyzed by SDS-PAGE and western blotting. Protein complexes on the western blot were detected by probing against anti-GFP and anti-Mover. GFP-F206R and GFP-4-mut failed to immunoprecipitate with Mover-myc from Hek293T cell lysates (C). L=total lysate, IP=immunoprecipitate.

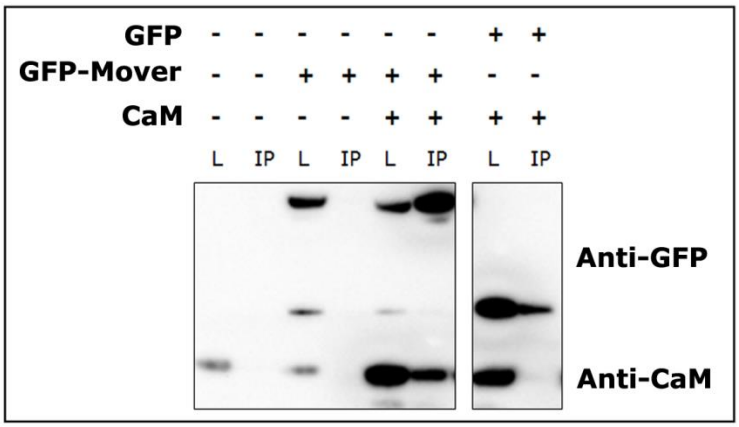
Another construct of Mover with point mutation at aa206 alone was also generated and labeled as F206R and the one with four mutations as 4-mut. Strikingly, expression of either N-terminal GFP or C-terminal monomeric GFP tagged version of Mover-F206R and Mover with four mutations in the predicted Calmodulin site in 14DIV cultured hippocampal neurons impaired the targeting of Mover to presynaptic terminals with a diffusely distributed pattern and a weak expression (Fig.3.5.1; B). Not only that, Mover-F206R and Mover with four mutations in the Calmodulin binding site also disrupt the self-interaction of Mover in Co-immunoprecipitation assay from HEK cell lysates (Fig.3.5.1; C).

3.5.2. Mover binds to Ca^{2+}- Calmodulin

We knew that Mover binds to Calmodulin in the presence of calcium (Saheeb Ahmed, ENI, Goettingen), unpublished data from pull-down experiments using GST-fusion proteins and synaptosomal extracts from rat brain).This was further corroborated by co-immunoprecipitation experiments using GFP-Mover. The mutation at aa206 from phenylalanine to arginine in the predicted Calmodulin binding site impaired presynaptic targeting and self-interaction of Mover. Next I tested to see whether the point mutation at aa206 has any effect on Calmodulin binding. To do this, Hek293T cell lysates expressing N-terminal GFP-tagged F206R were incubated with purified recombinant Calmodulin in the presence of calcium. Anti-GFP antibody was used to pull down the protein complexes and analyzed by SDS-PAGE and western blotting.

GFP-Mover binds to Calmodulin in the presence of Ca^{2+}, whereas GFP does not (Fig 3.5.2; A). Not only that the point mutation F206R is found to bind to Calmodulin in the presence of Ca^{2+}, eliciting no effect of F206R on Mover binding to Calmodulin (Fig 3.5.2; B).

A.



B.

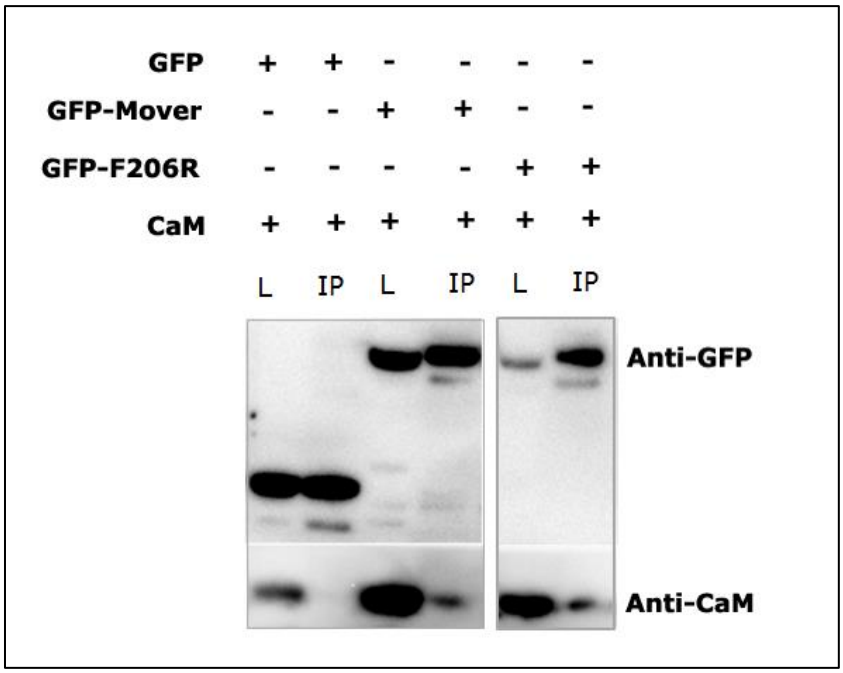


Fig.3.5.2 Mutation at aa206 does not affect the binding of Mover to Calmodulin. GFP-Mover binds to Calmodulin**.** HEK293T cell lysates expressing GFP or GFP-Mover were incubated with recombinant purified CaM in the presence of 2mM Ca^{2+}. Protein complexes were immunoprecipitated with monoclonal anti-GFP antibody, analyzed by SDS-PAGE and western blotting. GFP does not IP with CaM (A). Mutation at aa206 in the Calmodulin binding site had no effect on the binding of Mover to CalmodulinHEK293T cell lysates expressing GFP-F206R or GFP-Mover were incubated with recombinant purified CaM in the presence of 2mM Ca^{2+}. Protein complexes were immunoprecipitated with anti-GFP antibody, analyzed by SDS-PAGE and western blotting. GFP does not IP with CaM (B).

3.5.3. Mover does not inhibit Calmodulin *in vitro*

Knock-down of Mover at the Calyx of Held synapse increases release probability (C. Körber, University of Heidelberg, thesis published online). Knock-down of Calmodulin in hippocampal neurons decreases release probability (Pang et al, 2010). Therefore, Mover and Calmodulin may act in opposite directions regulating release probability, and Mover may inhibit Calmodulin in this scenario. To test whether Mover inhibits the role of Calmodulin an assay for Calmodulin was established based on the following facts: One of the targets of CaM is Calcium-Calmodulin dependent protein kinase II (CaMKII). Calmodulin is an EF hand member of calcium binding proteins that binds to four calcium ions. Ca^{2+}- bound Calmodulin activates CaMKII leading to auto phosphorylation of the enzyme.

A.

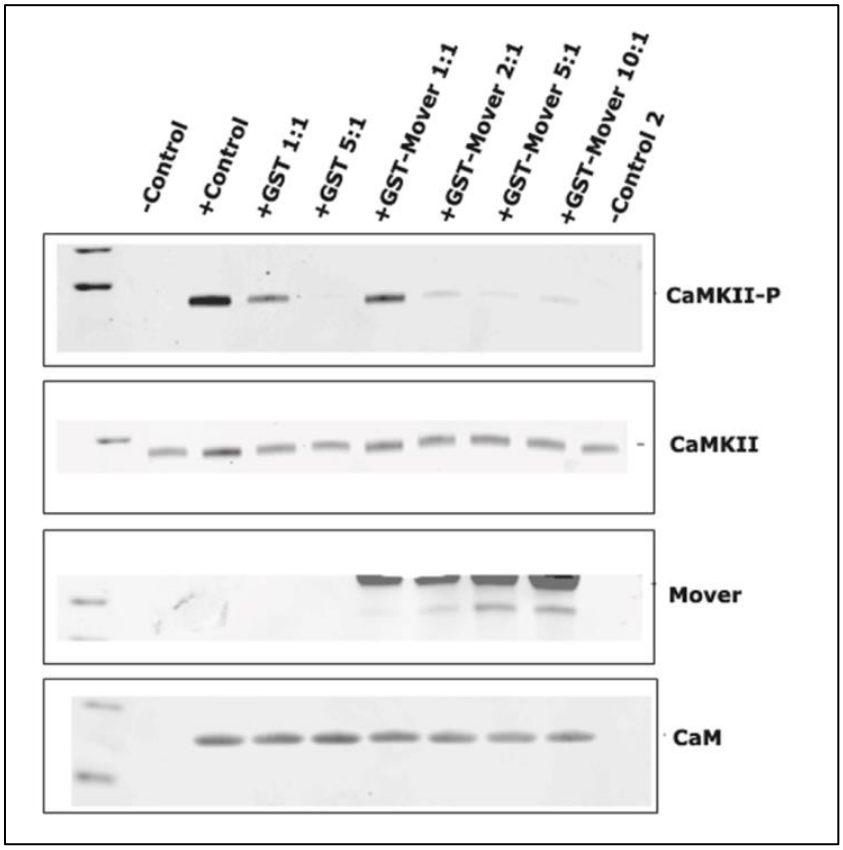


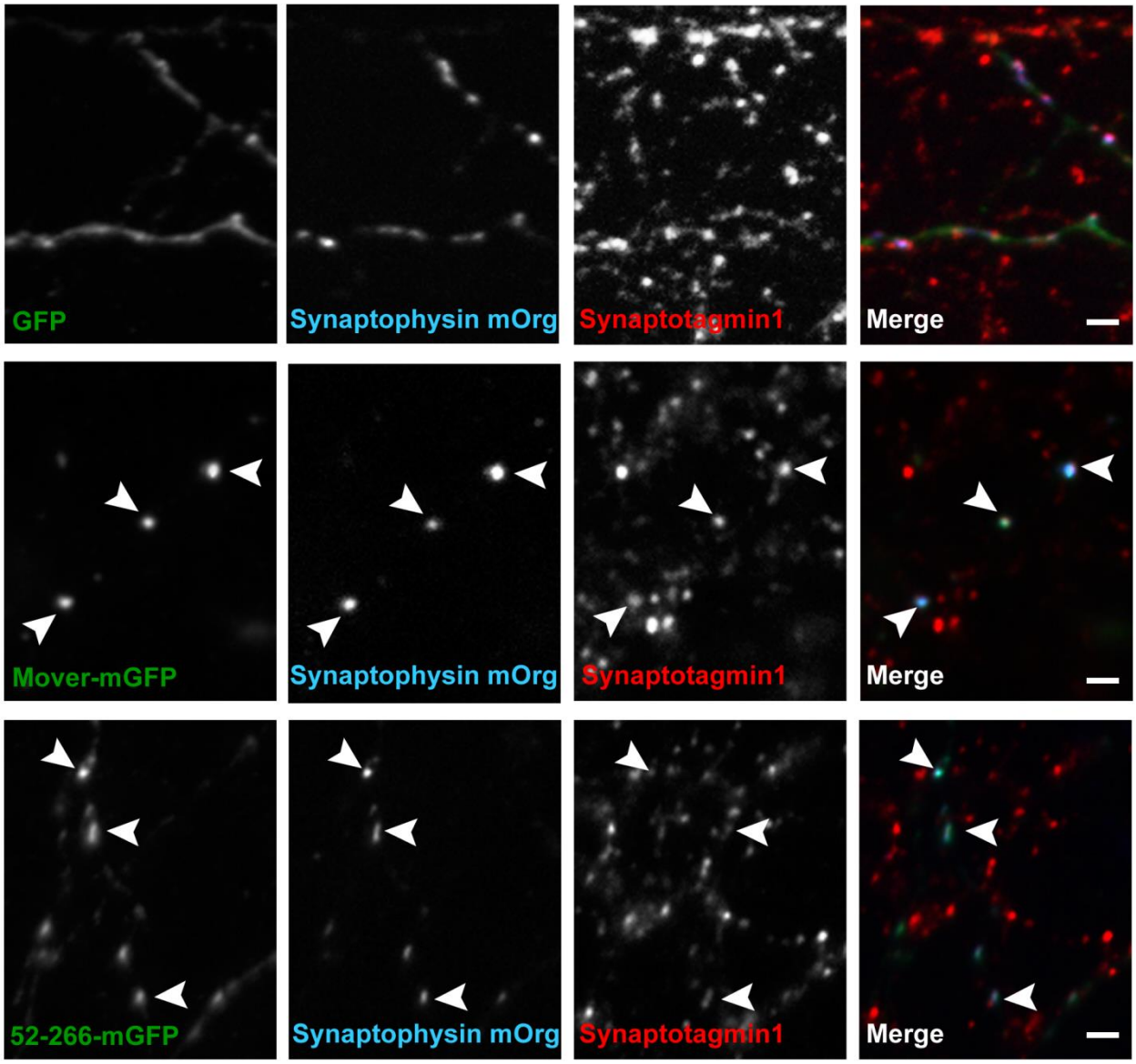
Fig.3.5.3. Mover does not inhibit Calmodulin *in vitro*. Purified GST-Mover and GST were used at varying concentrations and incubated with purified recombinant CaMKII and CaM in the presence of 2mM Ca^{2+}. Following incubation, analyzed by SDS-PAGE and immunoblotted with antibodies against Mover, Calmodulin and phosphorylated (CaMKII-P) and non-phosphorylated forms of CaMKII (A).

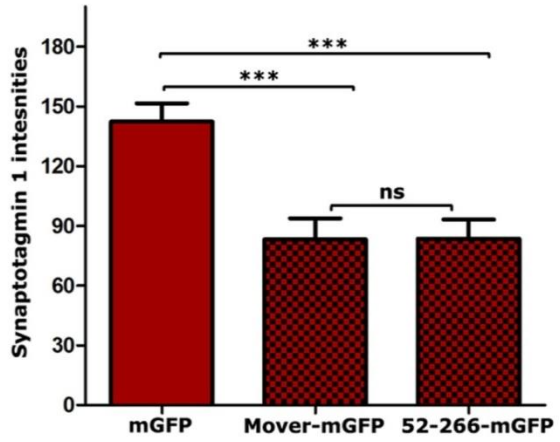
To this end, an *in vitro* biochemical assay was established and performed, keeping the concentration of CaMKII and Calmodulin constant but with varied concentrations of purified GST-Mover in the presence of calcium. This assay revealed no change in the phosphorylation of CaMKII both with GST-Mover and GST suggesting no effect on the phosphorylation of CaMKII by Mover-Calmodulin binding (Fig. 3.5.3; A).

3.6. Overexpressing Mover causes a reduction in the recycling pool of vesicles.

Knock-down of Mover at the Calyx of Held synapse increases release probability (C. Körber, University of Heidelberg, thesis published online), raising the possibility that Mover, and in particular overexpression of Mover, may decreases release. In order to investigate the role of Mover in synaptic vesicle recycling, a hypothesis was drawn from the preliminary unpublished data of Mover at the Calyx of Held. The hypothesis was to test whether over-expression of Mover decreases release probability (opposite to knock-down). To this end, recombinant mGFP tagged full-length Mover, mGFP and mGFP tagged aa52-266 version of Mover that is found to be presynaptic from the targeting experiments were co-expressed with Synaptophysin -mOrange in cultured hippocampal neurons. The Synaptotagmin1 antibody uptake assay (Syt1 assay) serves as readout for the extent of synaptic vesicle recycling. This is because Synaptotagmin1 antibody taken up by the synaptic vesicles during depolarization in cultured neurons is proportional to the total number of recycling vesicles. At 14DIV, a Synaptotagmin1 antibody uptake assay was performed, fixed and stained with respective secondary antibodies. Images were acquired using a 40X objective and Synaptotagmin1 fluorescence intensities were measured for Mover puncta positive for Synaptophysin mOrange. Synaptotagmin intensities measured for full length Mover and 52-266-mGFP revealed a statistically significant decrease in the total recycling pool of vesicles compared to mGFP (Fig.3.6; A and B), but no difference between full length and N-terminal deletion mutant of aa51 was observed. This indicates that both recombinant proteins reduce synaptic vesicle recycling with the same efficacy.

A.



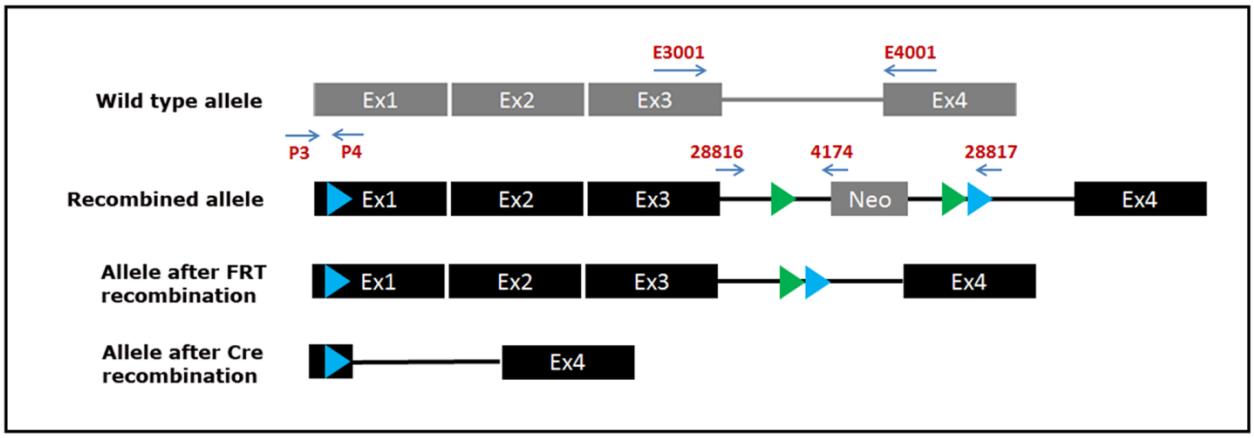


B. Fig.3.6. Mover decreases the total pool of recyling vesicles. Co-expression of Mover-mGFP, 52-266-mGFP and mGFP with Synaptophysin mOrange (SyPhy mOrg) in cultured neurons. Mover-mGFP, 52-266-mGFP and mGFP in green, Synaptophysin mOrange in blue and Synaptotagmin in red. Images acquired after Syt1 antibody uptake and analyzed for Synaptotagmin intensities (A). Scal bar 2µm (A). Bar graph representing the Synaptotagmin intensities for mGFP or Mover-mGFP or 52-266-mGFPpuncta positive for Synaptophysin mOrange puncta, that show a decrease with respect to mGFP but the Syt1 intensities between full length and 52-266-mGFP remain unchanged. One Way AOVA with Bonferroni's post hoc test. P≤0.0001 (***).Values represented as mean±SEM. N=2.

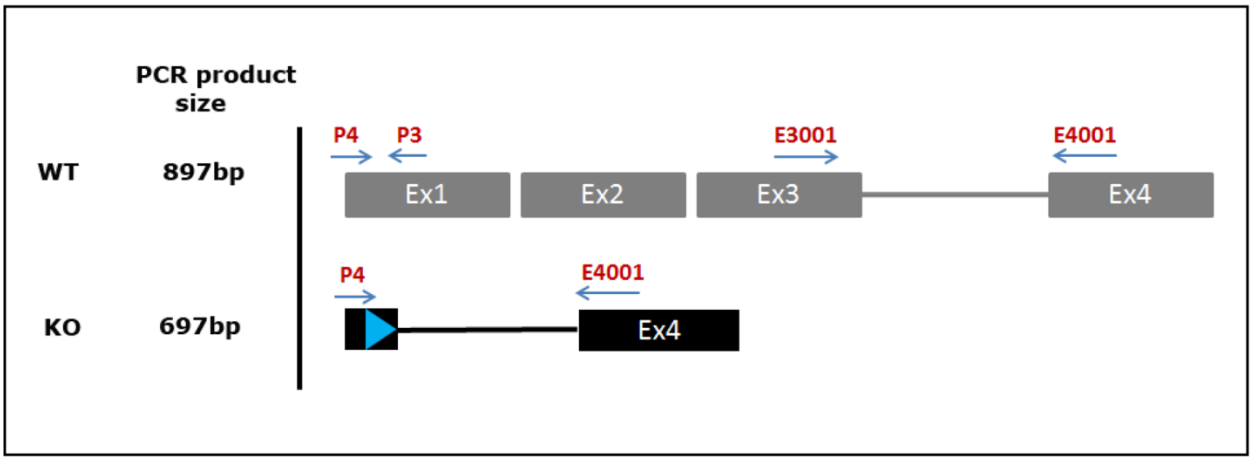
3.7. Generation of a Mover Knock-out mouse line

Chimeric mice with modified Mover gene were bred with wild type mice and further with Rosa FLIP mice allowing removal of Frt-flanked neo expression cassette. At this stage the mice have a Mover gene that is flanked by loxp sites, hence called as 'flox mice'. Heterozygous flox Mover mice were bred with Cre mice resulting in Cre positive heterozygous Mover mice, verified through genotyping. Finally the heterozygous Mover mice were inbred for the generation of global Mover knock-out mice line.

A.



B.



C.

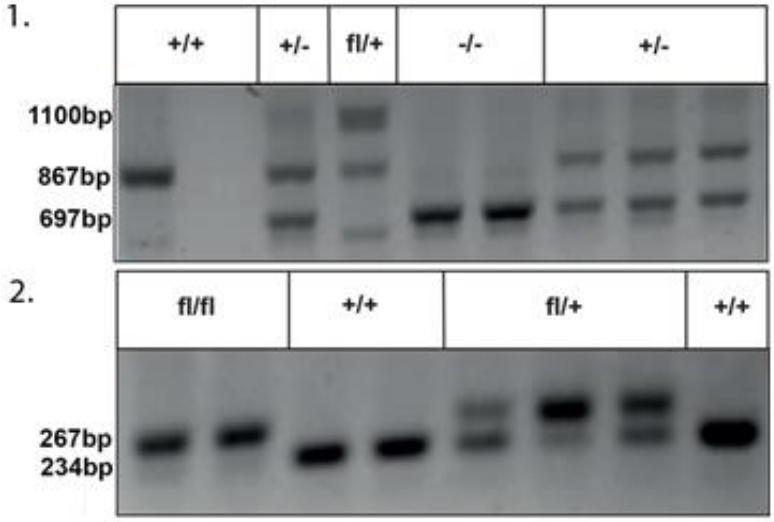


Fig. 3.7. Genotyping of Cre-lox Mover mouse population. Schematic representation of generation of Mover knock-out with the designed primers used for genotyping. Two primers (P3 and P4) were used to distinguish between wild type and recombined alleles of Mover. Primer pair 28816 and 28817 was used to identify 5' loxp sites. LoxP sites are indicated in by blue triangles and the neo cassette with green triangle. One of the LoxP site is in the untranslated region to the upstream of Mover gene (A). Schematic representation of PCR amplified loci for the identification of Mover knock-out. Three primers were used to distinguish (P4, E3001 and E4001) three alleles of Mover gene: wild type allele (+/+) heterozygous (+/-) and Cre-lox excised allele, KO (-/-). The sizes of the amplification products are 867bp for wild type (+/+), 697 bp (-/-) for knock-out and a double band with 867 and 697bp for heterozygous alleles (+/-) (B). Litter consisting of wild-type (+/+), heterozygous floxed (fl/+), and homozygous floxed mice (fl/fl) verified by PCR genotyping using two primers (P3 and P4) flanking the lower loxp site. A double band corresponds to heterozygote harboring a wildtype and floxed allele, single upper band represents the homozygous floxed mice and lower band a homozygous wild type animal (C1). Litter of a mouse population consisting of wild-type, heterozygous and knock-out alleles of Mover. A double band corresponds to heterozygote, a single lower band to knock-out and single upper band represents a wild-type allele (C2).

3.7.1. Characterization of Mover knock-out mouse line

Generating a conditional knock-out mouse line requires frequent genotyping at each stage of transgenic mouse populations. Genotyping requires establishing PCR protocols at each stage to identify the required transgene from mouse populations.

Brain homogenates of 5-week old Mover heterozygous (+/-), homozygous flox (fl/fl) and global Mover knock-out mice were analyzed through SDS-PAGE and western blotting for the presence and absence of Mover along with unchanged Synaptophysin and tubulin protein levels in the Mover knock-out (Fog. 3.7.1; A and B). Cortical neuronal cultures were from Mover knock-out mice were verified for the absence of Mover by immunofluoroscence (Fig. 3.7.1; C).

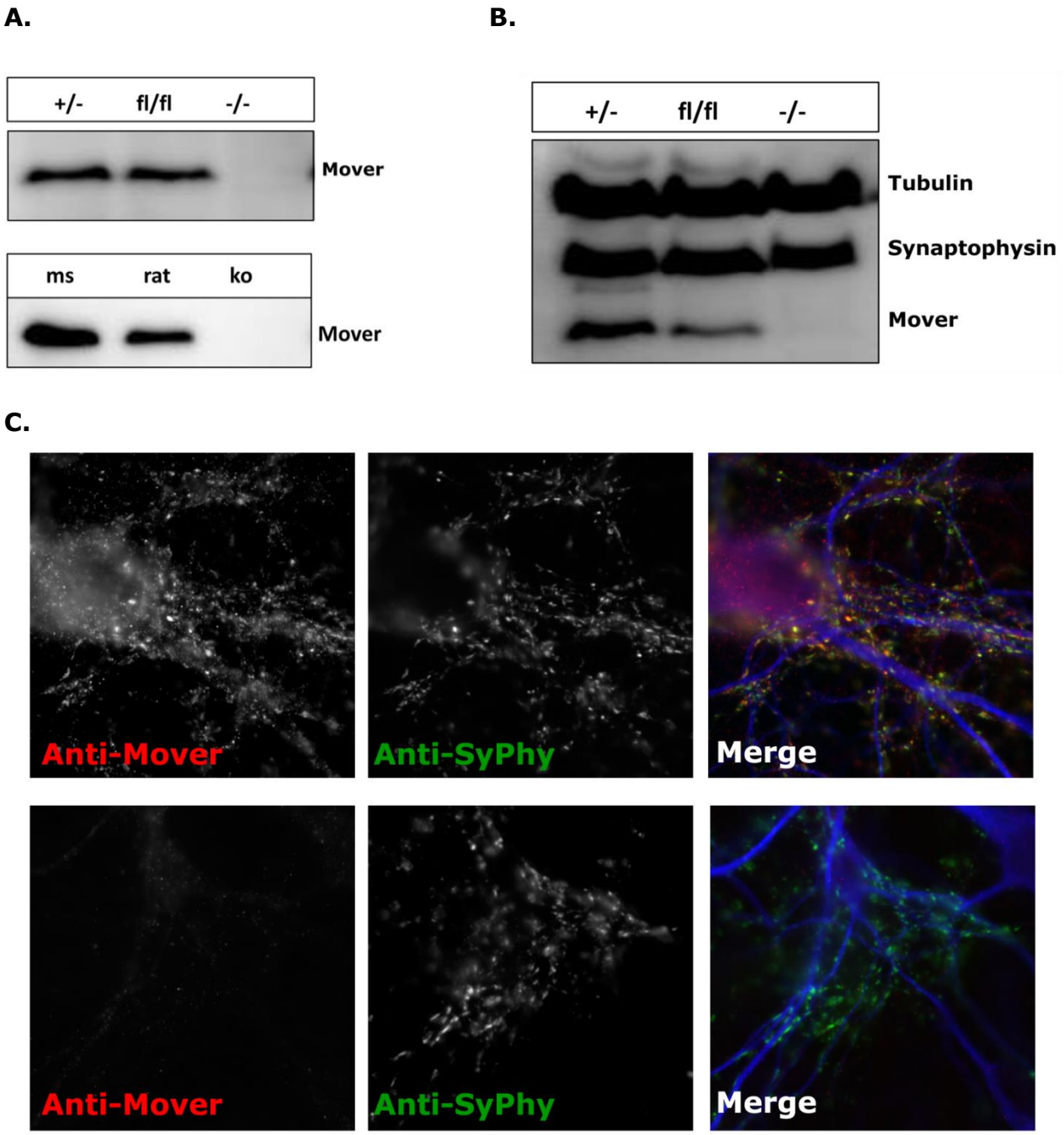
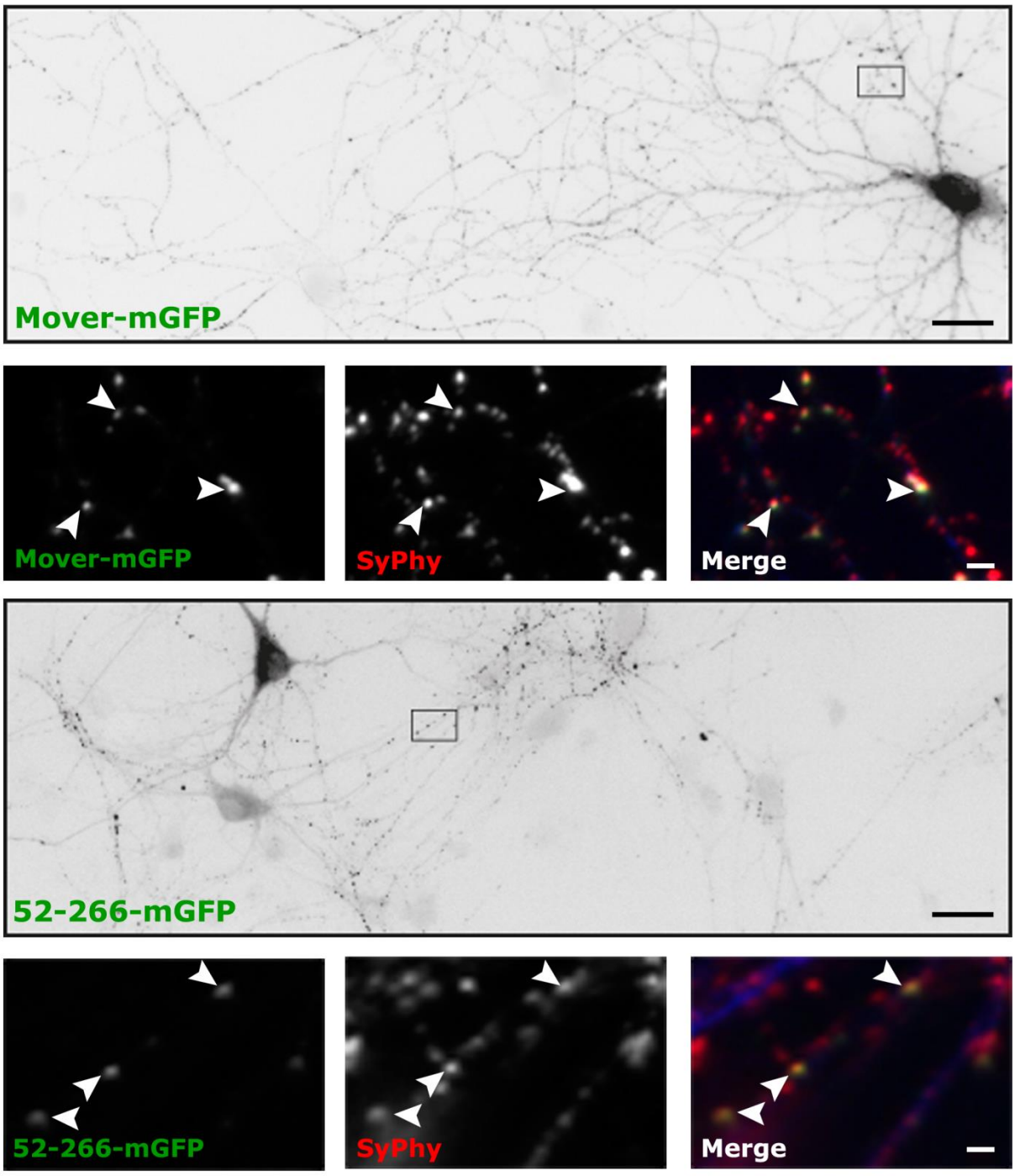


Fig 3.7.1. Characterization of Mover knock-out mouse line. Brain homogenates from heterozygous, flox Mover and Mover knock-out mice were analyzed through SDS-PAGE and western blot (top panel); and wild type mouse and rat, Mover knock-out mouse brains were homogenized and analyzed by SDS-PAGE and western blot (A). Western blot analysis showing the absence of Mover from 5-week old mouse brain homogenate with Synaptophysin and β-3-tubulin as control (B). 14DIV cortical neurons from Wild type and knock-out Mover mice were stained for Mover (red), Synaptophysin (SyPhy, green) and MAP2 (blue) (C).

3.7.2. Phosphorylation in presynaptic targeting of Mover

Mutations introduced at predicted phosphorylation sites did not show any impairment in the presynaptic targeting of Mover in wild type rat cultures. This could be because regulation of presynaptic targeting by phosphorylation may also

A.



B.

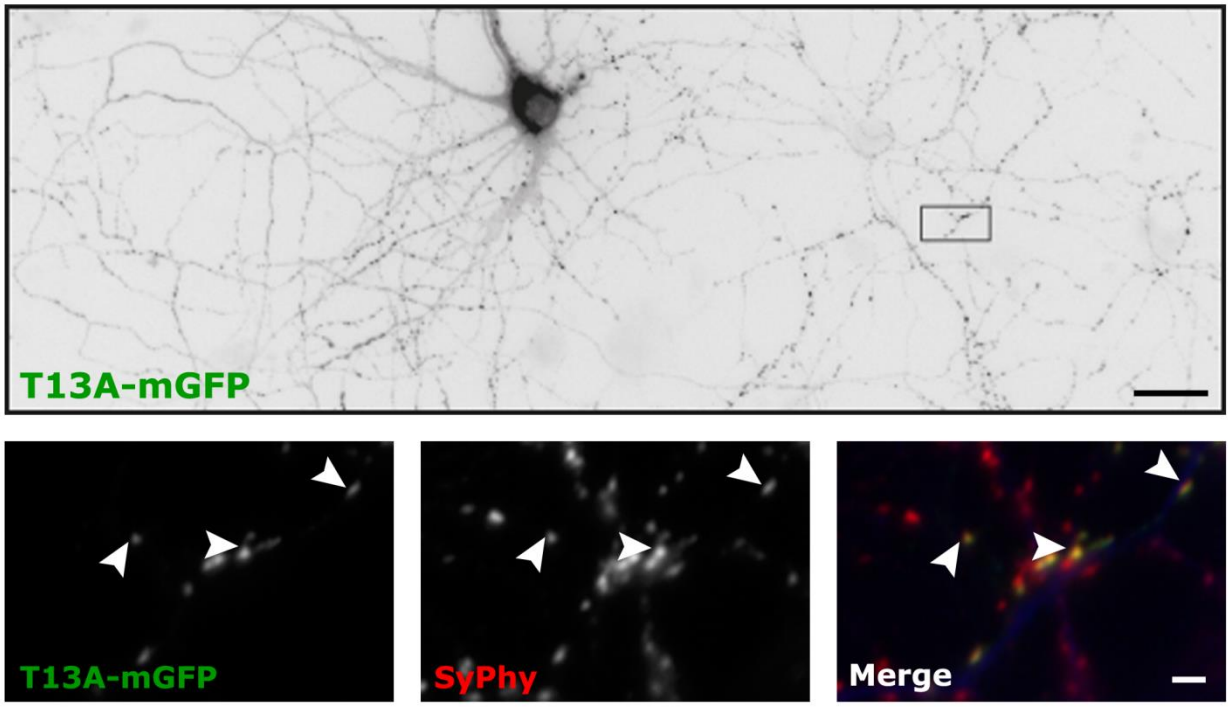


Fig.3.7.2. Point mutation at threonine 13 does not affect presynaptic targeting of Mover in Mover-deficient cultures. Expression of mGFP-tagged point full length Mover, 52-266-mGFP and the construct with a mutation at phosphorylation site threonine 13 (T13A) in 14DIV cultured hippocampal neurons showing a punctate pattern. Puncta co-localizing with Synaptophysin are indicated with arrow heads. Higher magnification images of the inlay are shown in individual channels (from left to right) - Over-expressed protein in green, Synaptophysin in red and a merge along with MAP2 in blue. Mover puncta co-localizing with Synaptophysin are pointed with arrow heads. Scale bar: 10µm for grey panel and 2µm for small black panels (A and B).

depend on self-interaction as Mover is a self-interacting protein. Therefore mutation at phosphorylation site 13 has been over-expresses in Mover knock-out hippocampal cultures along with full length Mover and N-terminal deletion mutant of aa51.Full length Mover and N-terminal deletion mutant of Mover produced discrete punctae that are presynaptic as they co-localize with endogenous Synapsin (Fig. 3.7.2;A). Mutating T13 did not impair presynaptic targeting of Mover even in Mover deficient cultures (Fig. 3.7.2; B). This could be due to the presence of this site (T13) within the variable N-terminal region of Mover that is unimportant for the presynaptic targeting and self-interaction of Mover. It will be interesting to test the expression of phosphorylation mutants at site 64 and 257 as they lie within the regions required for presynaptic targeting and self-interaction of Mover.

4. Discussion

Mover is a vertebrate specific presynaptic protein of 266 amino acids. Structurally and functionally nothing is known about Mover. In the current work, I aimed to characterize functional domains of Mover underlying its cellular properties, including presynaptic targeting, self-interaction and phosphorylation, and to determine the role of Mover in presynaptic terminals. To this end,

1. I used N-terminal or C-terminal GFP-tagged deletion mutants to identify the regions necessary for presynaptic targeting and self-interaction of Mover. This deletion analysis revealed different regions within the protein sequence including aa52-90, aa253-266, aa206 and 4 mutations in the predicted Calmodulin binding site to be necessary for presynaptic targeting and self-interaction.
2. I employed mutations changing threonine to alanine (to prevent phosphorylation) and mutations changing threonine to aspartate (to mimic phosphorylation) to investigate if phosphorylation may regulate the function of Mover.
3. I used immunoprecipitatation assays to test the interaction of Mover with Calmodulin, Bassoon and Munc13. No interaction of Mover was found with Bassoon and Munc13 (Appendix 7.4. A& B) but Mover bound to Calmodulin in the presence of Ca^{2+} *in vitro*.
4. I over-expressed full-length Mover and an N-terminal deletion mutant encoding amino acids 51-266 in cultured hippocampal neurons and found that both decreased the recycling pool of vesicles suggesting an inhibitory role for Mover in synaptic transmission.

4.1. Tprg, a homologue of Mover

Mover is found to share homology with Tprg (Transformation related protein 63 regulated), a protein expressed in skin. Because of this homology, Mover is also called Tprgl, i.e. Tprg-like (Antonini et al., 2008). Mover/Tprgl and Tprg are

vertebrate specific proteins that share 49% homology at the protein level. The former is associated with synaptic vesicles (Kremer et al., 2007) and the latter is predominantly expressed in skin with no expression detected in brain by PCR (Antonini et al., 2008). Expression of Mover-mGFP and Tprg-mGFP produces a punctate fluorescence pattern in primary hippocampal neurons that is typical of presynaptic targeting. These puncta have the same distribution as puncta created by expression of GFP-VAMP, a synaptic vesicle protein. Furthermore, the fluorescence puncta co-localize with endogenous Synaptophysin, indicating that they represent accumulation of the Mover and Tprg constructs at synapses. Antonini et al. (2008) did not detect Tprg mRNA in the brain, suggesting that Tprg is not expressed in brain tissue. However, antibodies that have been validated for western blot detection of endogenous Tprg are not available. At this point it cannot be excluded that Tprg is expressed in neurons at low levels. I found that recombinant Tprg accumulates in presynaptic terminals in transfected cultured neurons, indicating that the protein contains presynaptic targeting information. Tprg displays 49 percent amino acid identity with Mover overall, and the identity is 51 percent in the region downstream of amino acid 69 of Mover. Further inspection of the sequence alignment reveals that the identity is evenly distributed across the entire protein. Thus, sequence comparison does not hint to any individual domain that might be particularly important for presynaptic accumulation of Mover and Tprg. It rather seems that the overall structure of Mover and Tprg mediates targeting. Measuring presynaptic targeting of Mover-mGFP and Tprg-mGFP revealed that recombinant Mover is enriched twice the level of recombinant Tprg at presynaptic sites, but the sequence comparison does not hint to any amino acid sequences that could account for this difference in targeting efficacy. Since I cannot exclude that Tprg is expressed in the brain it was important to know if Mover can interact with Tprg. No interaction of recombinant Mover with recombinant Tprg is observed with co-immunoprecipitation experiments indicating a role for Mover by itself in the brain. Importantly, in overexpression experiments carried out in Mover knockout mice it is unlikely that recombinant Mover binds to endogenous Tprg, if it is expressed at all. Therefore Mover and Tprg are two different proteins that may have different roles to play. In future experiments; it would be interesting to test the expression of Tprg at protein level by using rat or mouse brain homogenates and

also by immunofluoroscence on neuronal cultures. In addition, to see if Mover and Tprg are in different set of synapses or brain regions, and to see if Tprg is upregulated in Mover knockouts.

4.2. Presynaptic targeting of Mover

Not much is known about the targeting of proteins to presynaptic sites. Predicted domains within protein sequence and structure would provide an insight into its functional role. Mover is a vertebrate specific presynaptic protein that has been identified as a binding partner of C-terminal region of Bassoon (Kremer et al., 2007). It has been further detected as a synaptic vesicle associated protein 30 (SVAP30) in a proteomics screen for synaptic vesicles (Burre et al., 2006). Absence of transmembrane domains and association of Mover to synaptic vesicles indicate that Mover is a peripheral membrane protein associated with SVs. This observation was confirmed by several assays including membrane floatation assay, TritonX-100 treatment, carbonate stripping and immunogold labeling of SVs (Ahmed et al., 2013). Several lines of evidence conclude that Mover is a peripheral membrane protein associated with SVs.

Structurally and functionally, little is known about Mover. Mover has no predicted homology domains except for a HSac2 domain spanning amino acids 53-163 and a predicted Calmodulin binding domain including amino acids 206-218. HSac2 belongs to family of inositol phosphatases and functions as inositol polyphosphate 5-phosphatase that are involved in several functions like synaptic vesicle recycling, synaptic vesicle trafficking and actin polymerization. Database analysis suggests that Mover gene is alternatively spliced producing two isoforms, a long form harboring Exons 1-4 and a short form lacking Exon2 (93-151).

Recombinant full length Mover is targeted to presynaptic terminals in cultured hippocampal neurons. But the regions or domains of Mover necessary for targeting to presynaptic terminals are unknown. In this study, regions required for presynaptic localization of Mover were dissected via truncation mutagenesis.

Monomeric GFP (mGFP) tagged versions of deletion mutants have been used to identify the domains of Mover required for localization to presynaptic terminals. A similar strategy has been used to study two other presynaptic proteins, α-synuclein and Synapsin (Yang et al., 2010; Gilter et al., 2004). Synapsins are the most abundant phosphoproteins present on synaptic vesicles and are involved in multiple functions of synaptic vesicle cycle. Domains B, C and E of Synapsin Ia are required binding sites involved in targeting of Synapsin Ia to presynaptic terminals. This observation indicates that multiple binding sites are required for targeting of Synapsin Ia to presynaptic terminals (Gilter et al., 2004). α-synuclein is a vertebrate specific presynaptic protein regulating neurotransmission in dopaminergic neurons. α-synuclein protein is unstructured with an amphipathic lysine rich N-terminus adopting a α-helical secondary structure upon binding to phospholipid membranes. Using deletion mutants of α- Synuclein, it was found that the N-terminal helix binding loop is necessary for the targeting of α-Synuclein to presynaptic sites (Yang et al., 2010). Mover as such has no predicted domains like α-synuclein or Synapsins and the recruitment of Mover to presynaptic sites is unknown. Similar to Synapsins, different sites have been found for the localization of Mover to presynaptic terminals in the current study and are discussed as follows:

The presence of either N-terminal GFP (Appendix 7.1 & 7.2) or C-terminal mGFP tag to the deletion mutants did not have any effect on their targeting to presynaptic sites. However, a full-length version of Mover with an N-terminal GFP tag aggregated upon expression in neurons. The study therefore focused on full-length Mover with C-terminal tags. All deletion constructs were produced with N-terminal and C-terminal tags, with no difference in targeting. Monomeric GFP tagged full length Mover and the N-terminal deletion mutant of aa51 were found to produce punctae along the axon that synaptic as they co-localize with endogenous Synaptophysin. Co-localization analysis with endogenous Synaptophysin shows no significant difference in the localization of full length Mover and the N-terminal deletion mutant to presynaptic terminals. In addition, the full length Mover and the N-terminal deletion mutant of aa51 were no different in their targeting to presynaptic terminals. These data suggest that Mover can still be targeted to presynaptic terminals without the N-terminal aa51. Amino acids 1-51 are predicted

to produce an unstructured conformation. This N-terminal region of Mover contains several parts where sequences are less well conserved across species than in the remaining parts of Mover. Thus, the N-terminal area of Mover may be relevant for other functions than a key property such as synaptic accumulation.

A construct lacking the sequences encoded by exon2 was generated to mimic a short splice isoform of Mover in which exon 2 is missing. This construct did not target to synapses in cultured neurons. A cDNA encoding such a splice isoform is found in data base searches. Interestingly, if such a splice isoform exists in the brain it is probably not synaptic. A construct encoding the HSac2 homology domain of Mover did not target to synapses, suggesting that the HSac2 domain is not sufficient for presynaptic targeting. In addition, several Mover constructs including the sequence encoded by exon2 (e.g. Mover-F206R-mGFP) do not target to presynaptic terminals. Thus, these two domains do not by themselves carry sufficient targeting information. However, both regions are required, because deleting parts of these regions impaired targeting. Deletion of N-terminal aa1-90 and the C-terminal 13 aa (253-266) impaired presynaptic targeting of Mover, generating a diffuse distribution along the axon. Thus, N-terminal aa51-90, a C-terminal region of aa13, a central exon2 region, and regions in the HSac2 domain of Mover are all necessary for the targeting of Mover to presynaptic terminals. These regions are widely distributed across Mover, suggesting that the entire protein (except for the N-terminal 51 amino acids) has to be present to adopt a functional conformation. The disruption of presynaptic targeting and the weak expression of these deletion mutants could be due to lack of proper folding or conformational changes, leading to protein degradation.

Not only that, a construct with four point mutations introduced into the predicted Calmodulin binding site also impaired presynaptic localization of Mover. Strikingly, a point mutation at aa206 from phenylalanine to arginine in the predicted Calmodulin binding site completely disrupts the localization of Mover to presynaptic terminals. Although different sites distributed over Mover protein sequence were found to be required for Mover to undergo presynaptic targeting, a single amino acid at 206 is sufficient for the presynaptic targeting of Mover. The fact that multiple regions are

required for presynaptic targeting of Mover can be attributed to the structure of Mover which is dominated by loops and possibly account for a highly dynamic structure prone to destabilization (PLYMOL prediction tool).Any change in the Mover protein structure except the N-terminal aa51 could disrupt the targeting to presynaptic terminals.

4.3. Role of Dimerization/ oligomerization in the presynaptic targeting of Mover

Dimerization/oligomerization of proteins is shown to be necessary for the targeting of presynaptic proteins like Synapsins, Bassoon, GAD65/GAD2and GAD67/GAD1 (Gitler et al., 2004; Maas et al., 2012; Kanaani et al., 2010). Very little is known about the targeting of proteins with no transmembrane regions. Multiple sites within the Bassoon Golgi binding region have been identified to undergo dimerization in addition to an oligomerization domain between aa2088-2563. A deletion mutant with aa1692-3263 harboring the oligomerization domain is found to be targeted to presynaptic sites but the expression of oligomerization domain alone disrupts the targeting of Bassoon in cultured hippocampal neurons (Dresbach et al., 2003; Maas et al., 2012). Synapsins are the peripheral membrane phosphoproteins associated with synaptic vesicles. Dimerization of Synapsin isoforms (Ia, IIa, and IIIa) allows their targeting to presynaptic sites. Heterodimerization with other isoforms of Synapsins is required for Synapsin Ib to be targeted to presynaptic terminals in cultured neurons (Gitler et al., 2004). Similarly, the region within the N-terminal 60 amino acids contains the information necessary for the presynaptic targeting of a peripheral membrane protein, GAD65 (Kanaani et al., 2002), and GAD65 can recruit the isoform GAD67 to synapses by hetero-dimerization (Kanaani et al., 2010). In most of the proteins, a combination of several areas distributed across the proteins and dimerization are involved in presynaptic targeting, but no consensus sequence seems to exist. Interestingly, the current study identifies multiple regions distributed over the Mover protein sequence are necessary for the self-association and presynaptic localization of Mover.

Yeast 2-hybrid assay suggests that full-length Mover undergoes self-interaction. The data that full length Mover undergoes self-interaction is further corroborated by a co-immunoprecipitation assay using two differently tagged recombinant Mover constructs in this study. Applying this assay, I next tested the regions required for self-interaction of Mover and compared them with the regions required for presynaptic targeting.

All the deletion mutants except for recombinant full length Mover and the N-terminal deletion of aa51 were found to self-interact forming a dimer (or oligomer) *in vitro.* The N-terminal deletion construct Mover-91-266-mGFP, the Exon2 deletion mutant, and further N- & C-terminal deletion constructs (aa53-163 and aa52-253) failed to dimerize, indicating that all the regions of Mover except the N-terminal 51 amino acids are required for the self-association of Mover. Not only that, four point mutations introduced into the predicted Calmodulin binding site also disrupt the dimerization of Mover. Interestingly, a single point mutation at aa206 was sufficient to completely disrupt the self-interaction of Mover, thus providing a link between the self-interaction and presynaptic targeting of Mover.

Overall, all the deletion mutants that impair presynaptic targeting also failed to dimerize in vitro. In particular, sites of aa52-aa90 and aa253-aa266, central Exon2 region, HSac2 domain, aa206 and the 4-mutations in the predicted Calmodulin binding site are required for Mover to undergo self-interaction and targeting to presynaptic terminals. Most importantly, a single amino acid at 206 was sufficient for Mover to undergo self-interaction and presynaptic targeting. Thus, regions necessary for the self-association and presynaptic localization of Mover are distributed over the entire Mover protein sequence.

4.4. Role of phosphorylation in the presynaptic targeting of Mover

Phosphorylation of proteins plays an important role in both pre-and post-synaptic plasticity. This is achieved through presynaptic modulation of neurotransmitter release by protein kinases and protein phosphatases. Most of the proteins involved

in multiple steps of synaptic vesicle cycling are found to be regulated by phosphorylation (refer to Fig 1.3). One best-studied protein is the Synapsin. Phosphorylation of Synapsin results in their dissociation from SVs in the reserve pool and mobilizing SVs to the active zone for exocytosis (Hosaka et al., 1999).

Mass spectrometry study of presynaptic proteins detected threonine phosphorylation at amino acid 13of Mover (Munton et al., 2007). Bioinformatical analysis also suggested additional predicted phosphorylation sites at aa14 (Serine), aa64 (Threonine) and aa 257 (Tyrosine). Threonine 13 is interesting because Mover phosphorylated at T13 is associated with SVs (Ahmed et al., 2013). Phosphorylation sites T64 (aa51-266) and Y257 (aa52-253) were also of interest as they lie within the regions required for self-interaction and targeting of Mover

I employed the expression of mutated forms of the Mover protein that were rendered non-phosphorylatable by changing threonine to alanine or tyrosine to phenylalanine, and phosphomimetic mutant where threonine was changed to aspartate. Each construct included one of the following mutations: a single point mutation introduced at threonine 13 or 64 (T13A, T64A), or tyrosine 257 (Y257F), double mutations at threonine 13 and 64(T13A-T64A) or threonine 13 and tyrosine 257 (T13A-Y257F), and a phosphomimetic mutation at threonine 13 to aspartate (T13D). Each of these constructs showed discrete punctae along the axon that co-localized with endogenous Synaptophysin, indicating that they accumulated at synapses. Co-localization analysis with endogenous Synaptophysin did not show any statistical difference between phospho-mutants of Mover and full length Mover. No statistical difference in the presynaptic targeting capacity between full length Mover and either the single or double phospho-mutants of Mover was observed. It is possible that over-expressed phospho-mutants bind to endogenous Mover protein present in cultured rat neurons and therefore are recruited to presynaptic sites. Hence it would be interesting to test the expression of either single or double phospho-mutants of Mover in Mover-deficient cultures. Alternatively, phosphorylation of Mover may have different functions other than targeting.

Co-immunoprecipitation experiments with all the phospho-mutants showed pull-down of all of the mGFP-tagged phospho-mutants of Mover with Mover-myc, indicating self-interaction. Therefore, the constructs may be deficient in presynaptic targeting but are recruited to synapses by the intact endogenous Mover protein present in cultured rat neurons. To test if the phosphorylation mutants still target to synapses in the absence of endogenous Mover, I expressed a phospho-mutant T13A in Mover knockout cultures. Unexpectedly, phospho-mutant T13A displayed a punctate fluorescence similar to full length and N-terminal deletion mutant. The puncta were synaptic as they co-localize with endogenous Synapsin. This could be due to the fact that the phosphorylation site at T13 lies with the N-terminal region of aa51 that is not required for Mover to undergo self-interaction and presynaptic targeting. Hence it would be interesting to see the phenotype of phosphorylation sites at aaT64 and Y257 as they are located within the sites necessary for the self-association and targeting of Mover (aa52-90 and c-terminal aa13). Future experiments would be to see how these two mutants (T64A and Y257A) either singly or double behave and this would provide a link to the role of phosphorylation in the presynaptic targeting of Mover.

Mover is lost from synaptic vesicles when synaptosomal fractions were incubated with bacterial lambda-phosphatase (Ahmed et al., 2013). This observation raised the possibility that the phosphorylation of Mover regulates its association with synaptic vesicles. If future experiments reveal that none of the predicted phosphorylation sites of Mover are required for targeting, one may conclude that phosphorylation of a direct or indirect binding partner of Mover is required for the association of Mover with presynaptic terminals. One possibility is that Mover binds to phosphorylated Bassoon (Collins et al., 2005; Schröder et al., 2013)

4.5. Interacting partners of Mover

Identifying interacting partners of Mover could provide a link to study the role and function of Mover in neurotransmitter release. The N-terminal region of Munc13-1 serves as common interaction region for active zone proteins including C-terminal

region of Bassoon (aa3601-3820) (Wang et al., 2009) and Calmodulin (Junge et al., 2004). Interestingly, Mover interacts with amino acids 3263-3938 of Bassoon in a yeast-2-hybrid assay, i.e. in the same region where Munc13 binds to Bassoon (Kremer et al., 2007). Knock-down of Mover at Calyx of Held synapse increases release probability and Ca^{2+} sensitivity of release without affecting the size of RRP (Körber C, published thesis). In contrast, knock-down of Calmodulin showed a decrease in release probability with unaltered RRP (Pang et al. 2010). I detected binding of Mover to Calmodulin with co-immunoprecipitation of recombinant protein. In addition, recombinant Calmodulin pulls down endogenous Mover from rat brain synaptosomes in the presence of Ca^{2+} (Saheeb Ahmed, unpublished data). Database analysis of Mover predicts a Calmodulin binding site between aa206-218. Together with these data, a point mutation was introduced at the hydrophobic phenylalanine residue of the Calmodulin binding site (206) to arginine to block Calmodulin binding. A similar mutagenesis strategy is published for Munc13-1, where a tryptophan to Arginine mutation blocked Calmodulin binding with no effect on the structural properties of Munc13-1 (Junge et al., 2004). In contrast to this study, no effect was observed on the binding of Mover to Calmodulin when phenylalanine 206 is mutated, but homomeric interaction and presynaptic targeting were abolished, indicating a profound effect on the structure and integrity of Mover. These data also indicate that monomeric and dimeric/ oligomeric Mover bind to Calmodulin, but only the dimerization / oligomerization – competent Mover is stable and accumulates at synapses in cultured neurons. Thus, binding of Mover to Calmodulin does not mediate targeting to presynaptic sites.

Because knock-down of Mover and Calmodulin individually have opposing effects on release probability, we assumed that Mover may regulate release probability by inhibiting Calmodulin. Inhibition of Calmodulin may reduce the action of Calmodulin targets such as CamKII (Ca^{2+}-Calmodulin dependent protein kinase II) (Popoli et al., 1993), Munc13-1 (Junge et al., 2004). However, in our assay, we found no effect of Mover on the CaM-dependent phosphorylation of CaMKII. It is possible that Mover may inhibit the action of Calmodulin on Munc13-1. Next, I tested if Mover inhibits Calmodulin and affects Munc13-1 interaction; preliminary experiments show decreased levels of either Calmodulin or the

Munc13-1. Although no interaction of Mover with Munc13-1 was found in co-immunoprecipitation experiments (Appendix 7.4, B).

4.6. Role of Mover in synaptic vesicle recycling

Knockdown of Mover at the Calyx of Held increases release probability, short-term depression and recovery from depression (Körber reference). Thus, the role of Mover may be to inhibit these events. To test if increased Mover levels affect the synaptic vesicle cycle, I employed a synaptotagmin1 antibody uptake assay. In this assay, antibodies against the intravascular domain of synaptotagmin are taken up into recycling synaptic vesicles during endocytosis after induced exocytosis. Because the N-terminal deletion mutant Mover-52-266-mGFP behaves like full length Mover in its cellular properties (targeting and dimerization), it was interesting to test if this deletion mutant also behaved like full-length Mover in the recycling assay. Measuring Synaptotagmin uptake intensities for the Mover-mGFP puncta positive for Synaptophysin-mOrange showed a robust decrease in the total pool of recycling vesicles compared to mGFP. The same decrease was observed withMover-52-266-mGFP compared to mGFP, but no significant difference was observed between full length Mover and Mover-52-266-mGFP, suggesting that Mover lacking the N-terminal 51 amino acids may be sufficient for Mover to regulate the synaptic vesicle cycle.

The reduction in synaptic vesicle recycling produced by Mover-1-266-mGFP and Mover-52-266-mGFP is due to a reduction in the Ca2+-dependent exocytosis, in endocytosis, or in replenishment of the releasable pool of vesicles. One possibility is that Mover inhibits one or more of these steps by inhibiting the action of Calmodulin. Calmodulin regulates multiple presynaptic events, including release probability, calcium channel inactivation, endocytosis, RRP replenishment and recovery from depression (Pang et al, 2010, Mochida et al, 2011, Lipstein et al, 2013, Rehger et al, 2015). Mover and Calmodulin act in opposite directions, raising the possibility that Mover inhibits Calmodulin. I tested if Mover inhibits the action of Calmodulin on activation of CaMKII and found no effect. An alternative option is that

Mover selectively inhibits the interaction of Calmodulin with Munc13. This interaction enhances replenishment of the RRP (Junge et al., 2004; Lipstein et al., 2013).

A reduction in the total pool of recycling vesicles could also result from interactions with proteins other than Calmodulin. For example, Tomosyn, a syntaxin binding protein is known to decrease synaptic transmission and release probability of vesicles by priming of synaptic vesicles and interfering with the SNARE complex formation, the essential machinery of vesicle fusion thereby inhibiting neurotransmitter release (Barak et al., 2010).Further knowledge about binding partners of Mover is needed to unravel the molecular mechanisms underlying its inhibitory role. In particular, a mutant construct that fails to bind to Calmodulin but targets to presynaptic terminals would help address these questions.

In summary, self-interaction of Mover is required for Mover to undergo targeting to presynaptic terminals. Sites distributed over the Mover protein sequence mediate both self-interaction and targeting of Mover to presynaptic terminals. Mutations introduced into the predicted phosphorylation site at T13 had no effect on the targeting of Mover but biochemical data suggests that phosphorylation is associated with binding of Mover to SVs (Ahmed et al, 2013). Phosphorylation of Mover may switch the transition of SVs from the reserve pool to active zones, where Mover binds to a C-terminal region of Bassoon. The C-terminal region of Bassoon acts as a common interaction region for the Munc13-1-CaM complex (amino acids 3601-3820; Wang et al, 2009) and Mover (amino acids 3263-3938;Kremer et al, 2007).Mover in turn also binds to Ca^{2+}-Calmodulin and inhibits SV recycling (this study). Mover may be present on SVs as a dimer, and one could speculate that one Mover copy would bind to Bassoon and the other to Calmodulin. This could inhibit the Munc13-1-CaM interaction, down regulating priming of synaptic vesicles by Munc13s resulting in reduced SV replenishment. Alternatively, Mover may regulate the interaction of Bassoon with voltage gated calcium channels and increase the distance between docked synaptic vesicles and sites of calcium influx, thus reducing release probability and short-term depression.

4.7. Future perspectives

In the future, it would be interesting to

1. test the expression of Tprg in the brain by western blotting using brain homogenates from wild type and Mover knock-out mice and also by immunofluoroscence on cultured neurons from wild type and Mover deficient mice.

2. test if Mover undergoes dimerization/oligomerization in the presence of Ca^{2+} using co-immunoprecipitation assay.

3. test the role of phosphorylation in the presynaptic targeting and synaptic vesicle recycling. This would allow identifying a role for phosphorylation of Mover.

4. test whether the deletion mutant harboring 4 exchanges in the predicted Calmodulin binding site affects the binding of Mover to Calmodulin and whether Mover affects the Munc13-1-Calmdulin interaction

5. unravel the role of Mover in synaptic vesicle recycling using wild type and Mover deficient cultures.

6. determine the expression levels of presynaptic proteins in Mover knock-out cultures as preliminary results from Mover knock-out cultures reveal a reduction in the Synaptophysin levels.

5. Summary

Synapses are asymmetric intercellular junctions. Targeting of synaptic vesicles to presynaptic sites is one of the most intricate examples of polarized trafficking and selective protein accumulation. Little is known about amino acid sequences or structural determinants mediating presynaptic targeting of synaptic vesicle proteins. Mover / TPRGL / SVAP30 is a 266 amino acid protein associated with synaptic vesicles as a peripheral membrane protein. Structurally nothing is known about Mover except for the presence of a predicted HSac2 domain, phosphorylation sites and a predicted Calmodulin binding site. The regions or amino acid sequences mediating targeting of Mover to presynaptic terminals are unknown.

I found that dimerization of Mover allows the targeting of Mover to synaptic vesicles. Sites widely distributed over large parts of Mover mediate both self-interaction and presynaptic targeting of Mover. The HSac2 homology domain of Mover and the part encoded by the alternatively spliced exon 2 are required but not sufficient for targeting. Despite strong homomerization Mover does not heterodimerize with its paralogue TPRG. Mover is a novel binding partner for Ca^{2+}/Calmodulin but this interaction does not mediate the presynaptic targeting of Mover, because a point mutated variant of Mover that still binds to Calmodulin is deficient for dimerization and targeting. Mutations introduced into the predicted phosphorylation sites had no effect on dimerization and targeting of Mover suggesting a function for phosphorylation other than targeting. Over-expression of Mover reduces the pool of recycling vesicles suggesting an inhibitory role in neurotransmitter release. A Mover knockout mouse was generated to explore the role of Mover for presynaptic function, and studies investigating synaptic vesicle recycling in cultures from these mice are underway.

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7. Appendix

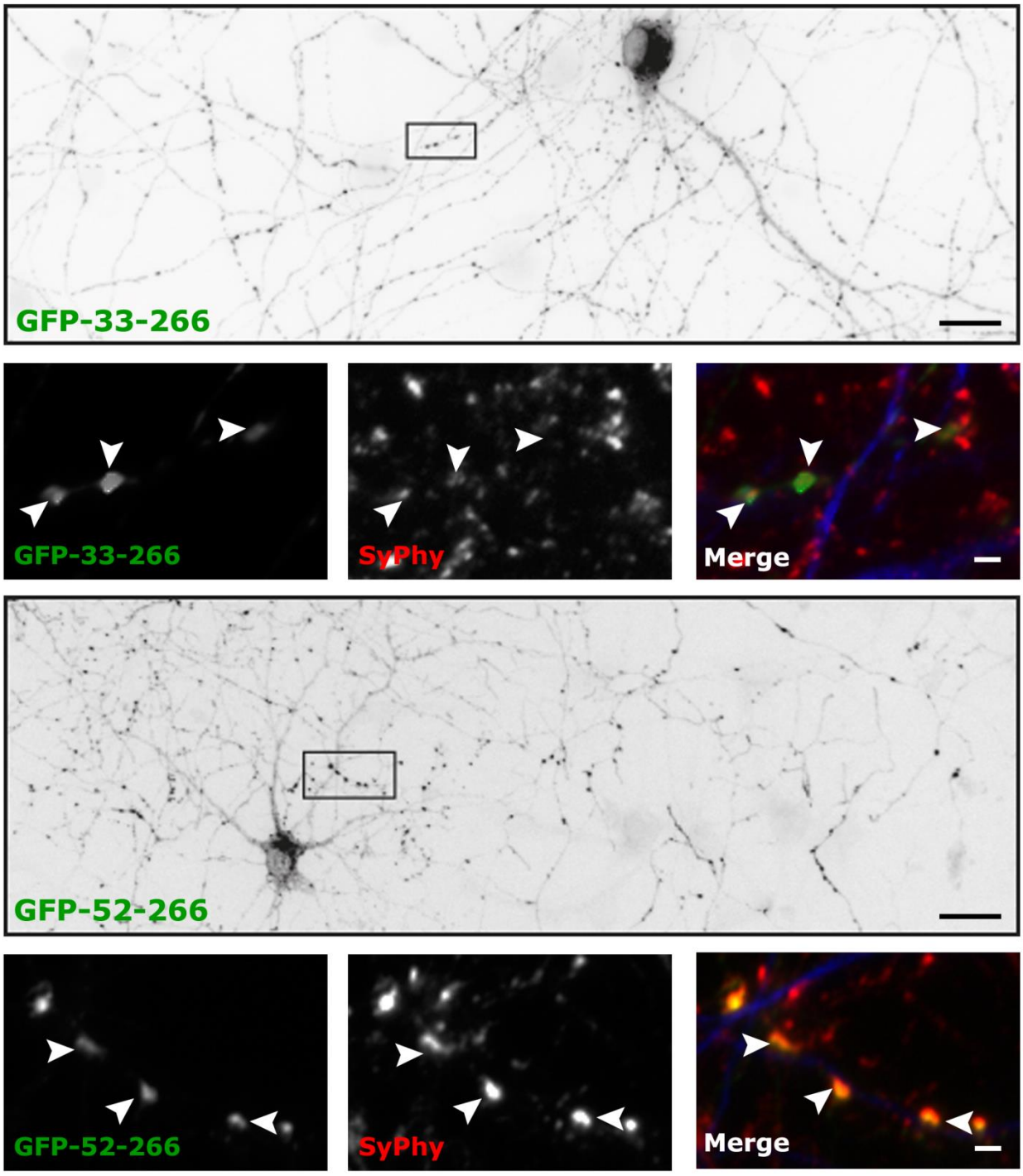

Fig.7.1. GFP-33-266 andGFP-52-266are accumulated at presynaptic sites. Expression of N-terminal GFP-tagged33-266and 52-266 showing a punctate fluorescence pattern in 14DIV cultured hippocampal neurons. Inlay is shown as higher magnification images (left to right) of the construct(green), endogenous Synaptophysin (red) and merge (along with MAP2).Arrow heads indicate synaptic puncta. Scale bar: 10µm for grey panel and 2µm for higher magnification images (A).

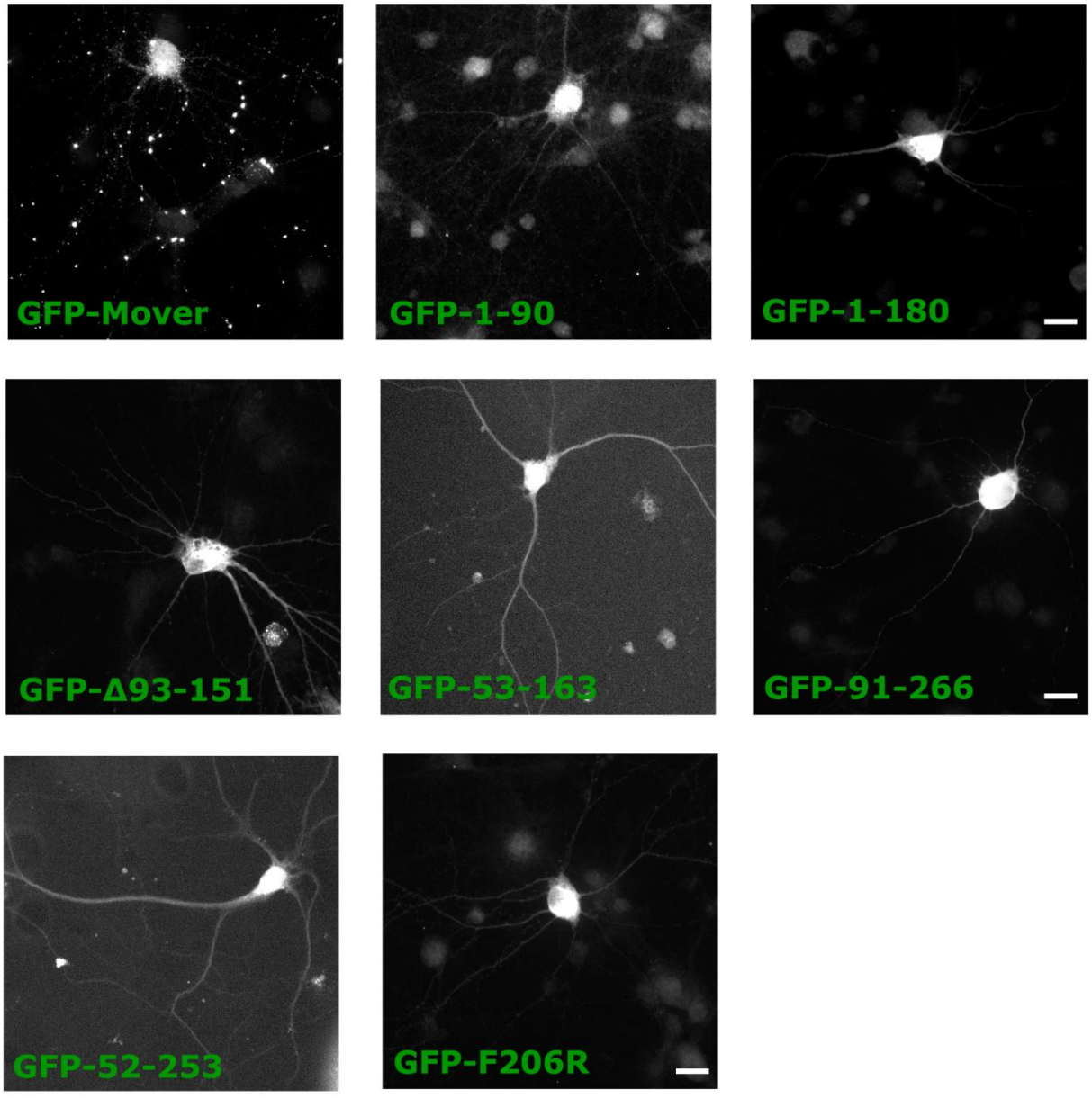

Fig.7.2. Localization of N-terminal GFP-tagged deletion mutants of Mover to presynaptic sites. Expression of N-terminal GFP-tagged1-90, 1-180, Δ93-151, 53-163, 91-266, 52-253 and F206R showing diffuse distribution in 14DIV cultured hippocampal neurons. Green fluorescent images shown in black and white .Scale bar: 10μm .

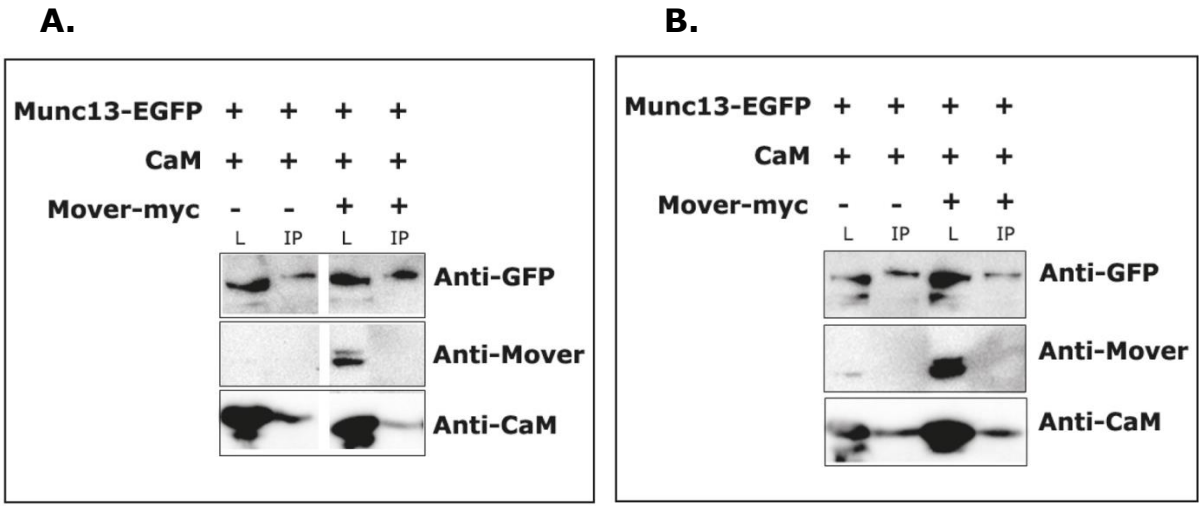

Fig.7.3. Recombinant Mover reduces the levels of either Calmodulin or Munc13-1 in vitro. Munc13-1-EGFP from Hek293T cell lysates is immunoprecipitated using mouse monoclonal anti-GFP antibody. Munc13-1 and CaM were immunoprecipitated upon IP with anti-GFP-antibody. Addition of Mover-myc resulted in decreased levels of either Munc13-1 (B) or CaM (A).

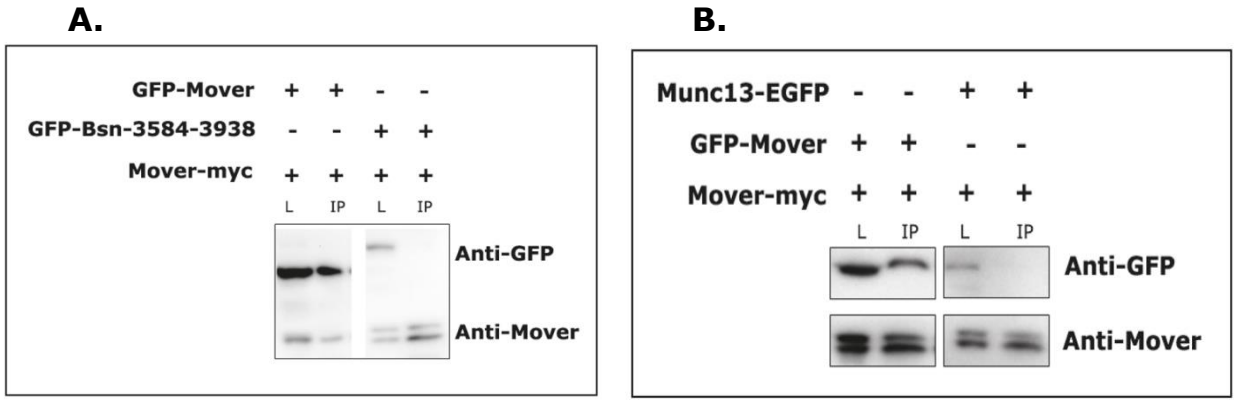

Fig.7.4. Co-immunoprecipitation experiments of Mover with Bassoon and Munc13-1. Mover-myc does not bind to GFP-Bsn-3584-3938 in co-immunoprecipitation experiments. Mover-myc from Hek cell lysates was immunoprecipitated using mouse monoclonal antibody against myc tag. GFP-Bsn-3584-3938 does not immunoprecipitate upon IP with Mover-myc (A). Munc13-EGFP co-expressed with Mover-myc does not immunoprecipitate upon IP with mouse monoclonal antibody against myc tag (B).

Curriculum Vitae

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Publications

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Conferences

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