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Postsynaptic machinery formation and remodelling - the roles of αdystrobrevin-1-interacting proteins and of extracellular matrix

PhD thesis

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Achievements

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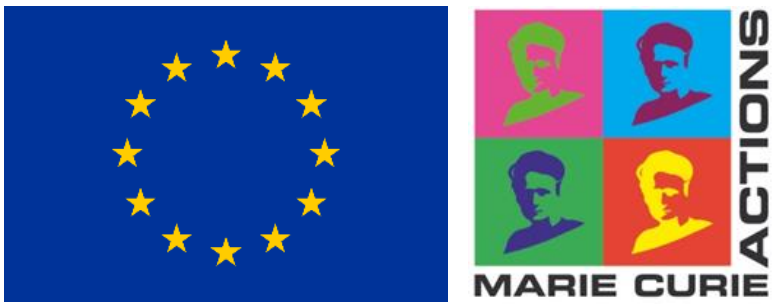

Abbreviations

Abbreviation	Descritpion
ACh	Acetylcholine
AChE	Acetylcholine esterase
AChR	Acetylcholine receptors
Amotl2	Angiomotin-like 2
AP	Action potential
APS	Ammonium persulfate
Arhgef5	Rho guanine nucleotide exchange factor 5
ATP	Adenosine triphosphate
AZ	Active zone
BL	Basal lamina
BSA	Bovine serum albumin
BTX	α-bungarotoxin
CaMKII	Calcium/calmodulin- dependent kinase
ChAT	Choline acetyltransferase
CLASP	CLIP-associated protein
CMS	Congenital myasthenic syndrome
CNS	Central nervous system
DG	Dystroglycan
DGC	Dystrophin Glycoprotein Complex
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
Dok7	Docking protein 7
DTT	Dithiothreitol
EB1	End binding protein 1
ECM	Extracellular matrix
ErbB	Epidermal growth factor receptor family
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HEK	Human embryonic kidney
HRP	horseradish peroxidase
HS	Horse serum
LL5β	Pleckstrin homology-like domain family B member 2
Lrp4	Lipoprotein receptor-related protein 4
MAC	Membrane attack complex
MG	Myasthenia Gravis
MT	Microtubules
MuSK	Muscle-specific kinase
NGS	Normal goat serum
NMJ	Neuromuscular junction
NP-40	Igepal CA-630
NRG	Neuregulin
N-Wasp	Wiscott-Aldrich Syndrome protein
PBS	Phosphate-buffered saline

PFA	Paraformaldehyde
PLA	Proximity ligation assay
PNS	Peripheral nervous system
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	short-interfering RNA
TA	Tibialis anterior
TBST	Tris-buffered saline with tween
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris-HCl	Hydroxymethyl aminomethane hydrochloride
Y730	Tyrosine 730
αDB1	α-dystrobrevin 1

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Abstract

The neuro-muscular junction (NMJ) is a chemical synapse between a motor neuron and a skeletal muscle fibre. The communication between the two cell types is endured by the release of neurotransmitter acetylcholine (ACh) from the presynaptic nerve terminal, which after binding to its receptor (AChR) induces exchange of ions between the myocyte and extracellular space, leading to depolarization of the muscle and its contraction. There are currently over 300 identified neuromuscular diseases. Among those with known etiology, aberrations in protein complexes, which stabilize the postsynaptic machinery within the plasma membrane such as the dystrophin-associated protein complex (DGC) appear to be a common cause of neuromuscular disorders. The DGC is responsible for anchoring the postsynaptic machinery to the cytoskeleton and extracellular matrix proteins. αDystrobrevin-1 (αDB1) is one of the key components of the DGC, as its absence leads to severe disruptions of the postsynaptic machinery organization. However, the exact function of αDB1 remains undiscovered. In order to understand how does αDB1 contribute to the organization of the postsynaptic apparatus, I performed an analysis of αDB1-interacting proteins and characterized some of their functions. Two proteins were identified though as mass spectrometry analysis that have not been yet studied at the NMJ before- liprin-α-1 and Tks5. Through my work I demonstrated that a scaffold protein liprin-α-1 is responsible for organization of cortical microtubules located below the postsynaptic machinery, allowing the AChRs to form clusters. On the other hand, I show that Tks5 is involved in formation of AChR cluster-remodelling structures known as synaptic podosomes, as well as recruitment of actin filaments to the postsynaptic machinery. Additionally, I developed an improved method of culturing myotubes *in vitro* leading to enhanced formation of the AChR clusters through the use of alternative laminin isoforms. I have also applied this method successfully to primary human muscle cells.

Streszczenie

Złącze nerwowo-mięśniowe jest chemiczną synapsą pomiędzy neuronem motorycznym a włóknem mięśnia szkieletowego. Do komunikacji pomiędzy dwoma typami komórek dochodzi przez uwolnienie neuroprzekaźnika acetylocholiny (ACh) z terminalu presynaptycznego, który po złączeniu się ze swoim receptorem (AChR) umożliwia wymianę jonów pomiędzy miocytem, a przestrzenią pozakomórkową co prowadzi do depolaryzacji mięśnia i jego skurczu. Obecnie rozpoznanych jest ponad 300 chorób układu nerwowo-mięśniowego. Pośród tych, których etiologia została poznana, zaburzenia często wynikają ze zmian w kompleksach białkowych które stabilizują molekularną maszynerię postsynaptyczną wewnątrz błony komórkowej takich jak kompleks dystroglicanu (DGC). DGC jest odpowiedzialny za zakotwiczenie maszynerii postsynaptycznej do filamentów cytoszkieletu oraz białek macierzy pozakomórkowej. Białko α-dystrobrewina 1 (αDB1) jest jednym z kluczowych elementów DGC, gdyż jego brak powoduje zaburzenia w organizacji całej maszynerii postsynaptycznej. Jednakże dokładna funkcja αDB1 jest nie odkryta. W celu zrozumienia, w jaki sposób αDB1 przyczynia się do organizacji maszynerii postsynaptycznej, podjąłem się identyfikacji oraz analizy funkcji białek oddziaływujących z αDB1. Moja praca skupiła się na badaniu dwóch białek- lipriny-α-1 oraz Tks5, gdyż analiza spektometrii mas wykazała, iż oba łączą się z αDB1, lecz żadne nie było wcześniej opisane w kontekście maszynerii postsynaptycznej. Dowiodłem, że białko rusztowania lipryna-α-1 jest odpowiedzialne za organizację mikrotubul znajdujących się bezpośrednio pod AChR, dzięki którym receptory dostarczone do błony komórkowej są w stanie tworzyć charakterystyczne dla maszynerii postsynaptycznej skupiska znane jako klastry AChR. W badanich nad Tks5 wykazałem, że jest odpowiedzialne za tworzenie się struktur znanych jako podosomy synaptyczne wewnątrz klastrów AChR, oraz za modulowanie oddziaływania cytoszkieletu aktyny z maszynerią postsynaptyczną. Dodatkowo, opisałem nową metodę hodowli komórek mięśniowych *in vitro* w celu uzyskania kultur ze zwiększoną ilością klastrów AChR, bazującą na pokrywaniu powierzchni hodowlanej alternatywnymi izoformami białka lamininy. Metodę tę, z sukcesem udało mi się również zastosowań w pierwotnej hodowli ludzkich komórek mięśniowych.

1. Introduction

1.1. The tripartite synapse

The key unit of signal transduction by the nervous system is a synapse. It connects either two neurons with each other, as is the case in the central nervous system (CNS), or a motor neuron of the peripheral nervous system (PNS) with another type of a tissue, such as the muscle. The appropriate functioning of these units enables a range of critical activities essential for life, such as perception, memory formation and cognition but also movement and other motor activities (Lai and Ip 2003, Grossberg 2015, Slater 2017). While the synapses that we observe in the brain have many similar features to the ones found outside of the CNS, there are also significant differences between them on the molecular as well as the physiological level (Lai and Ip 2003). Due to the fact that it is the largest synapse present in vertebrates, the neuromuscular junction (NMJ) is a widely used model for studying the biology of synapses (Harris and Littleton 2015). The NMJ is a complex system comprising of the motor neuron that stems out from a spinal cord and embeds itself on top of the muscle fibre. There is also a third component in this system, namely the Schwann cells, that are wrapped around the neuronal end (Auld and Robitaille 2003). Because of this arrangement, the NMJ is also referred to as the "tripartite synapse"(Li, Xiong et al. 2018). However, since the neuron is not in a direct contact with the muscle, the layer of extracellular matrix (ECM) between the two cells also plays an important role in regulation and maintenance of this synapse (Singhal and Martin 2011). Taken together, the NMJ is an enigmatic and elegant system, that reflects perfectly the biological notion of the structure ensuring function, which I will demonstrate in the following chapters.

1.1.1. The motor nerve

The neuron is a specialized cell that is used to transmit electrical impulses throughout the body. It is made up of an axon, soma and the dendrites. The axon is an elongated protrusion that reach is out of the soma to from a presynaptic terminal at the very tip of the filament. Dendrites are much shorter extensions that form a system of brunched structures that serve as the

postsynaptic terminals. The electrical signal, or an action potential (AP) directed to stimulate the muscle goes through the myelinated motor neuron in the spinal cord and travels all the way to the unmyelinated nerve terminal embedded on the membrane of a muscle fibre (Slater 2015, Nishimune and Shigemoto 2018). The APs arise as a result of membrane depolarization of a neuronal cell. This occurs through the initial hyperpolarization of the cell, which triggers opening of Na^+ channels, allowing the influx of Na^+ cations, which causes further opening of the channels and amplification of the signal (**Figure 1**) (Haimovich, Schotland et al. 1987, Martyn, Fagerlund et al. 2009).

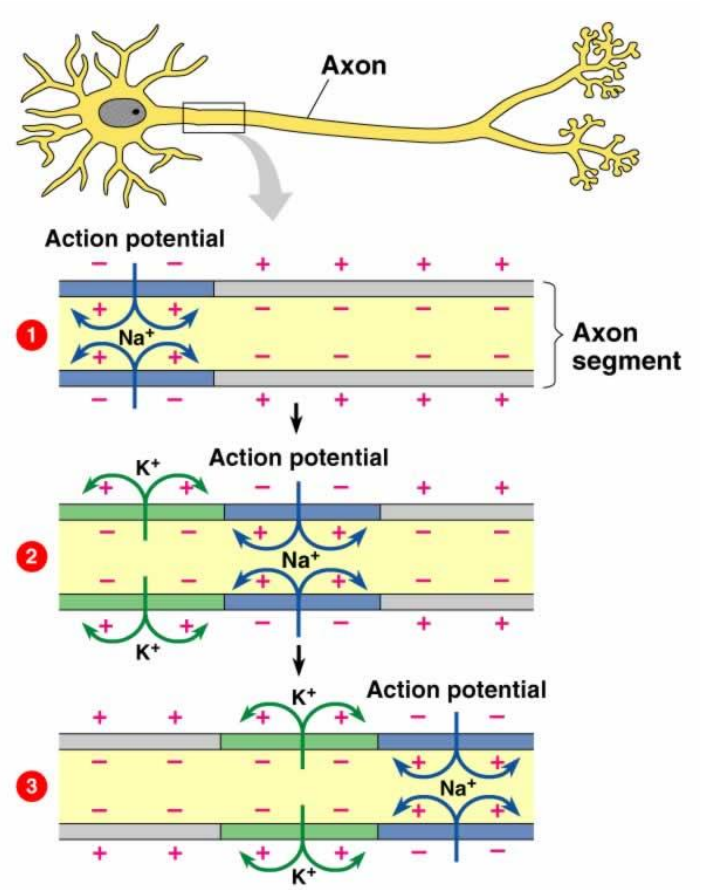

Figure 1: Propagation of the action potential through the neuronal axon. *As the electrical impulse reaches the soma of a neuronal cell, the depolarization begins to spread down the axon by opening of the Na^+ channels. As the signal travels further, the part of the neuron where the signalling was initiated begins to repolarize by closing of the Na^+ channels and subsequent opening of K^+ channels, thus preventing repeated depolarization for a short time, leading to a refractory period (Martyn, Fagerlund et al. 2009). Image from: https://psychology.stackexchange.com/questions/9173/explanatory-gaps-in-the-formation-and-propagation-of-action-potentials.*

The flow of Na^+ towards the cell is ensured by several-fold higher concentration of these ions outside of the neuron. Once the cell reaches an appropriate level of depolarization, the sodium channels begin to close, and Na^+ ions are

transported out of the neuron. This is followed by K^+ channels opening, which allows the potassium ions to flow out of the cell, as the K^+ concentration is lower outside of the neuron, leading to repolarization of the neuron to eventually bringing it to its resting potential (Slater 2015).

As the AP reaches the presynaptic nerve terminal of a motor neuron that innervates a muscle fibre, a change from an electrical into chemical signal is initiated. This occurs via the release of neurotransmitter acetylcholine (ACh), which is synthesized at the nerve terminal, and stored in synaptic vesicles (Del Castillo and Katz 1954, Del Castillo and Katz 1954, Whittaker, Michaelson et al. 1964, Hartzell, Kuffler et al. 1976). These chemicals will act as messengers in the communication between the neuron and the muscle fibre. When the AP reaches the nerve terminal, it again triggers membrane depolarization, which in this case leads to the opening of voltage-gated Ca^{2+} channels (Katz and Miledi 1967). Due to the fact that at a resting state, the Ca^{2+} ion concentrations outside of the cell is 10,000 times higher than inside of the cell, the opening of these channels leads to an extremely rapid influx of Ca^{2+} into the presynaptic nerve terminal (Rowley, Mantilla et al. 2007). The synaptic vesicles that contain neurotransmitter are primed for rapid release by forming a complex with the presynaptic membrane through v-SNARES (Vesicle-Soluble NSF [N-Ethylmaleimide-Sensitive Factor] Attachment Protein Receptor) and t-SNARES (Target-Soluble NSF Attachment Protein Receptor) (**Figure 2**) (Littleton, Chapman et al. 1998, Sutton, Fasshauer et al. 1998, Chen, Scales et al. 1999).

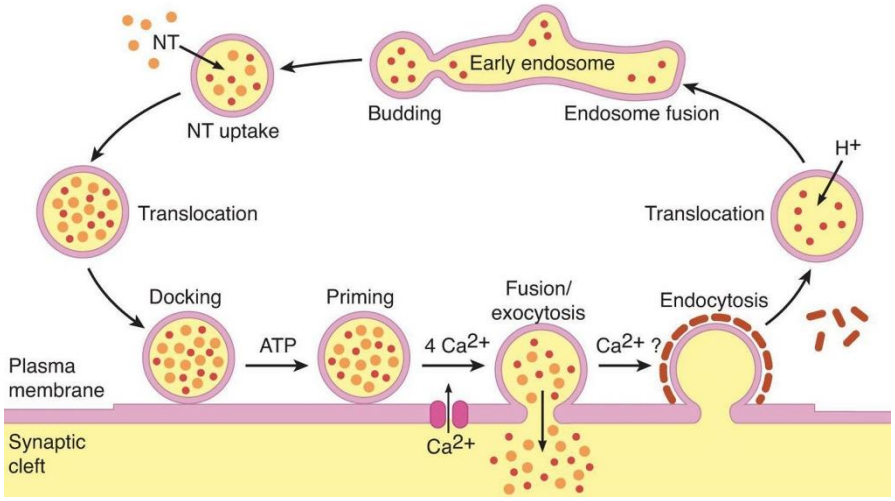

Figure 2: Synaptic vesicle cycle. *In order to ensure appropriate synaptic transmission, a stable amount of synaptic vesicles needs to be present at the nerve terminal. For this reason, the vesicles undergo a recycling process. After loading the vesicle with neurotransmitters, they get relocated and docked at the active zones. There, they undergo a process termed priming that involves ATP hydrolysis and allows for rapid exocytosis of the vesicles upon the arrival of the AP. Once the* Ca^{2+} *channels open, the neurotransmitter is released into the synaptic cleft. Then, vesicles are endocytosed through clathrin-coated pits, followed by their reacidification and refilling with neurotransmitters after going through an endosomal intermediate (Sudhof 2004). Image from: https://doctorlib.info/physiology/ganong-review-medical-physiology/7.html*

The Ca^{2+} that entered the cell bind to another vesicle protein known as synaptotagmin, which in response forms a complex with the v- and t-SNARES triggering exocytosis of the vesicles (Littleton, Stern et al. 1993, Broadie, Bellen et al. 1994). The ACh is then released into the synaptic cleft. Areas in the presynaptic membrane where the synaptic vesicles are primed for release are known as active zones (AZs) (Feeney, Karunanithi et al. 1998, Harlow, Ress et al. 2001). Once the neurotransmitter is released, it is picked up by acetylcholine receptors (AChRs) located on the muscle membrane. The ACh activity is then terminated by an enzyme acetylcholine esterase (AChE), which degrades the neurotransmitter to prevent overstimulation of the muscle (Silman and Sussman 2008).

1.1.2. The muscle

The muscle fibre contains multiple complexes involved in synaptic transmission, that are collectively referred to as the postsynaptic machinery. The most important protein in this assembly is transmembrane, ligand-gated ion channel the AChR (Fertuck and Salpeter 1974). The AChR is a transmembrane heteropentameric protein, made up of two α subunits, one β subunit, one δ

subunit as well as one γ or ε subunit, depending on the developmental stage. The extracellular N-terminal domain is responsible for binding to ACh, while the cytoplasmic C-terminal loop binds various regulatory proteins (Cetin, Beeson et al. 2020). The interaction of AChR with the neurotransmitter leads to a conformational change of the receptor, allowing influx of Na^+ ions, which depolarizes the muscle membrane. This change in electrical potential triggers opening of voltage-gated sodium channels, leading to a further influx of cations and propagation of the signal, which results in muscle contraction (Flucher and Daniels 1989, Wu, Xiong et al. 2010). The ACh leaves the receptor immediately to prevent prolonged stimulation, and is hydrolysed in the synaptic cleft by AChE (Jasmin, Lee et al. 1993). Appropriate functioning of AChE is crucial for clearing the neurotransmitter from the synaptic cleft, and mutation in the gene encoding this protein are associated with sever neuromuscular defects.

In order to significantly increase the area of the postsynaptic endplate and amplify synaptic signalling, the surface of the muscle underneath the motor nerve contains structures known as synaptic folds (**Figure 3**) (Johansen, Halpern et al. 1989, Slater 2008). These are invaginations in the postsynaptic membrane that harbour the postsynaptic machinery, and their openings are perfectly aligned with the AZs of the presynaptic nerve terminal (Meinertzhagen, Govind et al. 1998, Rowley, Mantilla et al. 2007). The AChRs are located at the crests of the synaptic folds, while the voltage-gated Na^+ channels concentrate at the depths of the invaginations (Haimovich, Schotland et al. 1987). This arrangement of pre-and postsynaptic membranes allows the neurotransmitters

to be efficiently picked up by the receptors, and for the resulting current to optimally depolarize the membrane (Slater 2008).

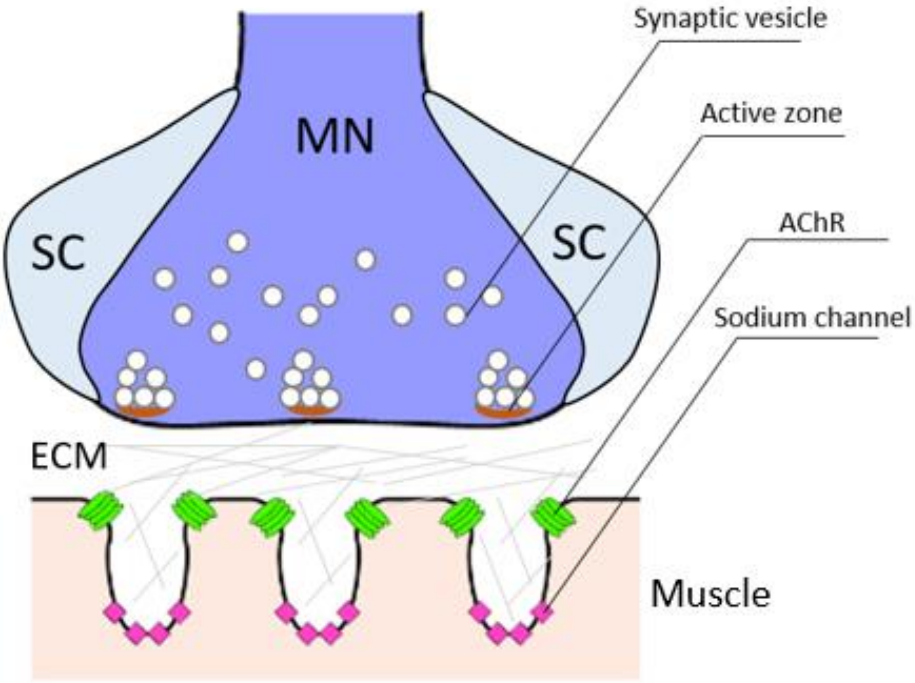

Figure 3: The transsynaptic alignment. *A diagram showing a cross-section through the NMJ. The important feature of the NMJ is the perfect alignment of AZs with the openings of postsynaptic folds. The AChRs are located at the crests of the invaginations, while the sodium channels at their depths. MN- motor neuron; SC- Schwann cell; ECM- extracellular matrix*

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1.1.3. Muscle contraction

The purpose of synaptic transmission at the NMJ is to evoke signals leading to contraction of the muscle fibre in order to generate movement or induce other motor activities such as maintaining posture. For this to happen, the electrical signal must travel through the motor nerves that then act on the muscle through ACh. Since a single neuron can innervate numerous muscle fibres forming a so-called motor unit, the individual fibres can be stimulated into a coordinated contraction (Lamb 2000). The basic unit of the contractile machinery is a large structure known as a sarcomere. The sarcomere is made up of actin filaments (thin) and myosin filaments (thick). This formation converts energy stored in the form of adenosine triphosphate (ATP) into mechanical work as changes in the length of a sarcomere are reflected by the contraction of a muscle (Lieber, Roberts et al. 2017). Muscle myosins are hexameric motor proteins composed of two heavy chains and two pairs of light chains with regulatory and support functions. The N-terminal part of the heavy

chains form globular, actin-binding head domain (motor domain) capable of ATP hydrolysis, followed by the neck domain with the light chain binding sites and C-terminal helical rod involved in dimerization and formation of the thick filament. ATP hydrolysis generates energy leading to sliding of both filament types against each other (Huxley and Niedergerke 1954, Huxley and Hanson 1954). Of note, there are also other types of myosins, termed as unconventional ones, that are not able to form filaments but contain a cargo-binding domain and are involved in transport of vesicles and other particles across actin filament (Irving 2017). The contraction is initiated by Ca^{2+} that enter the cell after stimulation with ACh. These ions then bind to the actin-binding troponin-tropomyosin, inducing a series of conformational changes that expose a binding site on actin for the myosin head (Sweeney and Hammers 2018). The myosin must hydrolyse ATP in order to bind to the thin filament and form a cross-bridge with the thick filament. Following a release of a phosphate group that induces a rotation of the catalytic head a force that is needed for the sliding of actin on the think filament is generated. The myosin can then bind to a new ATP molecule and the cycle is repeated (Sweeney and Hammers 2018).

1.1.4. Terminal Schwann cells

The Schwann cells that are wrapped around the presynaptic nerve terminal are non-myelinating glial cells that play an active role in synaptic transmission and regeneration (**Figure 3**)(Barik, Li et al. 2016). The evidence for this comes from the fact that upon electrical stimulation of the motor nerve, levels of Ca^{2+} elevate within the Schwann cells (Jahromi, Robitaille et al. 1992). This implies that they are able to sense synaptic transmission, which is further reflected by alterations in their gene expression upon blocking of a synapse (Jahromi, Robitaille et al. 1992). Interestingly, when a nerve is cut at the NMJ, Schwann cells begin to form protrusions that reach out to the denervated region and release ACh (Son, Trachtenberg et al. 1996, Yang, Cao et al. 2001). These processes then act as guides to the regenerating motor nerve, thus facilitating reinervation of the muscle fibres. They also regulate the growth of neuronal processes during development (Auld and Robitaille 2003). Collectively, they are an important component of the NMJ, involved in maintenance of the synapse (Alvarez-Suarez, Gawor et al. 2020).

1.1.5. The extracellular matrix

During synaptogenesis as well as synaptic maturation, the ECM proteins act as a stabilizing platform that binds receptors involved in cellular adhesion, and recruits signalling components that regulate the NMJ (Dani and Broadie 2012). The ECM produced by the muscle contains numerous proteins including laminins. Laminins are heterotrimers composed of α, β and γ subunits that make up for the majority of the basal lamina (BL) proteins (Gillies and Lieber 2011). Multiple isoforms of each subunit have been identified, however not all can form functional heterotrimers. The nomenclature of laminin heterotrimers is determined by their subunit composition. Thus a laminin, which is composed of α5, β2 and γ1 laminins would be described as laminin 521.

The individual α, β and γ subunits of the laminin trimer are encoded by different genes (Aumailley, Bruckner-Tuderman et al. 2005). The synaptic cleft harbours laminin isoforms α2, α5 and β2, which are mostly absent outside of the synapse (Patton, Miner et al. 1997). Interestingly, mutations in these genes lead to severe phenotypes resulting in decrease in synaptic transmission efficiency and aberrant folding of the postsynaptic membrane (Li, Xiong et al. 2018). Mutations in laminin β2 gene are especially detrimental. They lead to a severe reduction in the number of AZs, which results in a 50% decrease of the neurotransmitter release (Noakes, Gautam et al. 1995, Knight, Tolley et al. 2003). Importantly, mutations in this gene have been implicated in a type of a severe myasthenic syndrome, highlighting the importance of laminin β2 during synaptic development (Rogers and Nishimune 2017). The main receptors for the ECM proteins at the postsynaptic membrane are the dystrogylcan, and integrins (Ervasti and Campbell 1993, Heino and Kapyla 2009, Singhal and Martin 2011). The importance of these two transmembrane proteins will be thoroughly discussed in later chapters.

1.2. NMJ development

Since the NMJ is a tripartite system, its development requires a close cooperation between the muscle, the nerve and Schwann cells. All these elements feedback to one another from the earliest moments of development, and disruption of this signalling can be detrimental to the organism (Anderson and Cohen 1977, Liu and Westerfield 1992, Lin, Landmann et al. 2008, Alvarez-Suarez, Gawor et al. 2020). While there is some data concerning synaptogenesis in humans, most of the information that we have comes from studies done on rodents. Based on genetic analysis of people suffering from various neuromuscular disorders, this animal model proved to quite accurately identify common factors involved in the development of the NMJ (Li, Xiong et al. 2018, Swenarchuk 2019).

1.2.1. Embryonic development of the NMJ

In rodents, the muscles begin to form at E9.5 (embryonic development day 9.5), and become first innervated at E12.5 (Dennis, Ziskind-Conhaim et al. 1981, Brandon, Lin et al. 2003). The contact between the nerve and early muscle fibres triggers expression of certain muscle-specific genes including the AChR (Duxson, Ross et al. 1986, Salpeter, Cooper et al. 1986, McMahan 1990). This already illustrates how important the cooperation between the components of NMJ is for synapse formation. The AChRs begin to form small assemblies, known as ectopic clusters before they contact the first motor neurons at E13.5-E15.5 (**Figure 4A**)(Yang, Arber et al. 2001, Lin, Landmann et al. 2008). By E18.5 all of the ectopic AChR clusters disappear, although the ones that did get innervated are often contacted by more than one neuron (**Figure 4A**)(Lin, Burgess et al. 2001). Interestingly, the AChRs formed during the embryonic development have a different subunit composition than an adult receptor, resulting in a slight alteration of its electrophysiological properties (Mishina, Takai et al. 1986). While it is known that this switch is involved in centralizing the endplate to the middle of a myotube, the exact purpose of this transition is still debated (Lindstrom 2003).

1.2.2. Postnatal development of the NMJ

In rodents, the process of NMJ maturation extends into the first weeks of postnatal development (Slater 1982). During this time a number of events takes place to ensure efficient synaptic transmission and stabilization of the endplate components. One of the final steps in formation of a mature synapse is the reduction of neurons innervating the AChR cluster to a single motor nerve. This process is known as synapse elimination and is critical for establishing precise control over muscle contraction (Brown, Jansen et al. 1976, Tapia, Wylie et al. 2012, Tintignac, Brenner et al. 2015). While the factors that are involved in this reduction remain largely unknown, it was observed that this process relies to some extent on the activity of neurons (Brandon, Lin et al. 2003). As such, it was shown that synchronous stimulation of two neurons that innervated a single AChR cluster inhibited synapse elimination. In case where one neuron was more actively stimulated then the other, the synapse elimination was promoted and the less active neuron retracted from the synaptic site (Busetto, Buffelli et al. 2000, Turney and Lichtman 2012). While the terminal Schwann cells are not directly involved in initiating the synaptic elimination, they do play a role in separating the retracing nerve ending from the muscle (Smith, Mikesh et al. 2013).

The first two weeks after birth are also a time when postsynaptic membrane acquires its folds (Marques, Conchello et al. 2000). The AChRs become concentrated at the crests, while sodium channels at the depths of the folds (**Figure 3**)(Fertuck and Salpeter 1974, Stocksley, Awad et al. 2005). Interestingly, humans have one of the smallest NMJs among vertebrates, however our postsynaptic membrane exhibits one of the highest extent of postsynaptic folding (Slater 2017). This makes up for the lower amount of ACh released by the motor neuron, ensuring that the neurotransmitter binds efficiently enough to AChRs to elicit an AP in the muscle. This is known as the safety factor (Wood and Slater 1997, Wood and Slater 2001). Other NMJ features that contribute to the safety factor are the size of the nerve ending, amount of ACh within the synaptic vesicles as well as density of AChRs and of Ca^{2+} channels that regulate release of vesicles from AZs (Wood and Slater 1997). Importantly, there are multiple examples when the postsynaptic folds exhibit alterations in their size and arrangement in patients suffering from congenital myasthenic syndromes

(CMS) and autoimmune diseases. (**Figure 4A**) (Engel, Shen et al. 2015, Phillips and Vincent 2016).

In addition to the abovementioned changes to the NMJ, we also observe recruitment of postsynaptic nuclei closer to the synaptic membrane, likely to facilitate turnover of the postsynaptic machinery components (Slater 1982, Kues, Sakmann et al. 1995). During this time the AChR clusters increase in size and receptor density, but also undergo significant remodelling of their topology (Steinbach 1981). A useful tool for visualizing the AChRs both *in vivo* and *in vitro* is fluorescently labelled α-bungarotoxin (BTX), which can be extracted from a venom of *Bungarus multicinctus* snake. BTX binds specifically, and with high affinity to the AChRs. This causes a strong contraction of the muscle and leads to a paralysis. When fluorescently-labelled BTX is applied to the muscle tissue, it is possible to observe that the AChR clusters have an oval plaque shape at early developmental stages, and a complex, perforated, pretzel-like morphology at later stages (**Figure 4B**)(Marques, Conchello et al. 2000). While the purpose of such rearrangement is not understood, a failure to undergo through this process very often correlates to a neuromuscular disorder (**Figure 4A**) (Lee, Mikesh et al. 2011). Intersetingly, the pretzel-like shape of a mature postsynaptic machinery encompasses the motor nerve ending so that the AChRs localize directly below the presynaptic terminal. The areas between AChR branches harbour a number of proteins that are involved in adhesion and stabilization of the apparatus, such as nestin. This means that a fully developed postsynaptic machinery is made up of functional domains that are either directly involved in neurotransmission (such as the AChR-rich branches) or play supportive roles (the AChR-poor areas between the branches).

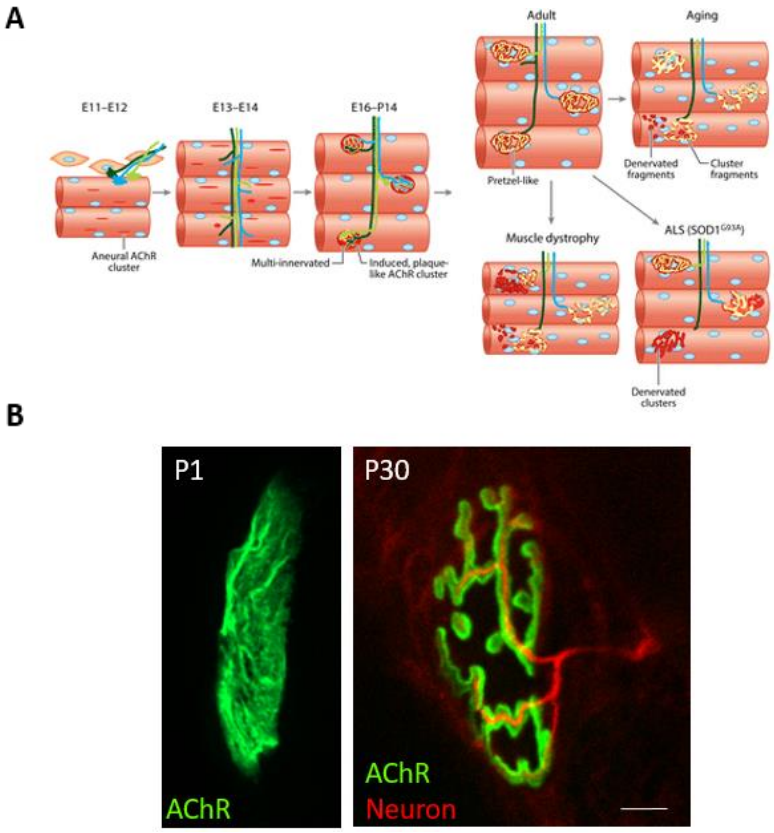


Figure 4: The development of NMJ. (A) *A schematic diagram showing key developmental stages of the synapse. The topology of an AChR cluster as well as the nerve-muscle alignment can be significantly disrupted as a consequence of aging or in muscular dystrophies.* **(B)** *AChR (green) cluster in tibialis anterior of a new born rodent (P1 [postnatal day 1]) assumes an oval shape. During the next two weeks the postsynaptic machinery undergoes a topological change from "plaque to pretzel", AChRs begin to cluster at the crests of synaptic folds at concentration close to 10, 000/µm^2. The invaginations are overlayed by the nerve (red). AChRs were visualized with BTX-488. Scale bar= 6 µm. Image in **A** from (Li, Xiong et al. 2018), changed.*

It may be that dividing the synaptic area into separate compartments or domains contributes to efficient membrane depolarization but also aids in maintaining stability of the synapse (Li, Xiong et al. 2018). Importantly, this topological change was inhibited when nerve was absent from the synaptic site, highlighting the interplay between neuron and the end plate during NMJ formation (Marques, Conchello et al. 2000).

1.2.3. Molecular factors involved in NMJ development

The processes that contribute to formation of a viable synapse are tightly regulated by proteins originating from the nerve, muscle, and Schwann cells (Wu, Xiong et al. 2010). They can be divided into two groups: those directly

involved in synaptic transmission such as AChRs or Na^+ channels, and those that organize these key components and maintain their stability.

The three most important signals released by the nerve that regulate AChR clustering are agrin, ACh and neuregulin (NRG) (**Figure 5**). Agrin is a large glycoprotein that not only promotes expression of AChRs but also directly regulates the organization of other postsynaptic machinery components that influence the stability, clustering and transport of the receptors (Nitkin, Smith et al. 1987, McMahan 1990, Meier, Hauser et al. 1997). Upon leaving the nerve and travelling through the synaptic cleft, it is recognized by transmembrane low density lipoprotein receptor-related protein 4 (Lrp4) (Zhang, Luo et al. 2008). This interaction triggers binding of Lrp4 to muscle-specific receptor tyrosine kinase (MuSK) (Kim, Stiegler et al. 2008). This allows MuSK to become activated by the Docking protein 7 (Dok7), which is an adaptor protein containing phosphotyrosine-binding domain, leading to dimerization and immediate internalization of the kinase (Okada, Inoue et al. 2006). However, data suggests that MuSK induction can be sufficient for inducing AChR clustering even in the absence of agrin (Jones, Moore et al. 1999). While not much is known about the pathways downstream of MuSK, its activation leads to clustering of other crucial components of postsynaptic machinery such as the scaffold protein rapsyn, which interacts directly with AChR and the laminin-receptor dystroglycan (DG) (Apel, Glass et al. 1997, Bartoli, Ramarao et al. 2001, Unwin 2013). Mutant mice lacking either agrin, Lrp4, MuSK or rapsyn are not able to form viable NMJs and usually die after birth due to inability to breathe (Gautam, Noakes et al. 1995, Gautam, Noakes et al. 1996, Weatherbee, Anderson et al. 2006, Kim and Burden 2008).

The neurotransmitter ACh acts as a negative regulator of AChR clustering, countering the activity of agrin (**Figure 5**). It activates a cyclin-dependent kinase 5 (Cdk5), which mediates disassembly of AChR clusters that are not supported by agrin (Lin, Dominguez et al. 2005). Diffused It is therefore responsible for mediating dispersal of pre-patterned clusters that were not innervated by the motor nerve.

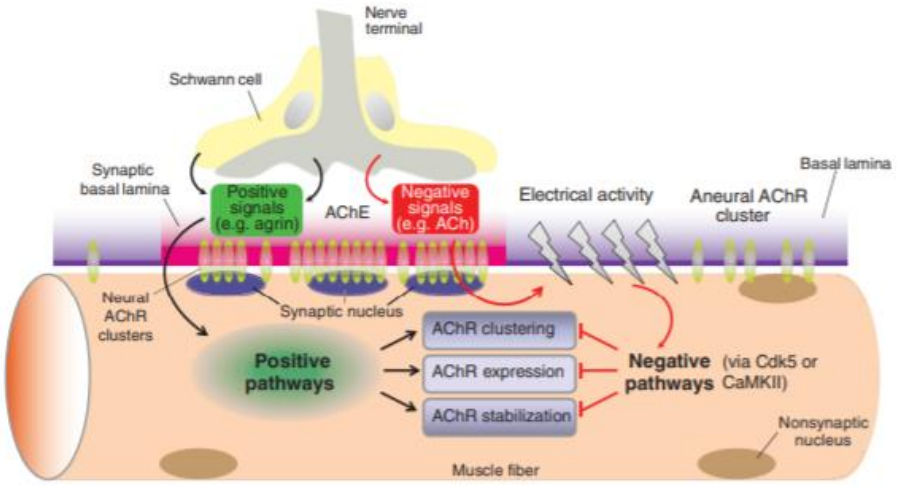


Figure 5: Neuronal regulation of NMJ development. *A schematic diagram showing how key synaptic regulators are integrated to form a functioning NMJ. Positive signals from the nerve (agrin) stimulate assembly AChRs clusters. Negative signals (ACh), which are released in response to nerve stimulation counteract agrin-mediated AChR assembly and thus a feedback mechanism regulating formation of the postsynaptic machinery is employed. Image from (Wu, Xiong et al. 2010).*

NRG can be synthesized and released into the synaptic cleft by either the muscle or the motor nerve (Moscoso, Chu et al. 1995, Meier, Masciulli et al. 1998, Rimer, Prieto et al. 2004). Its receptors- the epidermal growth factor receptor family (ErbB) family of proteins localize to the postsynaptic membrane as well as to the surface of terminal Schwann cells (Trinidad, Fischbach et al. 2000). It was thought that activation of ErbB family members is essential for initiating transcription of synaptic genes and development of a viable NMJ. This view was supported by the fact that systemic knock-out mice died early in development (Sandrock, Dryer et al. 1997) (Lin, Sanchez et al. 2000). However when a conditional knock out of ErbB was introduced into the muscle, the NMJs formed were only slightly affected and the animals were viable (Lin, Sanchez et al. 2000, Escher, Lacazette et al. 2005). This highlighted the redundancy of muscle ErbB receptors in the initial stages of synaptogenesis, but at the same time introduced the idea that NRG stimulates formation of AChR clusters mainly through regulation of the terminal Schwann cell activity (Woldeyesus, Britsch et al. 1999, Sugiura and Lin 2011). Later in development, muscle ErbB receptors are important in maintenance of postsynaptic stability, as they phosphorylate a key member of the Dystrophin Glycoprotein Complex (DGC)- α-dystrobrevin 1 (αDB1) (Schmidt, Akaaboune et al. 2011).

1.2.4. Muscle-derived postsynaptic organizers

During synaptogenesis, the muscle is responsible for responding to the neuronal signals but also for maintaining the stability of AChRs through direct anchoring of the assembly in the postsynaptic membrane (Kummer, Misgeld et al. 2006). These tasks could not be achieved without involvement of a great molecular machinery that would coordinate those functions. While no single protein could support all these actions, a large portion of such regulation is mediated by the DGC (Gawor and Proszynski 2018).

The DGC is a large protein complex that harbours multiple essential components of the postsynaptic machinery (**Figure 6**). Its first identified component, dystrophin is a crucial protein responsible for integrating actin, microtubule and intermediate cytoskeleton with the postsynaptic machinery (Campbell and Kahl 1989, Ervasti, Kahl et al. 1991, Gao and McNally 2015). It is also a platform for recruiting crucial signalling molecules (Allen, Whitehead et al. 2016). Mutations in the dystrophin are associated with Duchenne Muscular Dystrophy (DMD), a severe neuromuscular disease characterized by significant muscle loss and impairments of its regeneration (Monaco, Neve et al. 1986, Yiu and Kornberg 2015).

Another component of the DGC is the dystroglycan protein (DG), composed of two subunits: extracellular α-dystroglycan (αDG) and transmembrane β-dystroglycan (βDG) (**Figure 6**)(Pilgram, Potikanond et al. 2010). Their extracellular domain acts as a receptor for laminins present in the synaptic cleft (Nishimune, Valdez et al. 2008). It also binds neuronal agrin and interacts with MuSK (Sugiyama, Bowen et al. 1994, Apel, Roberds et al. 1995). Importantly, laminins α4 and α5 are essential for targeting of the DG to the postsynaptic membrane (Nishimune, Valdez et al. 2008). The C-terminal domain of βDG interacts with other major components of the DGC and other crucial postsynaptic machinery proteins including AChR and rapsyn (Apel, Roberds et al. 1995, Ishikawa-Sakurai, Yoshida et al. 2004). Interestingly, it has been demonstrated that while the DG is involved in synaptic machinery maturation

and remodelling, the AChR clusters can still forme in its absence and the knock-out mice are viable (Nishimune, Valdez et al. 2008).

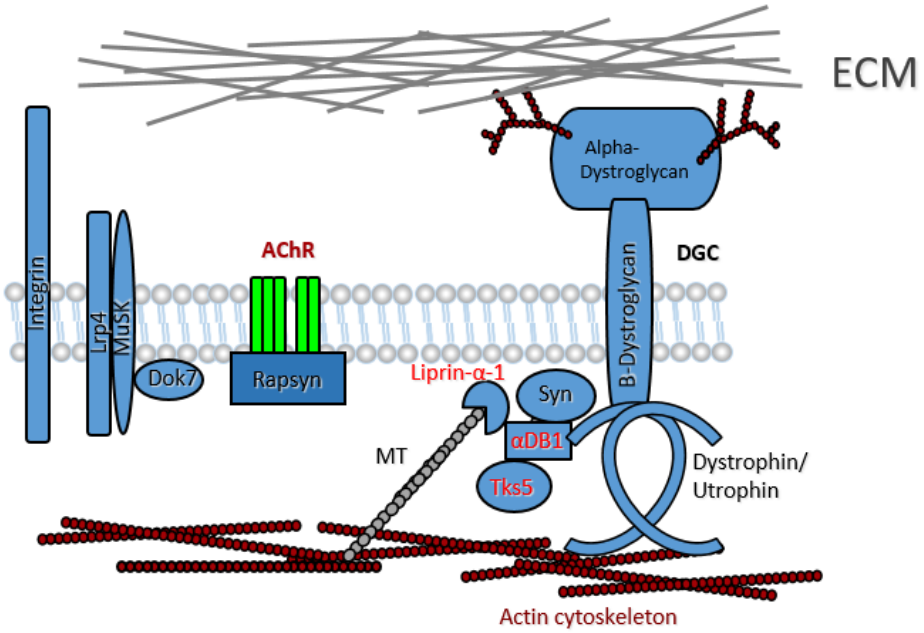


Figure 6: Muscle postsynaptic machinery. *A schematic diagram showing key components of the muscle postsynaptic machinery. Successful establishment of the NMJ requires activation of signalling cascades through intermediates such as Lrp4,* MuSK *and Dok7; but also stabilization of the entire complex in the muscle membrane. The latter is achieved through integrins and the numerous components of the DGC that link the postsynaptic machinery to the ECM and cytoskeleton filaments. SYN-syntrophin.*

Similarly to the DG, integrins act as laminin receptors that stabilize the postsynaptic machinery (Heino and Kapyla 2009). They are important constituents of focal adhesions, which act as cellular attachment hotspots (Bershadsky, Balaban et al. 2003). At the NMJ, integrin isoform α7β1 is recruited to the postsynaptic machinery in laminin-dependent manner during early development of the cluster, where it acts as a signal transducer between the ECM and the muscle fibre (Burkin, Kim et al. 2000). The binding of integrin α7β1 to the AChRs requires phosphorylation of tyrosine residues in the intracellular domain of the laminin receptor (Burkin, Kim et al. 2000). Interestingly it was shown that association of integrin with laminin significantly reduces the amount of agrin required for induction of AChR clustering (Burkin, Kim et al. 2000). This suggests that while laminin and agrin induce concentration of the receptor in the postsynaptic membrane via distinct pathways, their receptors are in close cooperation with each other.

αDB1 exhibits partial sequence homology with dystrophin, and similarly interacts with most of the major DGC components (Wagner, Cohen et al. 1993). (**Figure 6**). Errors in alternative splicing of this isoform are associated with myotonic dystrophy type 1 (DM1) in humans, while knock-out mice have unstable AChR clusters with aberrant shapes (Grady, Grange et al. 1999, Nakamori, Kimura et al. 2008). A lot of its function can be attributed to the three tyrosine phosphorylation sites in its C-terminal, which are critical for rescuing knock-out mice (Grady, Akaaboune et al. 2003) (**Figure 7**).

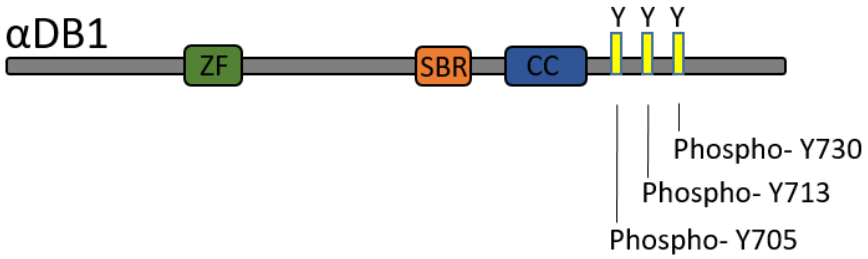


Figure 7: α-Dystrobrevin1 domains. *A schematic diagram showing functional domains and phosphorylation sites on αDB1. ZF- zinc finger domain; SBR- syntrophin-binding region; CC- coiled coil domain; Y- tyrosine residue. The CC domain is used to interact with utrophin.*

These residues can be phosphorylated by calcium/calmodulin- dependent kinase (CaMKII), a protein that is known to maintain postsynaptic stability (Martinez-Pena y Valenzuela, Mouslim et al. 2010). These phosphotyrosine sites are important for mediating interactions of αDB1 with a number of other proteins that are involved in formation of AChR clusters or their stabilization, such as Rho guanine nucleotide exchange factor 5 (Arhgef5), growth factor receptor-bound protein 2 (Grb2) or α-catulin. Additionally, these sites are used for binding of αDB1 to liprin-α-1 and Tks5, which are the main subjects of this thesis, and their functions are thoroughly described in later sections.

Syntrophin is an adaptor protein that also interacts with the majority of DGC components. It has been shown that its interaction with αDB1 is particularly important for maintaining a viable synapse (Butler, Douville et al. 1992, Dwyer and Froehner 1995, Adams, Kramarcy et al. 2000) (**Figure 6**). Mutants that lack syntrophin have reduced receptor density and their AChRs are more readily internalized (Martinez-Pena y Valenzuela, Mouslim et al. 2011). Similarly to αDB1, syntrophin is also phosphorylated by CaMKII. Interestingly, inhibition of this kinase prevents recycling of the receptors, which then start to accumulate

underneath the postsynaptic membrane (Martinez-Pena y Valenzuela, Mouslim et al. 2010).

Utrophin is a homolog of dystrophin, and is also a part of the DGC (Nguyen, Helliwell et al. 1995). It shares a lot of its functions with dystrophin. It stabilizes AChRs through direct interaction, recruits actin filaments to the postsynaptic membrane and mediates interactions between other proteins associated with the complex(Adams, Kramarcy et al. 2000).

While these are the core members of the DGC required to carry out its function, many other proteins are recruited to the complex to aid further with stabilizing the postsynaptic apparatus. One of such proteins is liprin-α-1, which binds specifically to phosphorylated αDB1 (Bernadzki, Gawor et al. 2017). Liprin-α-1 has been shown to be involved in stabilizing the components of a synapse in the CNS, but also to interact with CLIP-associated protein-pleckstrin homology-like domain family B member 2 (CLASP-LL5β) complex, which is known to play a role in various processes during development of AChR clusters *in vitro* (Spangler and Hoogenraad 2007, Asperti, Astro et al. 2009, Astigarraga, Hofmeyer et al. 2010, Astro, Chiaretti et al. 2014).

1.2.5. Muscle-derived presynaptic organizers

While the postsynaptic machinery induction by the motor nerve has been studied extensively, much less is known about the factors released by the muscle that aid in the presynaptic development (Lu and Je 2003). Initial *in vitro* studies identified Dishevelled (Dv1) as a muscle-derived factor required for AChR cluster assembly, and involved in modulation of synaptic currents (Luo, Wang et al. 2002, Li, Dong et al. 2008). This was followed up by *in vivo* studies of β-catenin, which acts downstream of Dv1. Muscle-specific knock-out mice that lacked β-catenin exhibited multiple aberrations in the presynaptic development as well as neurotransmitter release (Li, Dong et al. 2008). Interestingly, this phenotype did not occur when β-catenin expression was inhibited specifically in the neurons (Li, Dong et al. 2008). Since β-catenin is involved in regulating transcription, it is possible that this protein may be required for expression of other important components of the NMJ. However, as for today the events that take place downstream of β-catenin remain to be elucidated.

1.3. Neuromuscular disorders

Studying the biology of a synapse is highly relevant in terms of its translational applications. Currently it is estimated that there are over 300 neuromuscular diseases, and more than half of them have unknown etiology (Verschuuren, Strijbos et al. 2016). The common denominator shared between all of them is a significant reduction in the quality of patients life and health deterioration. As described before, the NMJ is a highly organized structure where its components need to be in close cooperation from the first day of its formation. The main theme in my thesis is the emphasis of the importance of synapse organization, and how its structure ensures function. The neuromuscular diseases are very often characterized by significant aberrations in architecture of the synaptic components, it is thus important to consider how certain changes in NMJ organization lead to its malfunction. Examples of some of the most common neuromuscular disorders are discussed below.

1.3.1. Myasthenia Gravis

Myasthenia Gravis (MG) is a severe neuromuscular disorder with either autoimmunological or congenital origin. The autoimmune MG affects 4-6 people per 10,000 and is characterized by significant reduction in the efficiency of neuromuscular transmission, muscle weakness and fatigue (Vincent 2002). While it can be caused by a range of autoantibodies, the most prevalent forms are characterized by the presence of anti-AChR; anti-Lrp4 and anti- MuSK antibodies (Higuchi, Hamuro et al. 2011, Zhang, Tzartos et al. 2012, Phillips and Vincent 2016). Since the AChR autoantibodies belong to either IgG1 or 3 subtype they are able to activate classical complement pathway triggering assembly of membrane attack complex (MAC) upon binding to the epitope (Lennon and Lambert 1980, Ruff and Lisak 2018). MAC damages the membrane by allowing Ca^{2+} influx, which leads to release of antibody-bound AChRs into the synaptic cleft, reducing the muscle's response to ACh. Additionally, MAC also indirectly attacks the Na^{+} channels located at the toughs of synaptic folds, which rises the threshold for the end plate potential-driven postsynaptic depolarization rendering the muscle even less sensitive to the presynaptic signal.

Interestingly, MG patients who produce autoantibodies against MuSK acquire the classical symptoms through a different mechanism. The MuSK antibodies belong mainly to the immunoglobulin G4 (IgG4) subtype, meaning that they do not lead to complement activation. Instead, they bind to MuSK and prevent formation of a viable agrin-Lrp4- MuSK complexes, which results in dissociation of AChR clusters (**Figure 8**) (Verschuuren, Huijbers et al. 2013, Phillips and Vincent 2016, Evoli, Alboini et al. 2018). This highlights the significance of the molecular architecture of the NMJ has on its function and how important it is to keep a balance between receptor clustering and dispersal.

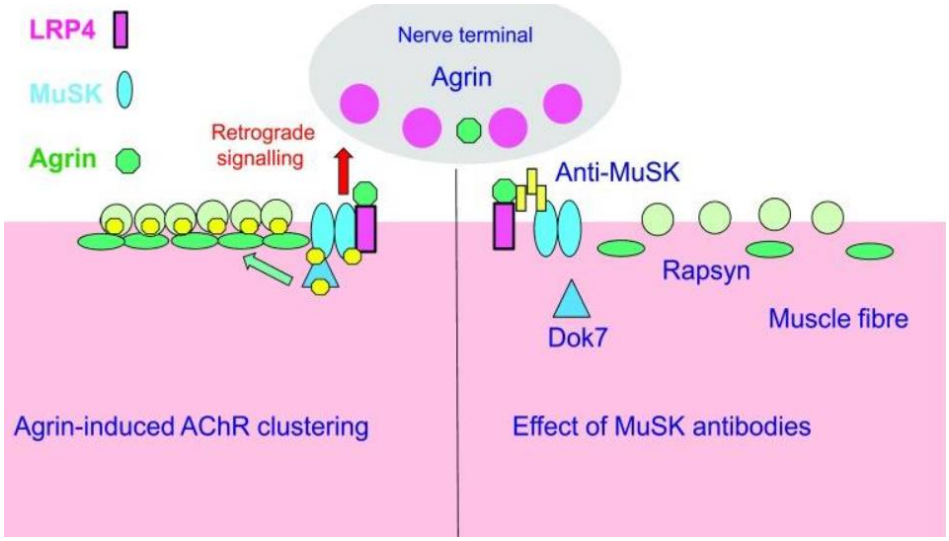


Figure 8: Effect of MuSK **antibodies on AChR cluster assembly.** *A schematic diagram showing how disruption of the postsynaptic architecture through binding of* MuSK *autoantibody can cause MG. Under healthy conditions (left side), agrin stimulation leads to clustering of AChR and other components of the postsynaptic machinery. When* MuSK *antibodies bind to their target (right side) they block formation of the complex with Lrp4 and agrin, leading to dispersal of AChRs. The AChRs are represented as pale green spheres. Image from: (Phillips and Vincent 2016).*

1.3.2. Congenital myasthenic syndromes

The hereditary neuromuscular disorders can be attributed to mutations in proteins that localize to the presynaptic nerve terminal, synaptic cleft or the postsynaptic membrane (Engel, Shen et al. 2015). An example of a CMS with presynaptic symptoms is the choline acetyltransferase (ChAT) deficiency. ChAT is required for catalysing the ACh synthesis reaction, and the most severe forms of disease manifest in patients with mutations in either the active site or the substrate binding domain of ChAT (Maselli, Chen et al. 2003). This leads to a

reduction in the end plate potential and results in muscle weakness (Schara, Christen et al. 2010).

Mutations in synaptic cleft proteins that lead to CMS can occur in laminin β2 AChE (Engel, Lambert et al. 1977, Hutchinson, Walls et al. 1993, Maselli, Ng et al. 2009). In both cases the nerve terminals are smaller than usual, leading to a decrease in the amount of released ACh (Engel, Shen et al. 2015). In case of AChE mutations in the triple stranded collagenic tail (ColQ) domain have been identified. The ColQ domain is responsible for anchoring of the AChE in the synaptic region by binding with the extracellular domain of MuSK (Massoulie, Pezzementi et al. 1993, Mihaylova, Muller et al. 2008). These mutations can either prevent assembly of the domain or reduce enzyme expression, leading to prolonged synaptic signalling attributed to the fact that the neurotransmitter is less efficiently hydrolysed and is able to act upon the postsynaptic membrane for a longer time (Engel, Shen et al. 2015).

The most common form of CMS occurs due to mutations in genes encoding different subunits of AChR (Engel 1993). Depending on their localization, they can cause a range of phenotypes. For example, mutations in the ε subunit lead to primary AChR deficiency, resulting in reduction in number of receptors at the postsynaptic membrane and thus a significantly decreased responsiveness to the presynaptic signalling (Engel, Ohno et al. 1996). Another type of mutations that can occur lead to kinetic defects of the AChR. As such mutation in the ligand binding domain of the receptor increases its affinity for ACh, prolonging the duration of channel opening and resulting in a drawn-out stimulation of the muscle (Engel, Lambert et al. 1982). This is known as the slow channel syndrome. As opposed to that, certain mutations in AChR cause brief opening of the channel in response to neurotransmitter, leading to generation of short lasting and weak currents as the physiological levels of ACh have a smaller chance to induce downstream signalling. Such condition is known as the fast channel syndrome (Sine, Shen et al. 2002, Engel, Shen et al. 2015). A range of mutations that cause CMS have been also described in proteins that are involved in development and maintenance of the synapse such as rapsyn, MuSK, Dok7, agrin and Lrp4 (Burden, Yumoto et al. 2013).

1.3.3. Duchenne muscular dystrophy

In line with the notion that the molecular architecture of the NMJ has to be tightly regulated to ensure efficient synaptic transmission, DMD is a good example of what happens when the integrity of the postsynaptic machinery becomes compromised. DMD is the most common neuromuscular disease affecting 1 in 3600 males (Chung, Smith et al. 2016). It is caused by mutations in the previously mentioned dystrophin gene located on the X chromosome. Several mutations have been identified as the cause of DMD, however they all lead to a loss of dystrophin expression (Yiu and Kornberg 2015). Absence of dystrophin leads to a number of muscle aberrations, and KO mice lacking this gene exhibit severe fragmentation of the postsynaptic machinery (**Figure 9**) (Muntoni, Torelli et al. 2003, Pratt, Shah et al. 2015). Patients develop significant respiratory complications along with cardiomyopathy that eventually lead to death. Interestingly, DMD is accompanied by activation of various pathological pathways resulting in Ca^{2+} ion influx, recruitment of proteolytic enzymes or cytokine activation, which promote progression of the disease (Shin, Tajrishi et al. 2013).

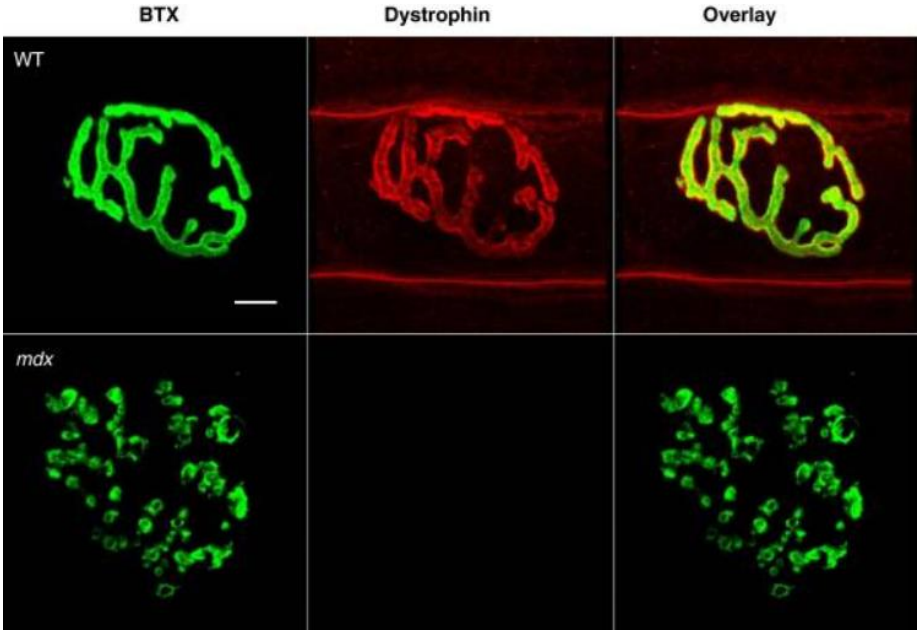


Figure 9: The NMJ in dystrophic mouse model. *BTX labelling of AChRs (green) and immunostaining of dystrophin (red) in wild type (top panel) and genetically modified mdx mice where the dystrophin gene was knocked out. Mdx mice are the most commonly used model for DMD. They have a point mutation in exon 23 that renders dystrophin absent from the muscle. This leads to destabilization of the postsynaptic machinery, reflected by the severe fragmentation of the NMJ. Scale bar= 6 µm. Image from: (Pratt, Shah et al. 2015).*

It has been observed that DMD patients have an elevated expression of utrophin (Kleopa, Drousiotou et al. 2006). Since this protein is a dystrophin homolog it compensates to a certain extent for the absent DGC component (Tinsley, Fairclough et al. 2011). While there is no cure for DMD yet, efforts are still made to find the optimal treatment. One of the approaches takes advantage of the fact that utrophin can be a viable substitute for dystrophin and focuses on modulation of utrophin expression through dosing with a small molecule that has been shown to increase utrophin mRNA (Tinsley, Fairclough et al. 2011, Vuorinen, Wilkinson et al. 2021). Another promising therapy is based on an exon-skipping strategy that aims to regain expression of a shorter dystrophin variant that can mediate most of a healthy protein's functions. So far, the main problem with this approach lies in the fact that there is no effective method that would deliver the therapeutic agent to all the muscle fibres (Echevarria, Aupy et al. 2018).

1.4. Models for studying the NMJ

While certain key components that regulate formation of the NMJ have been identified, there are still many gaps in our understanding of the synaptic biology. The fact that therapies for neuromuscular disorders are still lacking further highlights the need for deciphering the synapse. Nowadays scientists possess multiple tools that enable careful consideration of the biological phenomena that take place at the NMJ. These, present the investigators with an important choice regarding the model of their studies and in this section I will explore some of them, highlighting the benefits as well as limitations of each one. Because of the tripartite nature of the synapse, certain processes that regulate the NMJ are difficult to study in vivo. For this reason, minimalistic models have proven to be useful tools as they circumvent many compensatory mechanisms that arise due to the interaction of NMJ components with each other.

1.4.1. Mouse NMJ as a model for human synapse

While multiple studies have been carried out using invertebrate models of neuromuscular junction such as *C.elegans* and *Drosophila*, no other single model has provided as much information about the biology of a synapse as the mouse (Shi, Fu et al. 2012, Jones, Harrison et al. 2017). This is partially due to the fact that the murine NMJ is significantly larger and easily accessible, which

simplifies microscopic analysis as well as electrophysiological evaluation of the system (**Figure 10A**)(Shi, Fu et al. 2012). Although there is no data concerning developmental remodelling of human NMJ that could be used to further validate the murine model, certain parallels are known to exist between the two organisms (Jones, Harrison et al. 2017). For example, murine AChRs undergo the same developmental switch during postnatal period where the γ subunit is replaced by the ε subunit (Missias, Chu et al. 1996). A notable difference however is that while humans maintain the expression of γ subunit at low levels during adulthood, mice completely suppress it within 3 weeks after birth (Hesselmans, Jennekens et al. 1993). The fact that transgenic animal studies provided invaluable information about the mechanisms of many neuromuscular disorders and in some cases led to designing therapeutic methods is the main argument for considering the murine NMJ as a viable model of a human synapse (Jones, Harrison et al. 2017).

This, however, does not mean that the model is without limitations. As described in the earlier chapters, the NMJ is a tripartite synapse where the motor nerve, terminal Schwann cells and muscle fibres all feedback to each other at every developmental stage (Li, Xiong et al. 2018). This level of complexity means that *in vivo* functional studies can very often be tampered by various compensatory mechanisms. The denervation procedure partially solves this problem as it removes the nerve and Schwann cells from the muscle fibre allowing for observation of the postsynaptic machinery outside of the tripartite context (Vannucci, Santosa et al. 2019). The obvious problem with this approach is the fact that such invasive method triggers activation of other pathways that aim to repair the synapse, preventing studies of postsynaptic machinery in a healthy or undisturbed setting. For this reason scientists

developed various *in vitro* models where they are able to have more control over the samples.

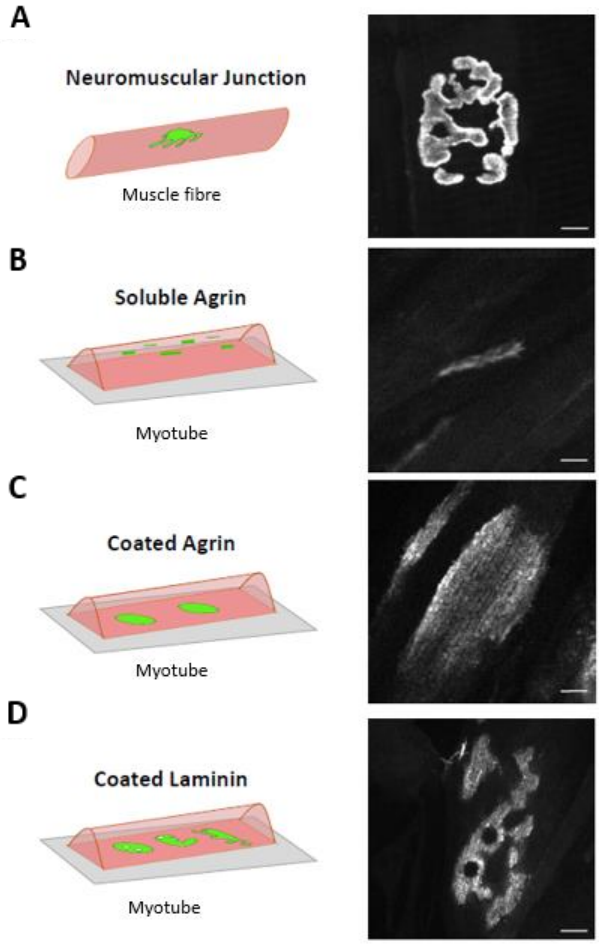


Figure 10: Models for muscle postsynaptic machinery. (A) *The murine NMJ. Postsynaptic machinery in mice acquires a complex pretzel-like topology. Each innervated muscle fibre has one NMJ.* **(B)** *Differentiated immortalized mouse myotubes (C2C12) were stimulated with soluble agrin, which triggered formation of numerous, small AChR hotspots at the surface of the cells.* **(C)** *C2C12 cells that were cultured on laminin coated surfaces with deposited agrin formed large, oval AChR clusters at the basal surface of the cells.* **(D)** *C2C12 myotubes that were cultured on surface coated with laminin developed complex AChR clusters that underwent developmental remodelling, similar to the postsynaptic machinery in vivo. The clusters also formed at the basal surface. Scale bar= 6 µm. BTX was used to visualize the AChRs.*

1.4.2. Agrin-based muscle cultures as models of postsynaptic machinery

Using *in vivo* model for studying the postsynaptic machinery is challenging due to the fact that other cell types present at the NMJ may interfere with the experiments. Luckily, scientists were able to develop methods for aneural culturing of muscle cells and stimulate them to form aggregates of the postsynaptic machinery (**Figure 10B**). The first such studies were done on

cultured chick muscles stimulated with isolated constituents of muscle ECM from the electric organ of Torpedo electric ray (Godfrey, Nitkin et al. 1984). This tissue was known to have a high concentration of cholinergic synapses, and the Godfrey et al., deduced that the insoluble fractions corresponding to the ECM of this organ may be enriched in AChR-aggregating molecules. Indeed, they observed a 20-fold increase in the number of AChR clusters when the chick muscles were incubated with this insoluble fraction (Godfrey, Nitkin et al. 1984). While they were not able to identify the active component that caused this effect, we now know that the muscles were stimulated with one of the most crucial AChR clustering factor- the glycoprotein agrin (Lin, Landmann et al. 2008). *In vivo*, agrin is released by the nerve to stimulate clustering of the postsynaptic machinery, thus this method to certain degree mimics presence of the nerve (Schmidt, Basu et al. 2017). This apprach became a standard procedure for generating *in vitro* models of the muscle end plates and remains the most widely used approach for acquiring AChR-producing cultures. Importantly, it was applied not only to primary cultures but also to immortalized cell lines such as mouse C2C12 myoblasts (Mlinaric-Rascan, Asa et al. 1994, Koneczny, Cossins et al. 2013). AChR clusters that are formed through agrin stimulation are numerous, however their topology largely differs from the postsynaptic machinery at the NMJ. These clusters reassemble small ectopic aggregates or AChR hotspots with an uncomplicated shape. Other than induction of clustering, agrin stimulation also triggers accumulation of muscle nuclei underneath the postsynaptic apparatus and prompts them into transcription of other components that are present at the synapse (Moransard, Borges et al. 2003). Thus, while agrin stimulation of cultured myotubes is a useful model providing the researcher with a tool to study AChR clustering and delivery, it fails to account for the crucial aspect of synaptogenesis, which is developmental remodelling (Pezinski, Daszczuk et al. 2020).

In 2017, Brenner and colleagues realized that the described method of administering agrin to the cell culture does not reflect the physiological way that this molecule is presented to the muscle *in vivo*. At the NMJ, agrin is released by the nerve and stably deposited within the BL which allows for exerting a lasting activity on the receptors located at the muscle membrane (Lin, Landmann et al. 2008). Agrin added to the culturing medium is more loose, resulting in a random AChR cluster distribution. To address this problem, the

scientists seeded Simian kidney (COS-7) cells on laminin substrate and transfected them with neuronal agrin-encoding plasmid. The cells secreted the glycoprotein, which deposited on the ECM substratum. After lysis of the agrin-expressing COS-7 cells, they cultured myotubes on the same surface and found much larger clusters of AChRs formed on top of the agrin patches (**Figure 10C**)(Schmidt, Basu et al. 2017). While it is true that this method reflects the physiological conditions of postsynaptic machinery clustering more closely, the assemblies that form assume a simple plaque shape that resembles the NMJ at the early postnatal stages of development. Thus, in order to investigate the process of postsynaptic machinery remodelling in aneural culture conditions, a different approach needs to be implemented.

1.4.3. Laminin-based muscle cultures as models of postsynaptic machinery

Laminins have three major physiological functions. First, they act as cellular exoskeleton by providing structural support to membrane-bound complexes and distribute mechanical stress through the cell (Colognato and Yurchenco 2000). Second, they form a complex network that serves as a platform for binding of other BL components (Charonis, Tsilibary et al. 1985). Lastly, they act as signalling molecules that regulate cell migration, adhesion and differentiation (Patton, Miner et al. 1997, Nishimune, Valdez et al. 2008). Multiple laminin isoforms have been identified and it has been shown that depending on the subunit composition of the laminin trimer, it has varying specificities for membrane-bound receptors and triggers activation of different signalling pathways (Hohenester, Tisi et al. 1999, Dempsey, Bigotti et al. 2019). In the muscle BL, laminin composition changes depending on the region. The extrasynaptic parts which make up for the most of muscle BL are mainly enriched with laminin chains α2, β1, γ1, with traces of other subunits. The synaptic cleft on the other hand, contains α2, α4, α5 and β2 domains, all of which except for α2 are specifically confined to the synaptic region. Thus the only laminin trimers that can be found at the adult NMJ are laminin-221,-421,-521 (Patton, Miner et al. 1997). Mutations in the α2 chain are associated with muscular dystrophies and studies that implemented laminin α2 knock-out mice showed that this subunit plays myo- and neuro-protective roles (Patton 2000, Barraza-Flores, Bates et al. 2020). As mentioned before, mutations in the β2 subunit lead to severe disturbances in neuromuscular transmission and affect

postsynaptic folding, while double knock out mice lacking the α4 and α5 domains do not develop mature NMJs (Noakes, Gautam et al. 1995, Lee, Chand et al. 2017).

Itwas shown that myotubes grown *in vitro*, solely on the ECM protein laminin-111 formed complex clusters of the postsynaptic machinery that had a similar topology to the NMJ, even in the absence of the nerve (Sugiyama, Glass et al. 1997, Kummer, Misgeld et al. 2004). Importantly, these assemblies underwent developmental remodelling that also reassembled the postsynaptic machinery *in vivo* (**Figure 10D**). At the early stages, these AChR clusters were plaque-shaped, and with time acquired numerous perforations leading to a branched, pretzel-like shape. This finding raised the possibility that a similar nerve-independent mechanism of postsynaptic remodeling might occur at the NMJ, where it could contribute to synaptic development, but is inhibited *in vivo* when nerve is present (Proszynski, Gingras et al. 2009). Interestingly, the AChR clusters induced by laminin did not require MuSK, highlighting that agrin and laminin trigger postsynaptic clustering through different mechanisms (Kummer, Misgeld et al. 2004). Aberrant maturation of an NMJ is a characteristic for many neuromuscular disorders, however the underlying mechanism for this process is still not understood (Baggi, Antozzi et al. 2012). It is likely due to redundant processes and compensation effects occurring *in vivo*, which make it significantly difficult to study synaptic maturation in skeletal muscles. Thus, *in vitro* system of myotubes cultured on laminin could be useful for identification of candidate proteins that are involved in the synaptic organization.

Interestingly, researchers who employed this method only used laminin-111 for induction of AChR clustering. As there are many isoforms of this protein present in vertebrates, and some of them are specifically enriched at the NMJ, I decided to investigate how culturing of immortalized mouse C2C12 myoblasts on alternative laminin isoforms would affect the efficiency and postsynaptic machinery aggregation and remodeling (Patton, Miner et al. 1997, Kummer, Misgeld et al. 2004, Pezinski, Daszczuk et al. 2020). I have also tested various culturing conditions involving laminin-coated surfaces for primary human myoblasts in order to evaluate the potential of these cells as a more accurate model for investigating synaptogenesis in humans. The results of these experiments are described in more detail in later section.

1.5. Synaptic domains

Muscle cells segregate synaptic components responsible for orchestration of signalling pathways, focal adhesion, and cytoskeleton regulation into specialized subsynaptic domains. This allows for a dynamic response to environmental cues as well as efficient transmission of information within the system. This segregation into specialized domains can be observed on many levels, from the arrangement of molecular postsynaptic apparatus components to apposition of the nerve with the muscle. In this section I will discuss how certain macro and micro domains present at the NMJ ensure its function, as they do in other types of synapses.

1.5.1. Macro domains present at the neuromuscular junction

The best example that illustrates how enrichment and special separation of certain synaptic components into specialized macro domains *in vivo* achieves efficient neurotransmission is the transsynaptic alignment of the nerve and muscle (Li, Xiong et al. 2018). As mentioned previously, the motor nerve ending is enriched with neurotransmitter release sites known as the AZs, that directly oppose the junctional folds present at the muscle membrane (Meinertzhagen, Govind et al. 1998). These areas contain much higher concentration of synaptic vesicles than anywhere else in the cell, and their localization is regulated by laminin β1 that binds to one of the most crucial components of the AZs- the voltage-gated Ca^{2+} channel (Carlson, Valdez et al. 2010). The AZs are regularly distributed across the presynaptic membrane, around 0.5 µm from each other, which provides an important regulatory mechanism as it ensures that opening of Ca^{2+} channel at one of these sites would not trigger release of synaptic vesicles from the neighbouring AZs. Thus, the spacing of secretory domains ensures that the nerve will have the highest possible concentration of neurotransmitter release sites that at the same time would not interfere with one another. Additionally, those domains are aimed at the openings of junctional folds to ensure efficient stimulation of AChRs, which further highlights the importance of spatial segregation of synaptic components (Rowley, Mantilla et al. 2007, Slater 2017).

At the junctional folds, two functional macro domains can be distinguished- the crests and toughs. They function as a hub for spatially segregating the AChRs from voltage-gated Na^{+} channels and their supporting complexes (Smith and

Slater 1983, York and Zheng 2017). Such distribution allows depolarization of the muscle membrane by amplification of the initial electrical signal generated by binding of ACh to its receptor. As described before, alterations to this arrangement reduce the efficiency of neurotransmission and are often a hallmark of neurodegenerative diseases (Li, Xiong et al. 2018).

Importantly, the postsynaptic machinery in *in vitro* cultured myotubes also has distinguishable macro domains that elicit different functions. A good example of it are the synaptic podosomes, present in the AChR-poor areas of the cluster, that form a large synaptic domain enriched with actin and machineries responsible for cellular adhesion (**Figure 11**) (Proszynski, Gingras et al. 2009). It was recently demonstrated that synaptic podosomes capable of degrading the ECM are involved in remodelling of AChR clusters in laminin-cultured myotubes (Proszynski, Gingras et al. 2009, Bernadzki, Rojek et al. 2014). It was the first time that these organelles have been shown to play a role in the biology of a synapse, however a complete evidence for their existence *in vivo* is still lacking. Several known podosomal markers have been identified to be present in the perforations of a mature NMJ, including Amotl2, γ-actin and LL5B, which suggests that a similar remodeling mechanism might occur *in vivo* (Proszynski and Sanes 2013, Bernadzki, Rojek et al. 2014).

Within this actin-rich structure we find proteins that are strictly involved in regulation of the cytoskeleton, such as the Wiscott-Aldrich Syndrome protein (N-Wasp) or Tks5, as well as those that constitute focal adhesions, for example paxillin and vinculin (Murphy and Courtneidge 2011, Bernadzki, Rojek et al. 2014). While the presence of podosomes *in vivo* has not been confirmed, many proteins that localize to these perforations within the AChR cluster are also found in equivalent areas of the NMJ. For example, actin-regulating protein angiomotin-like 2 (Amotl2) is a known podosome-associated protein that also localizes to the areas between AChR-rich branches of the NMJ (Proszynski and Sanes 2013). This holds true for other cytoskeleton regulators such as nestin (**Figure 11**). Taken together, these proteins form a domain that is completely separated from the complexes that are directly involved in synaptic transmission, while very often occupying two-thirds of the entire synaptic area. The evidence for the role of domain organization of NMJ components is largely missing, however the similarities between these areas and podosomes suggest that they may be involved in development and maintenance of synaptic

integrity. Alternatively, such domains may be specialized protein reservoirs that support the synapse according to either physiological or pathological conditions, as is the case of nestin re-localization upon denervation (Kang, Tian et al. 2007).

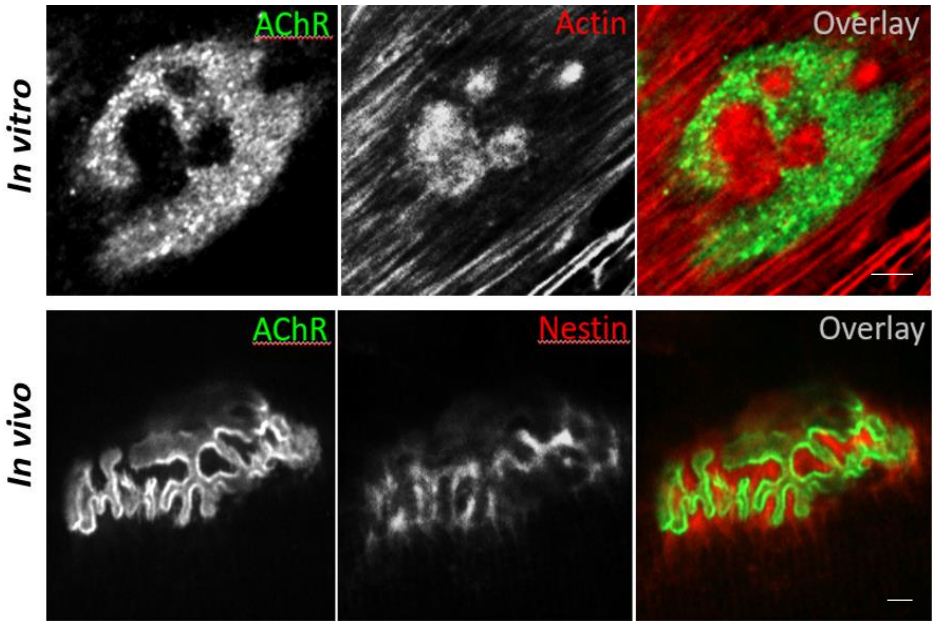


Figure 11: Postsynaptic machinery macro domains. *The most distinguishable functional macro domains at the postsynaptic machinery in vitro are the AChRs (green) and podosomes (red). While the AChR-rich areas harbour complexes involved in synaptic transmission, the podosome domain has a high concentration of actin as well as cytoskeleton-regulating proteins. Corresponding domains can be distinguished at the postsynaptic machinery of the NMJ. In this case the AChR-rich branches (green) are separated by domains that contain cytoskeleton and cytoskeleton-regulating proteins such as nestin (red). The muscle used in bottom panel was Tibialis anterior from P30 mouse. Images in the bottom panel were taken by Dr Marta Gawor. Scale bar= 6 µm*

1.5.2. The role of Tks5 protein in podosome formation

Tks5 (formerly known as FISH) is an adaptor protein that is regulated by proto-oncogene tyrosine-protein kinase Src (Src kinase) at early stages of podosome formation pathway (Seals, Azucena et al. 2005, Thompson, Kleino et al. 2008). Podosomes have also been described in multiple cell types including macrophages, dendritic cells, endothelial cells, vascular smooth muscle cells, osteoclasts, fibroblasts and cancer cells (Gimona, Buccione et al. 2008). Tks5 has been studied in the context of cancer metastasis, where it regulates invadopodia that support cancer spreading and it is considered the only known podosome-specific protein that is not associated with other actin-rich structures (e.g.) focal adhesions, filopodia, lamelipodia (Abram, Seals et al. 2003, Seals, Azucena et al. 2005). Tks5 regulates actin nucleation through

interaction with N-WASP that is responsible for polymerization of actin, it also interacts with a disintegrin and metallo-proteases (ADAM) family of proteases, and importantly, with the DG (Seals, Azucena et al. 2005)(Thompson, Kleino et al. 2008). Previously published data showed that Tks5 is enriched at the postsynaptic machinery formed by laminin-cultured myotubes and localizes to synaptic podosomes *in vitro* (Proszynski, Gingras et al. 2009). The function of Tks5 in cultured skeletal muscles, however, remains unknown. Similarly, the localization and function of Tks5 at the NMJ (or any other synapses) has never been studied.

1.5.3. Micro domains of the postsynaptic machinery

Segregation of synaptic components into specialized domains also occurs on a microscale, where complexes that support the postsynaptic machinery either colocalize with AChRs or occupy a separate region in their vicinity. This is the case for the major laminin receptors found at the AChR clusters. The DG, which stabilizes the receptors at the muscle membrane colocalizes with AChR aggregates, while integrins form a separate micro domain that doesn't overlap with this complex (**Figure 12A and B**). Since integrins are major components of focal adhesions that associate with actin and microtubules it is likely these microclusters are involved in stabilizing postsynaptic machinery and facilitating vesicular trafficking (York and Zheng 2017). Importantly, attachment of microtubules to the AChRs as well as exocytosis of the receptors are controlled by the LL5β-CLASP2 complex that binds the cortical filaments while Liprin-α-1 stabilizes this interaction (Astro and de Curtis 2015, Bernadzki, Gawor et al. 2017). Similarly to integrin, LL5β concentrates at subregions that do not overlap with the AChRs (Kishi, Kummer et al. 2005). Collectively, we observe that on a microscale level the postsynaptic machinery components that are directly involved in synaptic transmission or clustering of AChRs are spatially separated from complexes responsible for adhesion and exocytosis and this is reflected by both *in vivo* and *in vitro* localization of protein complexes (**Figure 12A and B**). However, how such arrangement is achieved remains unknown.

1.5.4. Architecture of other types of synapses

It is worth noting that special separation of adhesion hotspots from complexes involved in synaptic transmission into subdomains is a theme observed across multiple types of synapses and is not limited to the NMJ (**Figure 12C**). For example, the synapses formed between the neurons of the CNS have a

perforated structure where complexes mediating adhesion circumvent the neurotransmitter receptors (Spacek and Harris 1998). Similarly, the immunological synapse between an antigen-presenting cell and the T-cell forms a core enriched with signalling components that recognize the antigen, surrounded by adhesion proteins, for example talin (Grakoui, Bromley et al. 1999). This highlights that special separation of synaptic proteins is maintained throughout various cell types and that it directly correlates with their function.

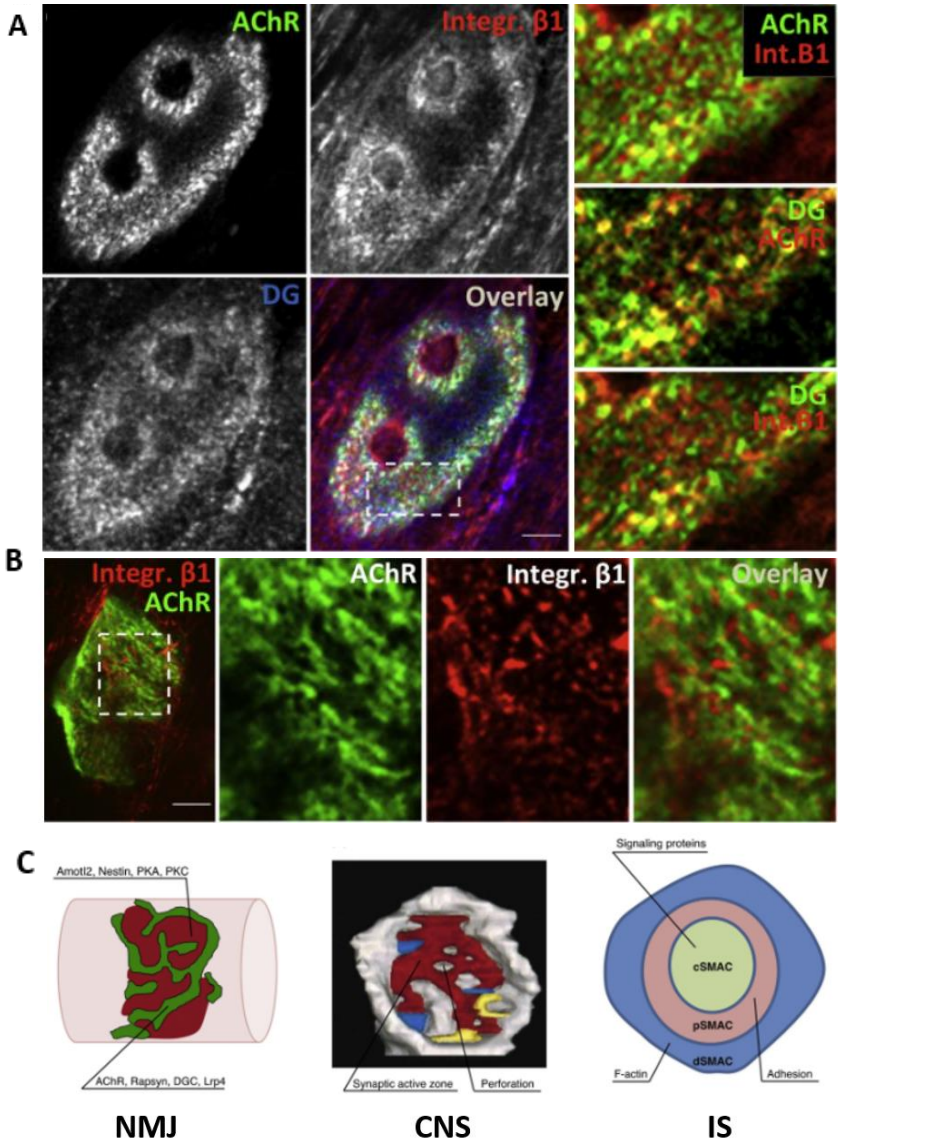


Figure 11: Micro domains at the postsynaptic machinery. *(A) Localization of major laminin receptors DG and integrin β1 at the postsynaptic machinery in vitro. Three panels on the right represent a higher magnification of the boxed area. While AChR and integrin are segregated into separate domains, immunoreactivity of DG overlaps with the former. (B) Integrin β1 localizes to the AChR-poor regions in the developing postsynaptic machinery. The image was taken from a tibialis anterior muscle from a P5 mouse prior to formation of postsynaptic folds. (C) Multiple synapses exhibit domain segregation of their components based on their function. This phenomena is observed at the NMJ (left diagram), dendritic spines in hippocampus (middle, image taken from: (Spacek and Harris 1998))* as well as the immunological synapse (right).

2. Aims

There are no cures for the majority of neuromuscular disorders and the existing therapies are mainly focused on symptomatic treatment. One of the reasons for it is that in many cases the etiology of these diseases remains largely unknown. In order to better understand the mechanisms of these diseases a constant effort must be put into investigating synaptic biology and finding new *in vitro* platforms for screening of therapeutic agents.

αDB1 is a crucial component of the postsynaptic machinery and and together with my colleagues we have identified a number of its novel interactors through mass spectrometry analysis providing possible insights into the mechanisms that regulate the AChR clusters. During my studies I have focused on deciphering the role of two of these interactors: liprin-α-1 and Tks5.

Liprin-α-1 has been previously implicated to play a role in maintenance of synaptic components in the CNS, however there was no evidence for its involvement in regulation of the NMJ postsynaptic machinery. Tks5 is a known marker of podosomal structures in cancer and other cell types. Based on the recent findings that postsynaptic machinery *in vitro* undergoes developmental remodelling via synaptic podosomes and the fact that maturation of a synapse if often compromised in various neuromuscular disorders, Tks5 can be an important regulator of this mechanism. I have identified both proteins as interactors of αDB1, thus deciphering their roles at the NMJ may provide valuable insights into the function of this protein.

Importantly, while multiple *in vitro* models for studying the biology of postsynaptic machinery are available, they all come with a number of limitations. Thus, I decided to improve existing laminin-based methods of culturing muscle cells for obtaining robust AChR clusters that undergo developmental remodelling, and apply this method to primary human muscle cells obtained from patient biopsies, providing a more appropriate platform that could be used for drug screening.

Thus, the aims for my studies are following:

Aim 1: To study the mechanism of postsynaptic machinery regulation by liprin-α-1

Aim 2: To determine a role of Tks5 in the AChR cluster maintenance and development

Aim 3: To create a reliable method for obtaining high yields of AChR clusters in *in vitro* cultures of immortalized and primary muscle cell lines.

A wide spectrum of methods were used during my studies, including *in vitro* muscle cell culturing, immunocytochemistry as well as sophisticated approaches for determining protein interactions such as proximity ligation assay and mass spectrometry analysis.

3. Materials & Methods

3.1. Cell lines

3.1.1. C2C12

C2C12 myoblasts were acquired from American Type Culture Collection (catalog no. CRL1772). Cells were cultured on 10 cm petri dishes covered with air-dried 0.2% gelatin in Dulbecco's Modified Eagle Medium - DMEM (catalog no. D6046, SIGMA) supplemented with 20% fetal bovine serum (FBS; catalog no. E5050-02, EURx) , 4,5 g/l glucose, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and fungizone and incubated at 37°C and 5% CO_2. The cells were passaged every three days before reaching 30% confluence to avoid excessive cell-cell contact that could trigger entering into differentiation prematurely. Once thawed, C2C12 myoblasts were passaged up to five times in total.

3.1.1.1. Passaging C2C12 cells

When C2C12 cells grown on a 10 cm gelatin-coated plate reached 30% confluence, the growth medium was aspirated and the plate was rinsed once with sterile phosphate-buffered saline (PBS) to wash out any remaining media that would inhibit trypsinization. In order to detach C2C12 myoblast from the surface of the culturing dish, 3 ml of trypsin were added to the plate followed by incubation at 37°C for 2-5 minutes. Once all the cells were suspended in the trypsin solution, 2 ml of growth medium containing 20% FBS were added in order to prevent the protease from damaging the cells. To collect the cells, they were centrifuged at 419 g for 3 minutes and resuspended in 6 ml of fresh growth medium. The suspension was then split in a 1:6 ration onto gelatin-coated plates and 9 ml of warm media were added. After 2-3 days the cells reached 30% confluence and were ready for a next passage.

3.1.1.2. Freezing C2C12 cells

Each gelatin-coated 10 cm plate designated for freezing contained 4.0×10^5 cells C2C12 myoblasts. The cells were trypsinized as described in the abovementioned paragraph, with the exception of being resuspended in 1 ml of the growth medium and split equally into two freezing vials. 0.5 ml of pre-chilled freezing medium (14% dimethylsulfoxide [DMSO; catalog no. A994.1, Roth]), 20% fetal bovine serum, DMEM with glutamine) was then added to each

cells-containing vial, which were placed in a cold freezing container and transported without delay to -80°C. For long term storage, the vials were located in liquid nitrogen 1-2 days after being placed in the -80°C freezer.

3.1.1.3. Thawing C2C12 cells

Freezing vials containing cells were carried on ice and placed in a 37°C water bath in order to speed up the thawing. This was crucial as prolonged exposure of cells to liquid DMSO leads to significant damage. Once thawed, the cells were transferred onto a gelatin-coated 10 cm dish with 10 ml of 37°C growth medium. As mentioned before, liquid DMSO damages the cells thus the medium was replaced one day after plating to allow the cells to attach to the plate and minimize the adverse effects of the chemical.

3.1.1.4. Differentiation of C2C12 cells on laminin

In order to provide appropriate format for differentiation and subsequent biochemical or microscopic analysis of C2C12 cells, Permanox slides (catalog no. 160005, Thermo Fisher Scientific) with ethanol-washed flexiperm 8-well grids (0.9 cm^2 surface area per well) (catalog no. 6032039, Sarstedt) attached to them were used for all experiments on *in vitro* AChR clusters. To stimulate formation of complex assemblies of the postsynaptic machinery, the surface of one well on a flexiperm slide was covered with 2 µg of either mouse laminin 111 (catalog no. L2020-1MG, Sigma) or with recombinant human laminins (111; 121; 211; 221; 411; 421; 511; 521) (catalog no. LNKT-201, Biolamina), diluted in 200 µl of DMEM. If a mixture of laminins was used, the total amount of laminin added per well was unchanged. Each laminin used was slowly thawed on ice to avoid aggregation what would result in asymmetrical spreading on the slide. An uneven distribution of laminins affects the ability of C2C12 myotubes to form AChR clusters, as it can lead to detachment of the myotubes. Once evenly covered with laminin solutions, Flexiperm slides were incubated at 37°C and 5% CO_2 overnight in order to allow the laminins to stick to the surface. The following day, C2C12 myoblasts were trypsinized as described in the previous section, and 1.0-1.2 × 10^5 cells were seeded per well. For the next 24h C2C12 myoblasts were allowed to grow and form an even mesh on the surface, so that individual cells were impossible to distinguish. Once this was achieved, myotube differentiation was induced by gently placing the slides on a heat block set to 37°C and replacement of the growth medium with 700 µl of the differentiation medium (DMEM with glutamine, 2% horse serum [HS; catalog no. 16050122, Thermo

Fisher Scientific], 0.1% fungizone and 1% penicillin/streptomycin). At this stage, the cells were allowed to undergo undisturbed fusion for 4 days (in case a protein knock down was performed this step is slightly different, see section 4.3.1), and special care was taken to avoid any kind of agitation as it would affect attachment of myotubes and in turn result in poor yield of AChR clusters.

3.1.1.5. Differentiation of C2C12 cells on gelatin

Myotubes used for agrin experiments were seeded the same way as described above for laminin cultures, except the flexiperm slides with Permanox grids attached to them were covered with 0.2% gelatin instead of laminins. Similarly, the cells were allowed to proliferate for 24h, followed by gentle replacement of the growth medium with the fusion medium. Four days after initiation of myoblast fusion, 10 nM of soluable agrin (catalog no. 550-AG-100/CF, Biokom) was added directly per well in order to stimulate the myotubes into formation of surface AChR clusters.

3.1.2. Human primary myoblasts

Human primary myoblasts were a gift from EuroBioBank. They were sourced from a healthy, 29 years old female during tissue sampling. Upon arrival the cells were at passage 4. For propagation of the culture the cells were plated and covered with F-10 Ham nutrient mixture (catalog no. N6908-500ML, Sigma). The medium was additionally supplemented with L-glutamine, 20% FBS, sodium bicarbonate, 0.1% fungizone and 1% penicillin/streptomycin. Importantly, 0.5 mg/ml of fetuin (catalog no. F3385-100MG, Sigma) as well as 0.39 µg/ml dexametazon (catalog no. D4902-25MG, Sigma) and 20ng/ml basic fibroblast growth factor (catalog no, G507A) were also added to the medium. The cells were passaged the same way as described for C2C12 cells, with the notable exception of using 0.7% gelatin-coated plates. For differentiation into myotubes, the cells were seeded on laminin-coated Permanox slides with 8-well flexiperm grids at density of 1 x 10^5 cells/well. After two days, the culturing medium was replaced with fusion medium (2% horse serum DMEM). This was followed by an 8 day incubation period to allow uninterrupted fusion. After that time the cells were either fixed for microscopy or lysed for biochemical analysis.

3.1.3. Human embryonic kidney (HEK) cells

HEK293T cells were obtained from American Type Culture Collection (catalog no. CRL3216). The cells were cultured in DMEM supplemented with 4.5 g/l

glucose, 10% fbs, 2 mM l-glutamine, 1% penicillin/streptomycin and 0.1% fungizone. HEKs were incubated at 37% and 5% CO_2 on sterile 6-well plates. When cells reached 90% confluence, they were treated with 3 ml of trypsin and split into new plates that contained fresh medium in a 1:6 ratio.

3.2. Mice

3.2.1. Sacrifice and tissue extraction

Wild type C57BL mice were sacrificed by placing them on cotton pads soaked with 1000mg/g of isofluoran inside a beaker. To extract *tibialis anterior,* the leg was cut off and skin together with fascia were removed. The procedures were conducted in accordance with all applicable laws and regulations.

3.3. Reagents and media

Name	**Ingredients**
C2C12 growth medium	DMEM, 20% FBS, 4.5 g/l glucose, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and fungizone
C2C12 fusion medium	DMEM, 2% HS, 4.5 g/l glucose, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and fungizone
HEK293T growth medium	DMEM, 10% FBS, 4.5 g/l glucose, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and fungizone
Human primary myoblast growth medium	F-10 Ham, 2mM L-glutamine, 20% FBS, sodium bicarbonate, 0.1% fungizone and 1% penicillin/streptomycin, 0.5 mg/ml of fetuin 0.39 µg/ml dexametazon and 20 ng/ml basic fibroblast growth factor
Tissue lysis buffer	50 mM Tris-HCl [Hydroxymethyl aminomethane hydrochloride; pH 8,0], 1 mM dithiothreitol [DTT, 1% Igepal CA-630 [NP-40], 0.5% sodium dodecyl sulfate [SDS], 10% glycerol,], 1 mM NaF, 150 mM NaCl and Mini Protease Inhibitors cocktail
Co-immunoprecipitation (Co-IP) lysis buffer	50 mM Tris-HCL [pH 8], 10% glycerol, 0.5% NP-40, 150 mM NaCl , 1 mM DTT and 1 mM NaF supplemented with Mini Protease Inhibitor cocktail

C2C12 lysis buffer (MS/MS)	50 mM Tris-HCl [pH 8,0], 150 mM NaCl, 0.1% NP-40, 10% glycerol, 1 mM DTT, 1 mM NaF, and Mini Protease Inhibitors cocktail
C2C12 wash buffer (MS/MS)	50 mM Tris-HCl [pH 8,0], 500 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM NaF, and Mini Protease Inhibitors cocktail
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer	25 mM Tris-HCl pH = 7.4, 0.1% SDS, 192 mM glycine pH = 8.3
5 x Transfer buffer	1.5 M glycine, 1.5 M Tris-Base, 0.15% SDS; diluted before use 1:3:1 (5 x transfer buffer : H_2O : ethanol)
Tris-buffered saline (TBS)	50 mM Tris-HCl pH = 7.5, 200 mM NaCl
Tris-buffered saline with Tween (TBST)	0.1% Tween-20 in 1 x TBS
Phosphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4; 1.8 mM KH_2PO_4, pH = 7,4
Blocking buffer for immunostaining	2% bovine serum albumin (BSA), 0.02% normal goat serum (NGS), 0.05% Triton x100, all in PBS

3.4. Antibodies and fluorescent markers

All antibodies used for immunostaining were diluted in the blocking buffer, while those used in western blot analysis were diluted in 10% milk. The respective dilutions and host species are listed in the table below.

Primary antibodies		
Name and host species	**Dilutions**	**Supplier and catalog number**
anti- AChR-α1 (rabbit)	1:1000 for western blot	Proteintech, catalog no. 10613-1-AP
Anti- Rapsyn (rabbit)	1:1000 for western blot 1:250 for immunostaining	Abcam, catalog no. ab156002
Anti- Tubulin (rabbit)	1:5000 for western blot 1:500 for immunostaining	Abcam, catalog no. ab18251
anti-liprin-α-1 (rabbit)	1:1000 for western blot	GeneTex, catalog no. 115098

	1:200 for immunostaining	
Anti LL5β (mouse)	1:250 for immunostaining	Proszynski et al., 2013
Anti- EB1 (mouse)	1:250 for immunostaining	BD Biosciences, catalog no. 610534
Anti-GFP (goat)	1:1000 for western blot	GeneTex, catalog no. GTX26673
Anti-Myosin II heavy chain (fast) (mouse)	1:250 for immunostaining	DSHB, catalog no. AB2147781
anti-Neurofilament (mouse)	1:250 for immunostaining	Developmental Studies Hybridoma Bank, catalog no. 2H3
Anti-β-dystroglycan (mouse)	1:250 for immunostaining	GeneTex, catalog no. GTX635178
Anti- Integrin β (rat)	1:250 for immunostaining	Milipore, catalog no. MAB1997
Anti-Vinculin (mouse)	1:250 for immunostaining	Sigma, catalog no. V4506
Secondary antibodies and fluorescent markers		
Goat anti rabbit IgG (H+L) conjugated with Alexa-Fluor-488	1:500	Thermo Fisher Scientific, catalog no. A-11034
Goat anti rabbit IgG (H+L) conjugated with Alexa-Fluor-568	1:500	Thermo Fisher Scientific, catalog no. A-11011
Goat anti mouse IgG (H+L) conjugated with Alexa-Fluor-488	1:500	Thermo Fisher Scientific, catalog no. A28175
Goat anti mouse IgG (H+L)	1:500	Thermo Fisher Scientific, catalog no. A-11004

conjugated with Alexa-Fluor-568		
Goat anti mouse IgG (H+L) conjugated with Alexa-Fluor-647	1:500	Thermo Fisher Scientific, catalog no. A-11004
Actistain-555 (phalloidin conjugated with Alexa fluor-555)	1:30	Cytoskeleton, catalog no. PHDH1
BTX (unlabelled)	1:1000	Life Technologies, catalog no. B1601
BTX conjugated with Alexa-Fluor-488	1:1000	Life Technologies, catalog no. B13422
BTX conjugated with Alexa-Fluor-555	1:1000	Life Technologies, catalog no. B35451
BTX conjugated with Alexa-Fluor-647	1:1000	Life Technologies, catalog no. B35450

3.5. Cell transfection

3.5.1. siRNA

To perform short-interfering RNA (siRNA) transection and avoid drastic temperature changes that would affect the ability of C2C12 myoubes to form AChR clusters, the permanox slides were taken out of the incubator and placed immediately on a heat block set to 37°C, under the laminar hood. C2C12 myotubes were transfected using 20 nM siRNAs, 72h after replacing the growth medium with differentiation medium. The siRNAs were incubated with Lipofectamine RNAiMAX (catalog no. 13778075, Life Technologies)for 10

minutes at room temperature. Each protein knock down was performed using multiple different siRNA sequences. The siRNA-Lipofectamine mixture was added dropwise to the designated wells and verified using Western Blot analysis. The cell lysis or fixation for microscopy was carried out 48h after the addition of siRNAs. The negative control was a scrambled sequence (catalog no. 12935300, Invitrogen)

siRNA	Sequence	Catalog no. (QIAGEN)
liprin-α-1 siRNA-1	CTGCTTGACGGAAACCATGAA	SI04927860
liprin-α-1 siRNA-2	CACGTCTGTGCATGACCTCAA	SI04927867
liprin-α-1 siRNA-3	ATCCTGTCGATTGGCCTTAAA	SI04927874
Tks5 siRNA-1	CTGGATAAGTTTCCTATTGAA	SI01417395
Tks5 siRNA-2	AAGATGGATATTAGTTCCTTA	SI01417402
Tks5 siRNA-3	TCCGGTGGAGATCATAGGGAA	SI01417409
Tks5 siRNA-4	CACCGTGGAGGTGATTCGAAA	SI01417416

3.5.2. Plasmid DNA

The HEK293T cells were transfected with a Tks5-GFP plasmid using Lipofectamine 2000 (catalog no. 11668027, Invitrogen). 2 µg of plasmid were used to transfect one well in a 6-well plate. The plasmid was diluted accordingly in OPTI-MEM (catalog no. 51200-046, Life Technologies) and 4 µl of Lipofectamine 2000 were added to the mixture. The plasmid solution was the added to the well in a drop-wise manner and cells were incubated at 37°C for 1-2 days before lysis.

3.6. Fixation of cells and tissues for microscopy

3.6.1. Paraformaldehyde fixation

A 4% paraformaldehyde (PFA) solution was prepared by diluting a 20% stock in PBS. Permanox slides were placed on a heat block set to 37°C, and the fusion medium was gently aspirated. The cells were removed from heat block, and 300 µl of 4% PFA were added to each well. Fixation was allowed to proceed for 7 minutes. The PFA was then gently aspirated from the wells, and cells were washed 3 times using PBS.

3.6.2. Cold methanol fixation

In order to preserve microtubules for microscopy, myotubes in some experiments were fixed using cold methanol. For this, cells were taken out of the incubator and placed under the laminar hood in room temperature. The fusion medium was gently removed and wells were covered with methanol that was pre-chilled to -20°C. Fixation was allowed to proceed for 3 minutes and was followed by 3 washes with room temperature PBS.

3.6.3. Tibialis anterior fixation

After extracting the tissue, the muscle was placed in 4% PFA solution for 1h at room temperature, followed by 3 x 5 min. washes in PBS with shaking.

3.7. Immunocytochemistry and immunohistochemistry

3.7.1. Blocking

In order to prevent unspecific binding of antibodies, fixed cells were blocked using a blocking buffer (2% BSA; catalog no. SC-2323, ChemCruz]; 2% NGS; 0.5% Triton X-100 [catalog no. T8787-100ML, Sigma], diluted in PBS). After 1 hour incubation, the blocking buffer was removed and antibodies were added to the wells. If only the proteins present on the surface of the cells were to be stained, the blocking buffer did not contain Triton X-100.

3.7.2. Primary and secondary antibody binding

Primary antibodies were diluted in blocking buffer using 1:250 dilution. 200 µl of the antibody solution were added to each Flexiperm well and the cells were incubated at 4°C overnight. The following day primary antibodies were gently

aspirated and cells were washed 3 times with PBS. Secondary antibodies diluted in the blocking buffer according to a 1:500 ratio (1:1000 for BTX) were then added to appropriate wells and incubated for 1 hour at room temperature. After that period, the antibodies were gently aspirated and the cells were washed 3 times with PBS.

For staining of muscle tissues, the fixed extracts were cut into single fibres and incubated in the blocking buffer. Antibody binding was preformed using the same antibody concentrations and the same buffers as described above.

3.7.3. DAPI staining and preparation of slide for microscopy

Once the last PBS wash was completed, the flexiperm grid was removed the permanox slide and 130 µl of fluoromount containing DAPI (diluted 1:1000), a fluorescent dye specifically binding to DNA, was added on top of the slide. A cover glass was then placed on the slide making sure that no air bubbles are present. The edges of the cover glass were then sealed using transparent nail polish to prevent fluoromount evaporation and securing the slide that was now ready for microscopy and could be stored at 4°C.

3.7.4. Nocodazole-mediated microtubule disruption

For disrupting the microtubules a total concentration of 10 µg/ml of nocodazole (catalog no. M1404, Sigma) was added per well.

3.7.5. Microscopy and image processing

Microscopic analysis was performed at the Laboratory of Imaging Tissue Structure and Function, Nencki Institute, using a Zeiss Spinning Disc confocal microscope that was equipped with a diode. Images were collected using ZEN software (ZEISS International) and analyzed using ImageJ/Fiji software.

3.8. Protein detection

3.8.1. Isolating proteins from cells

In order to extract proteins, cells cultured on the Permanox slides were lysed using a lysis solution (50 mM Tris-HCl [pH 8,0], 150 mM NaCl, 0,1% NP-40, 10% glycerol, 1 mM DTT, 1 mM NaF, and Mini Protease Inhibitors cocktail [catalog no. 11873580001, Roche]), the surface of the slide was additionally scraped using a pipette tip to maximize the amount of extracted proteins. The lysates were then placed on ice and were centrifuged at 20, 000 x g for 30 minutes at 4°C. After that the supernatant was removed and used for further analysis while the pellet was discarded.

3.8.2. Western Blot

Prior to performing the Western Blot analysis the protein concentration of used lysates was evaluated using NanoDrop 2000C spectrophotometer (Protein A280 program) to optimize amounts of loaded samples. Each time enough lysate was loaded on the gel to achieve around 10 µg of protein per well. Every sample analysed was diluted in 2x sample loading buffer + DTT and boiled at 95°C for 10 minutes before loading on the gel

3.8.2.1. Protein electrophoresis

The SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out using a home-made gel of one of the following compositions. For an 8% separating gel, 3.7 ml of water, 2.1 ml of 30% acrylamide, 2 ml of 1.5M Tris HCl (pH 8.8), 0.08 ml of 10% SDS, 0.08 ml of 10% ammonium persulfate (APS) and 0.008 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) were used. For the 4% stacking gel, 3.8 ml of water, 0.7 ml of 30% acrylamide, 0.5 ml of 1M Tris HCl (pH 6.8), 0.5 ml of 10% SDS, 0.5 ml of 10% APS and 0.005 ml of TEMED were used. After pouring the separating gel into the glass covers, it was overlayed with isopropanol to get rid of air bubbles. After the separating gel solidified, the alcohol was washed away and the stacking gel fraction was added on top of it. Commercial protein markers were used (ColorPlus Prestained Protein Ladder, catalog no. P7711, New England Biolabs).

3.8.2.2. Protein transfer to nitrocellulose membrane

Once the electrophoresis was completed and the proteins were separated, the contents of the gel were transferred onto a nitrocellulose membrane (catalog

no. 66485, Pall Corporation). For this, the membrane and two cotton pads were soaked in a transfer buffer. The first cotton pad was then placed in a transfer cassette, on top of it a membrane was placed followed by the gel and the second pad. The cassette was then closed and placed in Trans Blot Turbo machine (catalog no. 1704270, Bio-Rad), which elicited a 1,3 A, 25 V current for 7 minutes, leading to a transfer of protein from the gel onto the membrane.

3.8.2.3. Antibody binding

After the transfer, the nitrocellulose membrane was incubated in 10% solution of milk (nonfat powder) dissolved in tris-buffered saline with tween (TBST), for 1 hour at room temperature with shaking. The milk was then discarded and replaced with a primiary antibody-containing 10% milk, and incubated at 4°C overnight. The following day, the antibody-containing solution was collected for future use, and the membrane was washed 3 times for 10 minute using TBST, at room temperature and with shaking. After that a secondary antibody conjugated with horseradish peroxidase (HRP), also dissolved in 10% milk, was added on top of the membrane which was incubated at room temperature for 1-2 hours with shaking. This was followed by 3 10 minute washes with TBST.

3.8.2.4. Film development

In order to visualize the proteins labelled by secondary antibodies, a chemiluminescent signal was induced by supplementing the HRP bound-secondary antibodies with a luminol substrate and peroxide buffer (catalog no. 1705060, Bio-Rad). This was carried out in the dark room, and once the reaction was initiated, a blue-light sensitive autoradiography film was placed on top of the membrane to imprint the emitted signal. The film was then developed in a medical film processor Fujifilm FPM 900A.

3.8.3. Proximity ligation assay (PLA)

The assay was used to visualize protein-protein interactions. Briefly, two proteins are labelled with primary antibodies, which then are hybridized to secondary antibodies conjugated to oligonucleotide sequences. If the two proteins are in close proximity, the oligonucleotides can ligate and be further amplified with polymerase. A fluorescent probe can then bind to the amplified sequence and be detected with a confocal microscope.

All of the reagents (except from primary antibodies) to perform the PLA were provided in a single kit (catalog no. DUO92101, Sigma). The assay was

performed according to the manufacturer's instructions, using anti-rapsyn (catalog no. ab156002, Abcam) and anti-βactin (catalog no. A1978, Sigma) primary antibodies. The kit that was used contained anti-mouse and anti-rabbit secondary antibodies.

3.9. Protein complex pulldown

3.9.1. Preparation of magnetic beads

Prior to the experiment, magnetic beads (catalog no. Dynabeads™ Protein G 10004D, Invitrogen) were resuspended by vortex. The total amount needed for the experiment (25 µl per sample) was transferred to an Eppendorf tube, and placed on a magnet in order to separate the beads from the buffer. The supernatant was then extracted and beads were rinsed 3 times with PBS + 0.1% NP40.

3.9.2. Coupling peptides

20 µg (per sample) of the appropriate αDB-1 peptide were diluted in 50 µl of PBS + 0.1% NP40 and added to the beads, followed by a 30 minute incubation with rotation at room temperature. Two peptides were used in the experiment, one unphosphorylated containing the sequence around Y730 αDB-1, and the second one containing the same sequence with phosphorylated Y730 (**Figure 12A**).

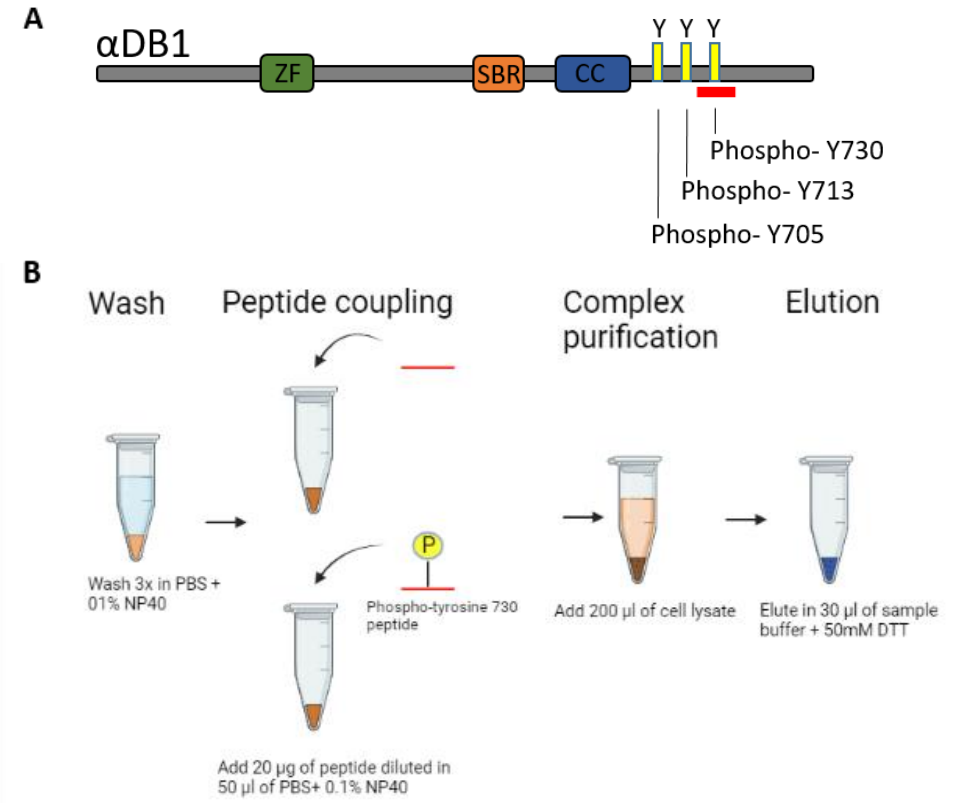


Figure 12: Schematic representation of protein pulldown protocol using αDB1 peptides. (A) *αDB1 contains three tyrosine sites at its C-terminal, that can undergo phosphorylation: Y705; Y713 and Y730. For my experiments, I used either phosphorylated or unphosphorylated (control) αDB1 peptides containing Y730, indicated with the red line on the schematic drawing.* **(B)** *Beads were first washed, followed by coupling of diluted peptides and 30 min incubation at room temperature. Then, beads were washed and lysate was added for overnight incubation at 4°C to purify the complexes. The next day, samples were eluted and subjected to electrophoresis.*

After that time, the tube was placed on the magnet and supernatant containing unbound peptides was removed. The bead-peptide complex was then washed 2 times with PBS + 0.1% NP40 and 1 time with the lysis buffer in order to calibrate the beads. αDB1 has 3 possible site of tyrosine phosphorylation. For this analysis peptides from the αDB1 region containing tyrosine-730 were used (indicated with a red line in the Figure below). Peptides phosphorylated at tyrosine-730 were used and the same unphosphorylated sequence was used as control (**Figure 12B**).

3.9.3. Purifying target protein complex

In order to purify the target complexes, 200 µl of cell lysate (per sample) were added to the beads-peptide complex, followed by an overnight incubation with rotation at 4°C. The next day, tube containing beads-peptide-protein complexes was placed on a magnet and the supernatant (flow-through) was removed and

stored to be used as control later on. The beads were then washed 4 times with the lysis buffer.

3.9.4. Eluting target protein complex

Once the last wash was discarded from the tube containing beads-peptide-protein complex, it was removed from the magnet and the beads were resuspended in 30 µl (per sample) of 2x Laemmli Sample Buffer + 50mM DTT, followed by a 10 minute incubation at 95°C. After denaturation the samples were immediately loaded on the gel, or frozen in -20°C.

3.10. Mass spectrometry

The mass spectrometry analysis was carried out in order to find interacting partners of αDB1, that bind to it either when the tyrosine-730 is phosphorylated or unphosphorylated. For this purpose, the mass spectrometry analysis was performed on eluates obtained by coupling the phosphorylated and unphosphorylated DB1-730 peptides to Dynabeads M-270 and incubation with C2C12 cell lysates.

3.10.1. C2C12 cell lysis

For this analysis, a combined lysate from ten 15-cm plates was used per sample. The whole procedure was carried out in at 4°C. The growth medium was aspirated and each plate containing cells was first gently washed with 20 ml of cold PBS in order to remove any remaining medium. 1.5 ml of lysis buffer were then added per plate and the cells were scraped in order to facilitate the lysis. The lysis was repeated for all the remaining plates and the lysate from all of them was collected into a single 50 ml tube. The lysate was then pumped through a 25 gauge needle 5 times followed by rotation for 30 minutes in the cold room. Next the lysate was centrifuged at 18 000xg in -4°C for 30 minutes and the supernatant was placed in a fresh, chilled 50 ml tube.

3.10.2. Coupling peptides

For each sample, 440 µl of Epoxy M-270 beads (catalog no. 14301, Invitrogen) were used. The beads were first placed on a magnet and the supernatant was removed, followed by 4 washes with PBS + pervanadate (PVD). Then, 40 µl of either DB1-730phospho or DB1-730 were added to the beads followed by 30 minute incubation at room temperature. After that, the tubes were placed on a

magnet, supernatant was removed and the samples were washed 3 times with PBS +PVD and 2 times with the lysis buffer.

3.10.3. Protein complexes precipitation

Once beads coupled with appropriate peptides were washed, they were added to C2C12 lysates, followed by an overnight incubation with rotation at 4°C.

3.10.4. Protein complexes elution and identification

Following the overnight incubation, the tubes were placed on a magnet, supernatant was collected and beads were washed 3 times with the lysis buffer. Then, 40 µl of 2x Sample Buffer +DTT were used to resuspend each sample. The samples were then placed on a heat block and incubated for 10 minutes at 95°C. The mass spec analysis was outsourced to the MS Bioworks facility (USA).

3.11 Statistics

One way ANOVA with Dunnett tests were used to determine the statistical significance if more than one sets of data were compared against control. If one set of data was compared against control, unpaired t test was used to determine the statistical significance. Error bars represent SEM.

4. Results

4.1. Liprin-α-1 as a novel NMJ protein

Liprin-α-1 is a scaffold protein that has been shown to have important functions in maintaining the neuronal synapses in the CNS (Spangler and Hoogenraad 2007). However, its role has never been studied at the vertebrate NMJ. Given that my colleagues, Dr Krzysztof Bernadzki and Dr Marta Gawor, have identified its interaction with an important postsynaptic protein- αDB1, I decided to investigate whether liprin-α-1 is involved in regulating the assembly of NMJ postsynaptic machinery (Bernadzki, Gawor et al. 2017).

4.1.1. Liprin-α-1 is required for AChR clustering

To first evaluate whether liprin-α-1 has any function at the postsynaptic specialization, I performed a knocked down of its expression through RNA interference, implementing 3 distinct siRNA sequences. Prior to siRNA transfection, the *in vitro* cultured C2C12 myoblasts were stimulated into production of AChR clusters by addition of soluble agrin. The AChR clusters were visualized by incubation with AlexaFluor488-bungarotoxin (BTX488). BTX is a protein derived from the paralyzing venom of *Bungarus multicinctus* snake, that binds specifically to the AChRs. And is therefore a useful tool for labelling the postsynaptic machinery (**Figure 13A**). The myotubes that were either untreated with the siRNA or treated with a scrambled siRNA sequence (negative control), formed numerous clusters of AChRs. However, the cells where liprin-α-1 expression was inhibited had produced a significantly lower amount of these assemblies. While transfection of all of the siRNA sequences led to the reduction of AChR cluster number, the most profound effect was observed for myotubes treated with the liprin-α-1 siRNA1 sequence that corresponds to the intrinsically disordered domain (**Figure 13B**). The efficiency of siRNA-mediated knockdown of liprin-α-1 was confirmed by my colleague, Krzysztof Bernadzki, by western blot analysis of HEK lysates transfected with liprin-α-1-GFP and the respective siRNA sequences (**Figure 13C**). Each siRNA sequence was equally efficient at silencing its target.

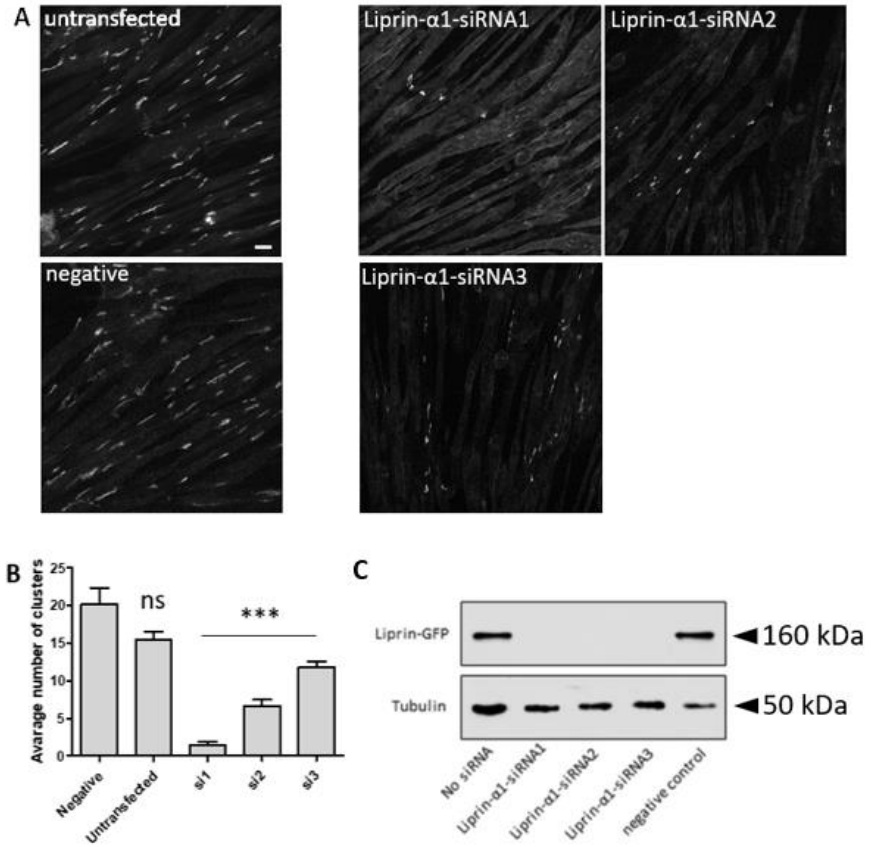


Figure 13: AChR clusters after liprin-α-1 knockdown. (A) *Control C2C12 myotubes untreated with siRNA (upper panel) and treated with non-targeting siRNA (lower panel). AChR clusters in C2C12 myotubes treated with 3 different siRNA sequences had significantly fewer AChR clusters with respect to controls.* **(B)** *Quantification of results from **A** representing an average number of clusters.* **(C)** *Western blot analysis showing level of liprin-α-1 protein in HEK lysates transfected with liprin-α-1-GFP plasmid and respective siRNA sequences. Tubulin was used as loading control. This western blot was performed by my colleague Dr Krzysztof Bernadzki. One way ANOVA with Dunnett test (negative control used as reference) were used to determine the statistical significance. A total of 30 areas was evaluated for each transfection in terms of the number of clusters. Error bars represent SEM. ***P< 0.0001. Scale bar= 20 µm. For visualizing AChRs α-BTX488 was used.*

4.1.2. AChRs and rapsyn proteins are expressed after liprin-α-1 knockdown

Aberrant formation of AChR clusters can be caused by defects in expression of postsynaptic machinery proteins, faulty delivery of the receptors to the cell surface or the inability of single, membrane-bound receptors to cluster (**Figure 14A**). Thus, in order to see whether the absence of postsynaptic machinery clusters on the surface of C2C12 myotubes, observed after liprin-α-1 knockdown is a result of a decreased expression of AChR or other crucial components of the postsynaptic machinery such as rapsyn, I performed a

Western Blot analysis (**Figure 14B**). This revealed that no significant differences in the expression of these proteins took place in the absence of Liprin-α-1.

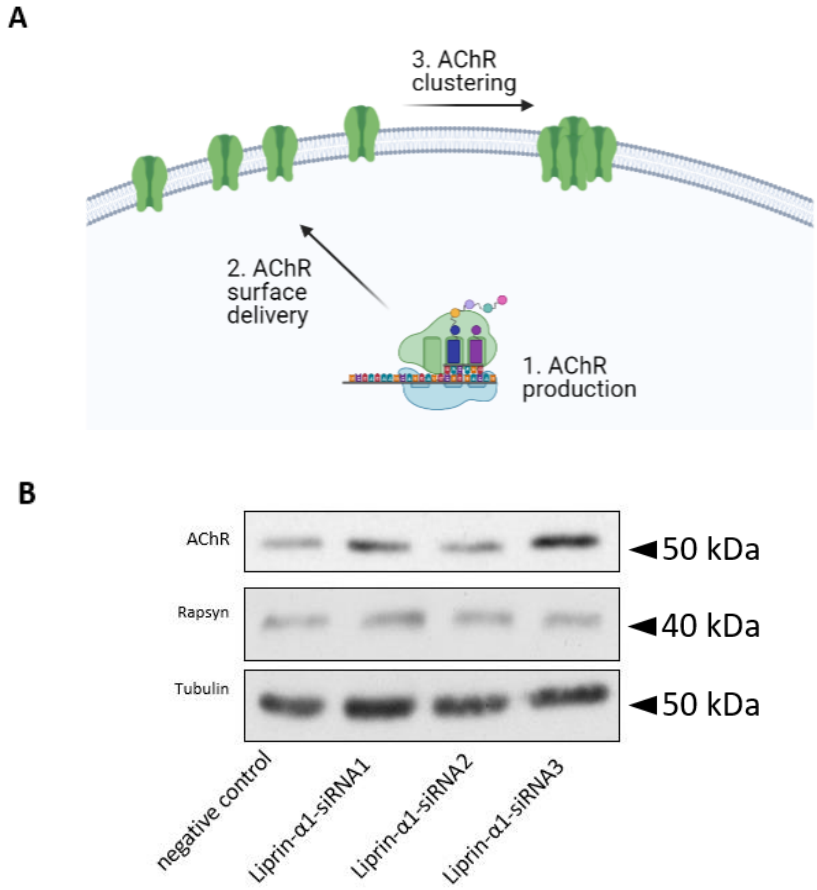


Figure 14: Liprin-α-1 knockdown does not inhibit expression of postsynaptic machinery proteins. (A) *Schematic representation of key steps involved in formation of AChR clusters.* **(B)** *The lysates of C2C12 myotubes treated with respective siRNAs were probed with antibodies for AChR and rapsyn. Tubulin levels were used as a loading control.*

4.1.3. AChRs are delivered to the surface of myotubes after liprin-α-1 knockdown

Since the expression of crucial postsynaptic machinery proteins was undisturbed upon of liprin-α-1 knockdown, the next possible explanation for the reduction of AChR clusters number in those myotubes was aberrant surface delivery of the receptors. In order to determine whether the AChRs are present at the cell surface but unable to cluster, myotubes where liprin-α-1 expression was inhibited were stained live with BTX488, followed by fixation, permeabilization with Triton-X100 and addition of AlexaFluor555-bungarotoxin (BTX555) for marking of the internal receptors (**Figure 15A**). While the levels of internal AChRs were the same in knockdown and control cells, myotubes where liprin-α-1 was silenced had a strong green fluorescence signal suggesting that

receptors were present at the cell surface, despite absence of clusters (**Figure 15B**).

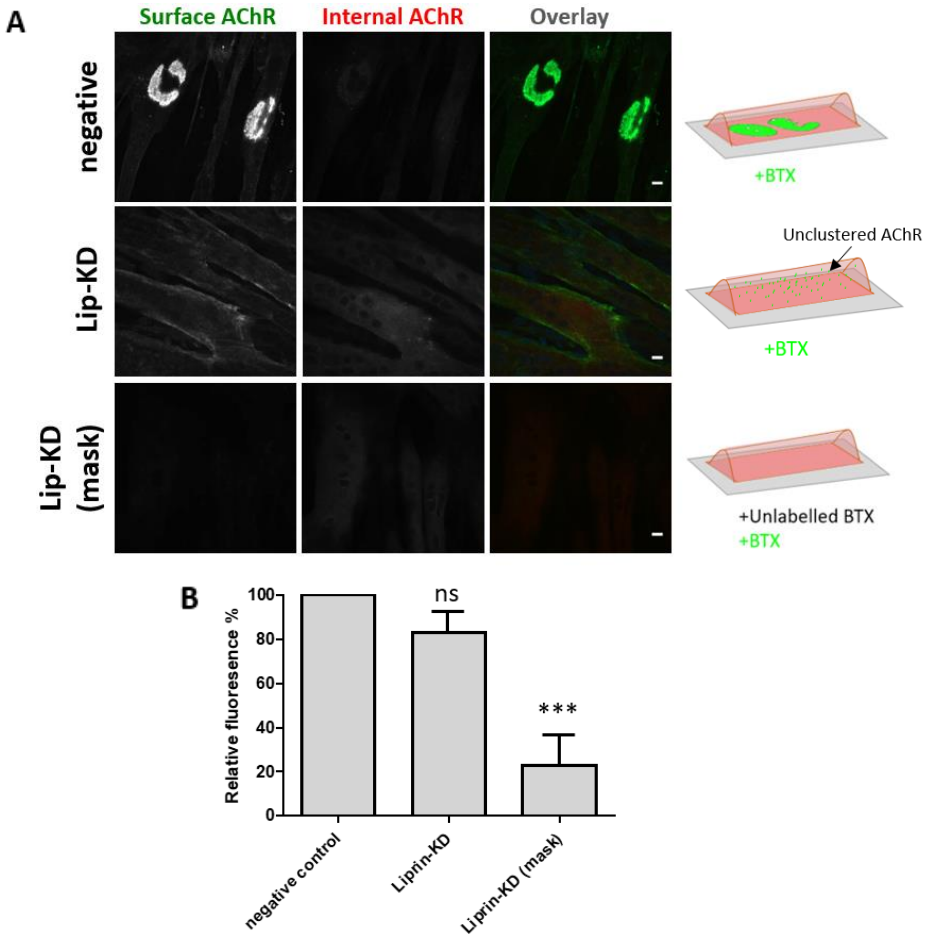


Figure 15: AChRs are still delivered to the cell surface after liprin-α-1 knockdown. (A) *BTX488 was used to label the surface receptors, while 0BTX555 was used after permeabilization to label internal AChRs. The bottom panel represents myotubes treated with unlabelled BTX prior to the addition of BTX-488. Schematic diagrams show the source of fluorescence in each experiment. In top panel most of the signal came from clustered AChRs, while in the middle from unclustered AChRs. Since epitopes in the myotubes from the bottom panel were saturated with unlabelled BTX, the recorded signal con only be attributed to autofluoresence.* **(B)** *Fluoresence quantification of results shown in A. Values were normalized to control. One way ANOVA with Dunnett test (negative control used as reference) were used to determine the statistical significance. A total of 30 readings from random areas was used for each point. Error bars represent SEM. ***P< 0.0001. Scale bar= 10 µm*

To eliminate the possibility that the signal comes from autofluoresence of myotubes, the experiment was repeated with an additional step of saturating the surface AChRs with unlabelled BTX prior to the addition of BTX488. As a result, myotubes where receptors were masked did not produce any green fluorescence, proving the specificity of the implemented labelling method (**Figure 15B**). Taken together, this data points out that despite the absence of

AChR clusters in myotubes depleted of liprin-α-1, significant amounts of the receptors are still delivered to the cell surface.

4.1.4. Liprin-α-1 co-localizes with LL5β at the neuromuscular junction

Given that the AChRs were expressed and delivered to the cell surface appropriately after liprin-α-1 knockdown, the reason for the absence of receptor hotspots **(Figure 13)** was the inability of AChR to form clusters. As mentioned in the introduction, liprin is a component of the LL5β-CLASP complex that regulates microtubule organization. It is possible that through interaction with this complex in C2C12 myotubes, liprin orchestrates cortical microtubules that are essential for maintenance of the AChR clusters. Thus, I performed immunohistochemical staining of tibialis anterior muscle to determine whether liprin co-localizes with AChR and the LL5B (**Figure 16**).

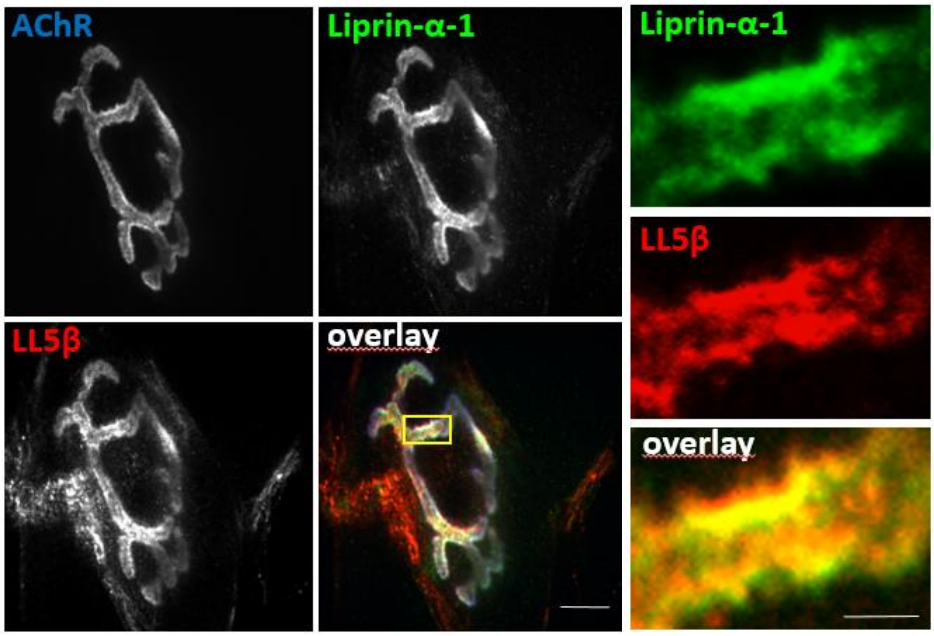


Figure 16: Liprin-α-1 co-localizes with LL5β and the AChRs *in vivo.* *Tibialis anterior muscle was surgically removed from P60 mouse, fixed in 4% PFA, incubated with blocking buffer and labelled with primary antibodies against LL5β and liprin-α-1. Following the incubation with secondary antibodies and BTX the muscle fibers were analysed under confocal microscope. Magnified image (4x) of the area indicated with a yellow box. Scale bar= 10 µm for entire-NMJ photos and 5 µm for magnified images.*

4.1.5. Liprin-α-1 regulates cortical microtubules in C2C12 myoblasts

Following the immunocytochemical staining for LL5β and liprin-α-1 at murine NMJ, a possibility arose that the two proteins could be part of the same complex in skeletal muscle given their strong co-localization. If that were the case, knockdown of liprin-α-1 could lead to instability of cortical microtubules, which would provide an explanation for the reduced amount of MT tips reaching the edge of cells (**Figure 13).** Thus, I transfected C2C12 myoblasts with siRNA directed towards liprin-α-1 sequence, and performed immunohistochemical analysis of the microtubules in both, transfected and control cell lines (**Figure 17**). What I observed was a significant reduction in the number of microtubules contacting the edge of myoblasts when liprin- α-1 was absent.

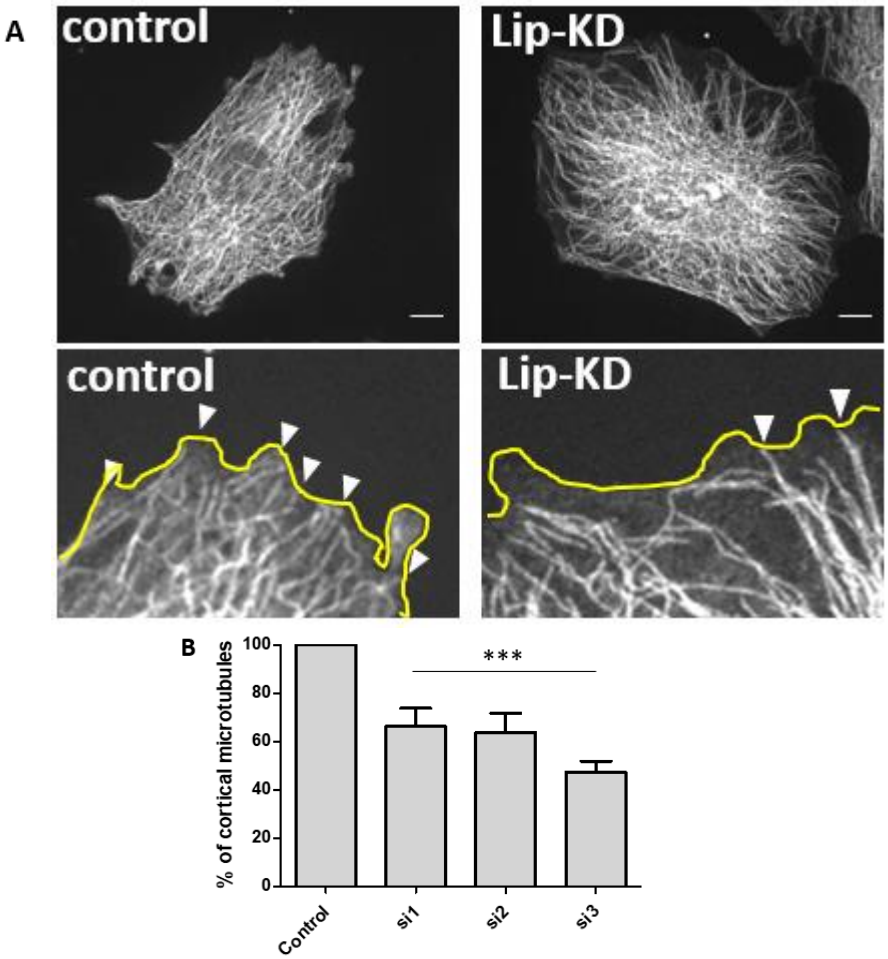


Figure 17: Liprin-α-1 regulates the number of cortical microtubules in C2C12 myoblasts. (A) *C2C12 myoblasts were fixed and stained with primary antibodies against β-tubulin. The bottom panels represent higher magnification of cortical areas from the top panels. The white arrowheads mark the cortical microtubules. The yellow line marks the edge of cells, only microtubules that contacted the yellow line were quantified* **(B)** *Quantification of data from* ***A****. Values were normalized to control. One way ANOVA with Dunnett test (negative control used as reference) were used to determine the statistical significance. A total of 30 cells was used for each point in the evaluation. Error bars represent SEM. ***P< 0.0001. Scale bar= 10 µm*

4.1.6. Liprin-α-1 regulates cortical microtubules in fused myotubes

Having found that in the absence of liprin-α-1 there are fewer cortical microtubules, I wanted to see if their number is also affected in fused myotubes in the synaptic area. Since myotubes have significantly denser microtubule system than myoblasts it would be extremely difficult to determine the exact number of filaments that are present underneath the AChR cluster. For this reason, I performed immunohistochemical staining for microtubule end binding protein 1 (EB1), which allowed visualization of microtubule tips (**Figure 18**). Each

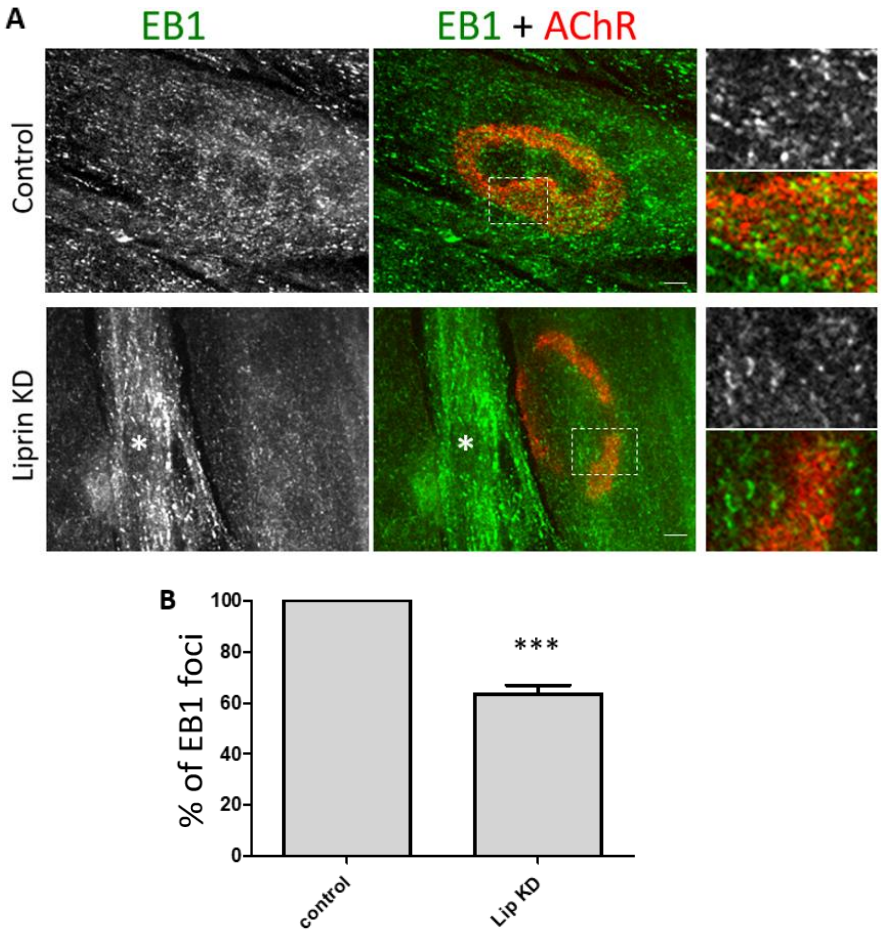


Figure 18: Liprin-α-1 knockdown leads to fewer EB1 foci in the synaptic region. **(A)** *C2C12 myotubess were fixed and stained with primary antibodies against EB1 to visualize the microtubule tips. Boxed areas are magnified on the right panels. The asterisk denotes a nucleus of an elongated myocyte.* **(B)** *Quantification of data from **A**. Values were normalized to control. Unpaired t test was used to determine the statistical significance. 30 AChR clusters were evaluated for each point of the analysis. Error bars represent SEM. ***P< 0.0001. Scale bar= 10 µm*

EB1 foci corresponds to a single microtubule tip, thus the images presented below show just how densely the filaments are packed within a myotube. I

found that after liprin-α-1 silencing, significantly fewer EB1 foci are found in the synaptic area, suggesting that liprin-α-1 is also involved in regulating the cytoskeleton in mature myotubes. In order to normalize the acquired data, only the EB1 foci that overlapped with AChR clusters were counted and then divided by the area occupied by the receptors. The graph shows the number of EB1 foci in myotubes treated with liprin-α-1 siRNA as percentage of the value obtained for control cells.

4.1.7. Microtubules are not involved in maintenance of AChR cluster but are needed for cluster growth.

Through the previous experiments I was able to hypothesize that the absence of AChR clusters in myotubes where liprin-α-1 was depleted was neither a result of aberrant AChR expression nor compromised delivery of the receptors to the cell surface. This leads to a conclusion that the observed phenotype is due to inability of surface AChR to cluster, likely because of cortical microtubule impairment. However, I had to confirm that microtubules play a role in assembling the AChR cluster. For this reason, I evaluated addition of new receptors to pre-existing clusters after treating the myotubes with microtubule-disrupting agent nocodazole **(Figure 19A)**. The result of this experiment showed that while the pre-existing AChR assemblies were not affected by the disruption of microtubules, no new receptors were added after the nocodazole treatment as opposed to the DMSO control (**Figure 19B, C and D**). Combined with the results of my colleague, Paula Mazurek, who showed through AChR precipitation experiment that the amount of surface AChR is unchanged after nocodazole treatment, this data suggests that while microtubules play an essential role in AChR clustering, their involvement in delivery of the receptors to the plasma membrane is marginal.

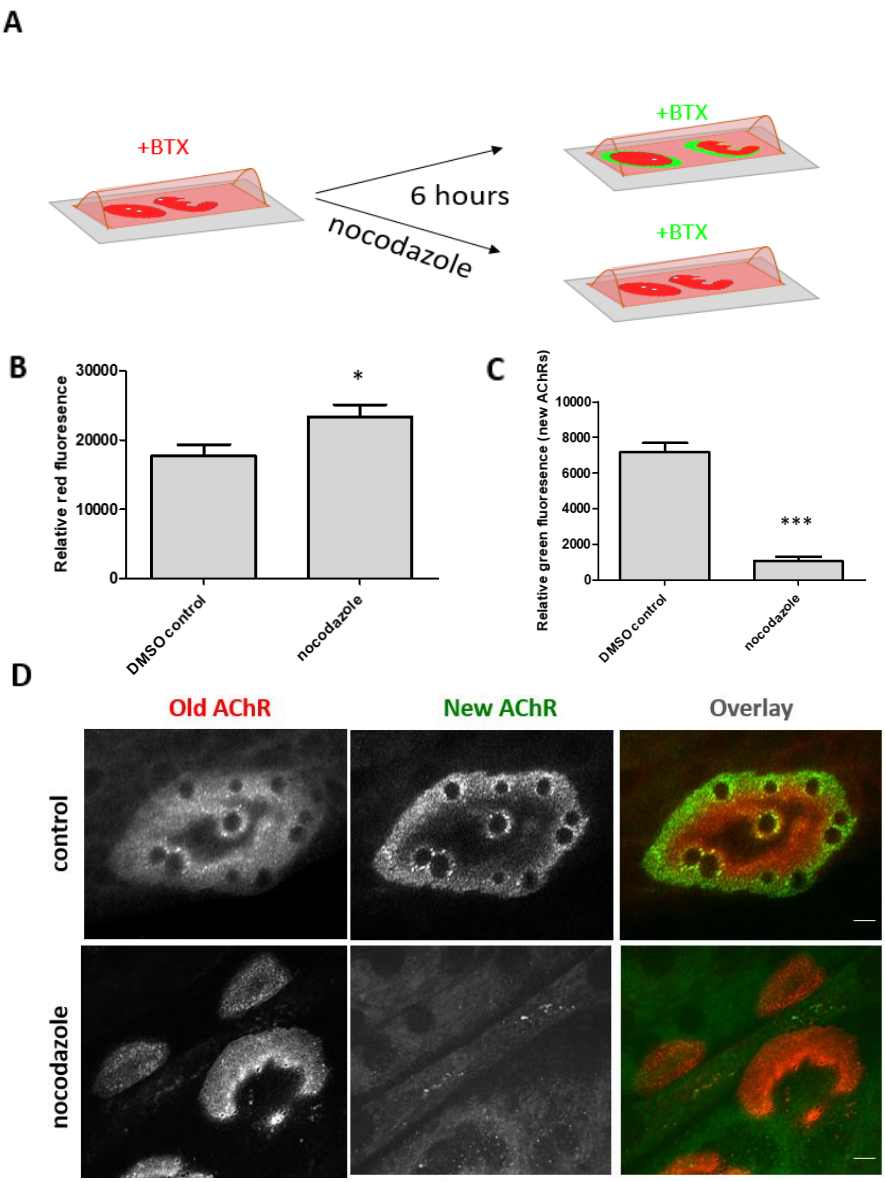


Figure 19: Microtubules are involved in AChR clustering. (A) *Schematic diagram showing experimental design for determining the role of microtubules in AChR clustering. Briefly, BTX555 was added live to C2C12 myotubes for 15 minutes, followed by addition of nocodazole. After 6 hours BTX488 was added to the cells for 15 minutes followed by fixation. As a result, control cells had visible green signal on the periphery of old (red) clusters, while nocodazole-treated cells had no apparent signal of new receptors around the synaptic areas.* **(B)** *Fluoresence intensity for old (red) AChR clusters.* **(C)** *Fluoresence intensity for new (green) AChR clusters unpaired t test was used to determine the statistical significance.* **(D)** *Fluorescent staining of AChR clusters labelled as described in* ***A.*** The image in **D** was taken by my colleagues Paula Mazurek and Dr Marta Gawor. *30 AChR clusters were used for each analysis. Error bars represent SEM. *P< 0.01; ***P< 0.0001. Scale bar= 10 µm*

Through my work on liprin-α-1, I was able to demonstrate that this protein is required for formation of AChR clusters in *in vitro* cultured C2C12 myotubes. It regulates cortical microtubules found at the postsynaptic machinery, which in

turn are involved in clustering of AChRs that got delivered to the cell surface. This is a first time when liprin-α-1 was shown to play a role in the formation of a muscle postsynaptic machinery.

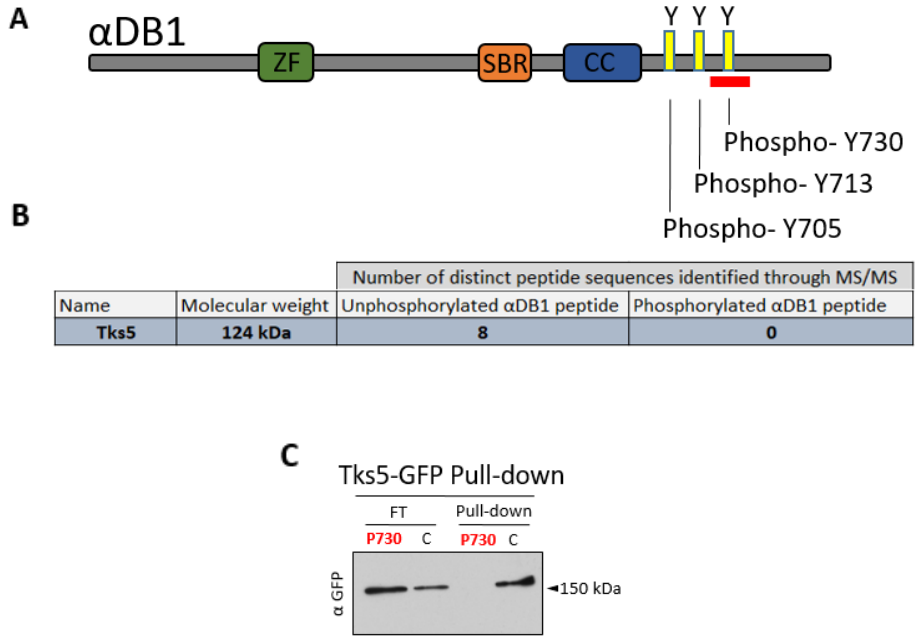
4.2. Tks5 as a novel NMJ protein

The DGC is an important molecular anchor that ensures stability of the postsynaptic machinery by linking it to the ECM via the αDG and to the cytoskeleton using intracellular complexes. αDB1 is a crucial component of the cytoplasmic DGC, however very little is known about its function in regulating the NMJ. Since the first mass spectrometry analysis of αDB1 interactors yielded valuable insights into the cytoskeleton organization at the postsynaptic machinery, in the form of identifying liprin-α-1 as a protein that regulates cortical microtubules, I decided to continue investigating other αDB1-interacting proteins to further dissect the role that these protein play at the NMJ (**Figure 6**). Tks5 is an adaptor protein known mainly for its crucial involvement in the formation of structures known as invadosomes. These protrusions resemble very closely actin-rich structures responsible for remodelling of the postsynaptic machinery *in vitro*, known as synaptic podosomes. However, the role of Tks5 has never been studied before in the context of the NMJ, making this protein a particularly interesting target for investigating the mechanism of postsynaptic machinery remodelling.

4.2.1. Identification of Tks5 as an interactor of unphosphorylated dystrobrevin

αDB1 has three potential sites of tyrosine phosphorylation: Y705; Y713 and Y730 located at its C-terminal. Since these sites are likely involved in regulating the function of this protein, it is worthwhile to investigate the interactors that bind to these tyrosines in either phosphor-specific or unphospho-specific manner (Grady, Akaaboune et al. 2003). To identify novel proteins that interact with αDB1, I performed a pulldown experiment using magnetic beads coated with either unphosphorylated or phosphorylated αDB1 peptide 730 as bait to extract its interacting partners from a lysate of fused C2C12 myotubes (**Figure 20A**). The eluted samples were then sent for mass spectrometry analysis. Through this analysis Tks5 protein was identified as an interactor of specifically

unphosphorylated 730 αDB1 peptide (**Figure 20B**). After obtaining the mass spectrometry results, I was able to confirm the mode of Tks5 interaction with αDB1 through a pulldown assay (**Figure 20C**). Briefly, HEK cells were transfected with Tks5-GFP encoding plasmid, lysed and placed on beads coated with the same peptides that were used for the mass spectrometry analysis.



		Number of distinct peptide sequences identified through MS/MS	
Name	Molecular weight	Unphosphorylated αDB1 peptide	Phosphorylated αDB1 peptide
Tks5	**124 kDa**	**8**	**0**

Figure 20: Tks5 interacts with α-dystrobrevin. (A) *Schematic representation of* αDB1 *phosphorylation sites. The red line represent portion of the protein used for generating the peptides used in experiments.* **(B)** *Mass spectrometry analysis results for interactors of phosphorylated and unphosphorylated αDB1 peptide 730. Tks5 was one of the interesting and unique hits.* **(C)** *Western blot for anti-GFP antibodies. HEK cells were transfected with Tks5-GFP construct followed by lysis and loading on beads coated with phosphorylated and unphosphorylated* αDB1 *peptide 730. The analysis showed that Tks5 only interacts with unphosphorylated peptide. FT- flow-through; Pull-down- eluted sample; P730- phosphorylated αDB1 peptide; C- unphosphorylated αDB1 peptide.*

4.2.2. Tks5 is essential for synaptic podosome formation

In order to investigate what is the potential role of the known actin-regulator Tks5, in terms of synaptic regulation, I performed an siRNA-mediated knockdown of this protein in C2C12 myotubes using four different siRNA sequences (**Figure 21A**). While the number of AChR clusters was unchanged upon silencing the Tks5 expression, other interesting phenotypes have appeared (**Figure 21B**). One of them was a nearly complete disappearance of synaptic podosomes (actin-rich organelles responsible for the remodelling of postsynaptic machinery) from the AChR clusters (**Figure 21C and D**). All of the

tested siRNAs evoked effect, while cells treated with the siRNA4 had the most profound reduction in the number of synaptic podosomes. The clusters still underwent some kind of remodelling, as they did not appear as simple plaques, which are considered to be the first developmental stage of the AChR cluster. This meant that Tks5 is specifically involved in podosome-driven remodelling of AChR cluster topology and other pathways involved in this process are likely unaffected by silencing of this protein's expression.

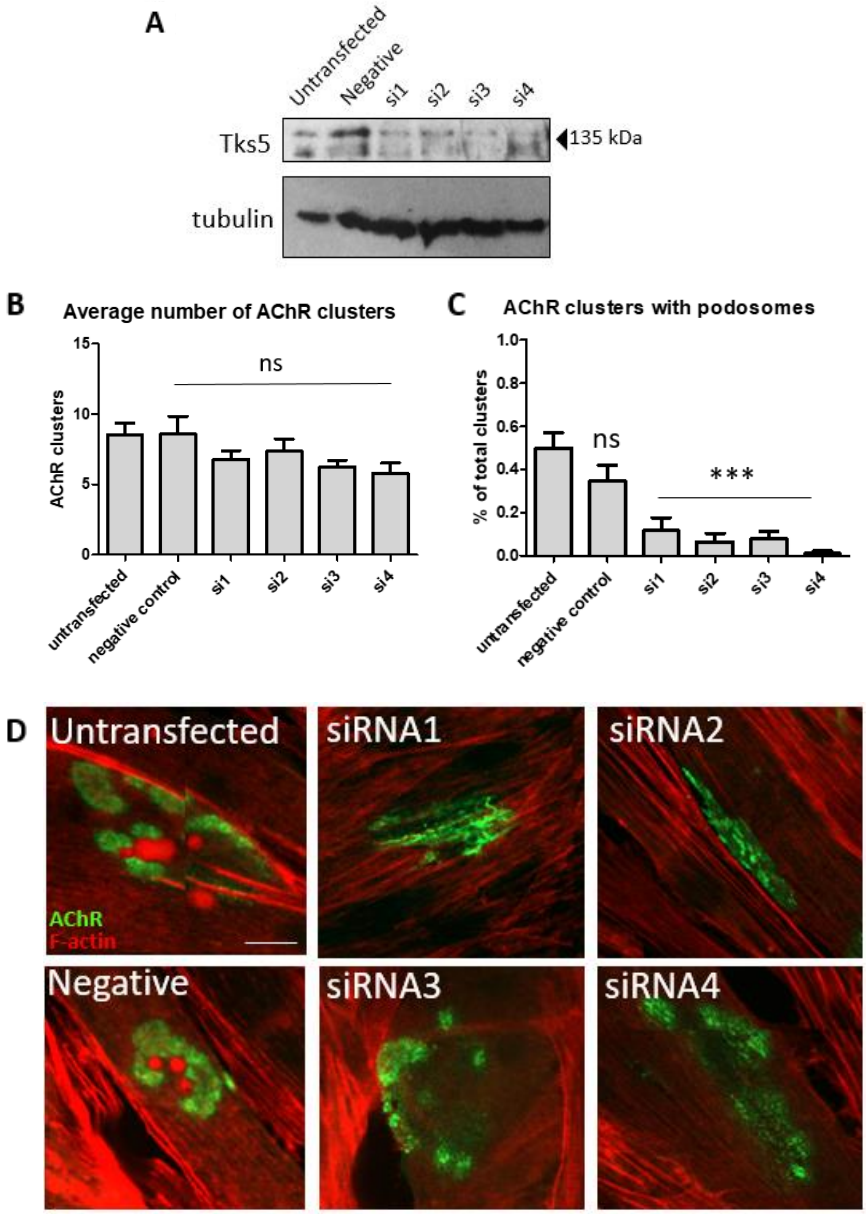


Figure 21: Tks5 regulates formation of synaptic podosomes. (A) *Western blot analysis of Tks5 protein in lysates of C2C12 proteins treated with either unspecific (negative) or Tks5 siRNAs. Tubulin was used as a loading control.* **(B)** *Graph represents the average number of clusters found in C2C12 myotubes in control as well as after treatment with the indicated siRNA.* **(C)** *Graph represents the percentages of clusters containing synaptic podosomes in control cells and those treated with the indicated siRNAs.* **(D)** *Example images of AChR clusters in*

*control and Tks5-depleted C2C12 myotubes. AChRs were visualized with BTX488 and actin with Alexa555-phalloidin. One way ANOVA with Dunnett test (untransfected control used as reference) were used to determine the statistical significance. 40 AChR clusters were used per point in the analysis. Error bars represent SEM. ***P< 0.0001. Scale bar= 10 µm. Initial observations were made by Dr. Kamila Olejniczak.*

4.2.3. Absence of Tks5 leads to AChR cluster dissociation

Another interesting phenotype observed after Tks5 knockdown in C2C12 myotubes was the apparent instability of the AChR clusters represented by fragmentation of the postsynaptic machinery. All except one of the tested siRNAs elicited this effect (**Figure 22A and B**).Similar aberrations are observed at the postsynaptic machinery *in vivo* when members of the DGC, such as dystrophin are silenced.

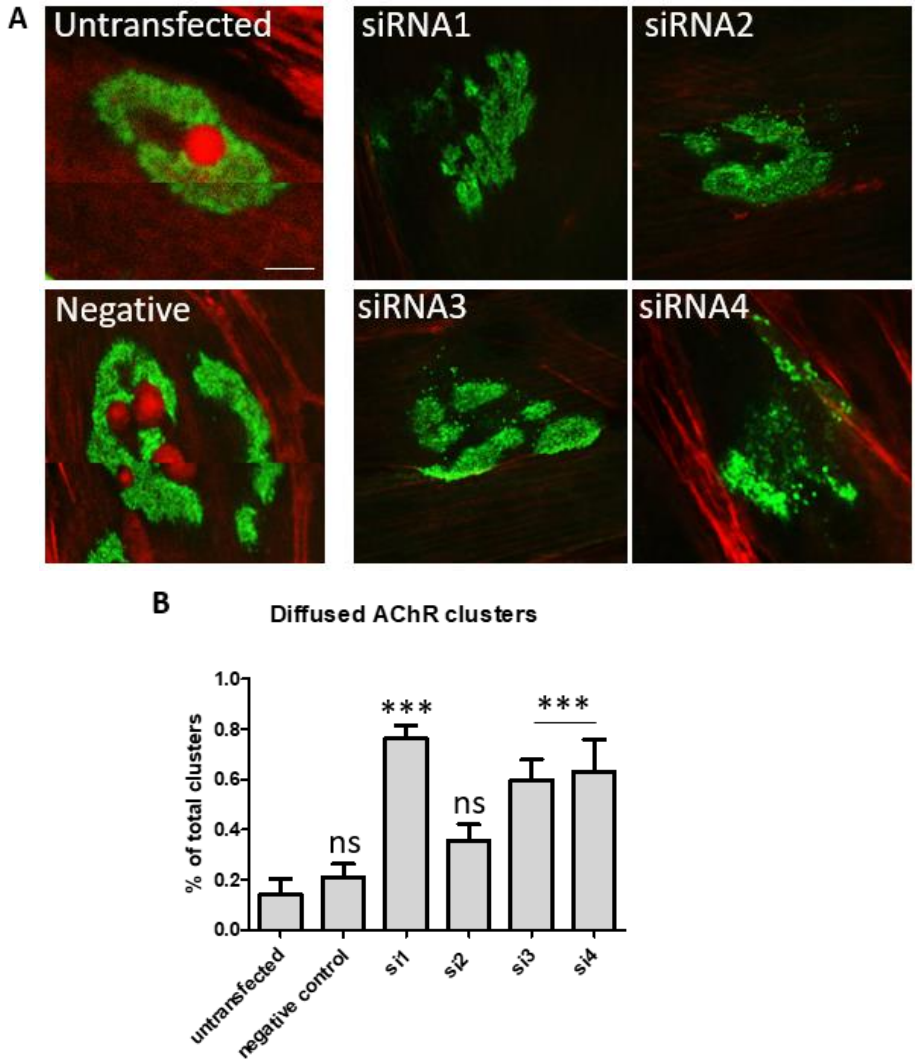


Figure 22: AChR clusters become fragmented after depletion of Tks5. (A) *Examples of AChR clusters in control and after treatment with siRNA against Tks5. AChRs were visualized with BTX488 and actin with Alexa555-phalloidin.* **(B)** *Graph represents the percentages of fragmented clusters in control cells and*

*those treated with the indicated siRNAs One way ANOVA with Dunnett test (untransfected control used as reference) were used to determine the statistical significance. 30 AChR clusters were used per point in the analysis. Error bars represent SEM. ***P< 0.0001. Scale bar= 10 µm.*

4.2.4. New AChRs are inappropriately added to the existing cluster when Tks5 expression is silenced

While the absence of synaptic podosomes and significant fragmentation of the AChR clusters are prominent phenotypes of C2C12 in the absence of Tks5, I went on to investigate how the dynamics of the postsynaptic machinery is affected by the lack of this protein. For this reason I incubated the cells with BTX555 for 10 minutes to label the pre-existing clusters, and added BTX488 6 hours after the visualize the newly added receptors (**Figure 23A**).

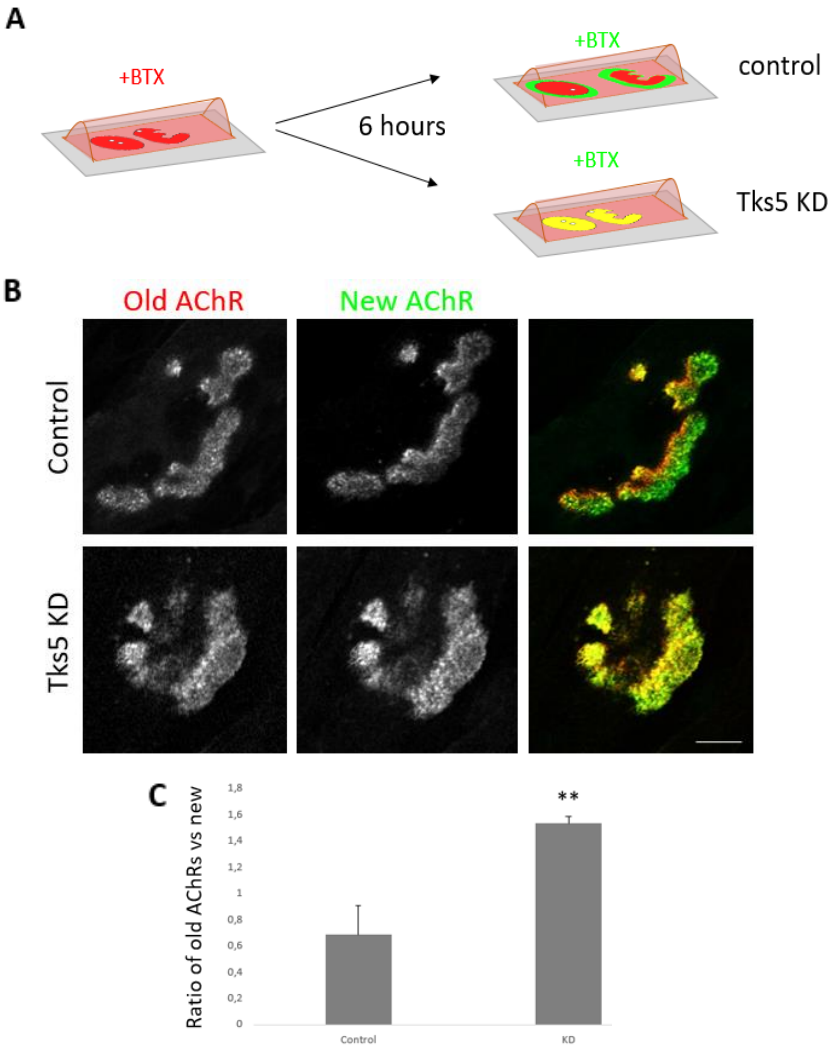


Figure 23: New AChRs are inserted randomly into the cluster after Tks5 silencing. (A) *Schematic diagram showing the experimental design. Briefly, BTX555 was added live to C2C12 myotubes for 10 minutes and washed away. After 6 hours the cells were fixed and BTX488 was used to label the new receptors.* ***(B)*** *Example AChR clusters after transfection with siRNA1. Staining as*

described in A. New receptors were added peripherally to the pre-existing clusters in control cells, however after Tks5 knockdown the two signals overlapped. ***(C)*** *Quantification of fluorescence intensity shown as ratio of old AChRs to new ones. Unpaired t test was used to determine the statistical significance. 30 AChR clusters were used per point in the analysis. Error bars represent SEM. **P< 0.001. Scale bar= 10 µm.*

As a result, I observed that while new receptors are being added to the clusters, they do so in an unorganized manner randomly inserting themselves in the entire area of the cluster (**Figure 23B**). In normal conditions, new receptors are added peripherally to the pre-existing assemblies and the signals from old and new labelled receptors do not overlap. Additionally, calculating the ratio of the fluorescence intensity of old receptors to the new ones revealed that after Tks5 knockdown significantly fewer receptors are added to the pre-existing clusters (**Figure 23C**).

4.2.5. Tks5 is required for association of actin with the postsynaptic machinery

In the absence of Tks5 the AChR clusters cannot undergo complex topological remodelling, loose their integrity and exhibit aberrations in the receptor turnover. From the literature, we know that these three processes are largely regulated by the components of cytoskeleton. Thus, the next step was to evaluate if Tks5 is needed for recruitment of actin to the postsynaptic machinery. For this reason, I performed a PLA using anti-rapsyn and anti-βactin primary antibodies in C2C12 cells where the expression of Tks5 was knocked down (**Figure 24A**). This assay revealed a detrimental decrease in the number of actin-rapsyn interactions when Tks5 is absent, suggesting that this protein is indeed required for associating the acitn cytoskeleton with the postsynaptic machinery, which could explain the phenotypes discussed in the previous sections (**Figure 24B**).

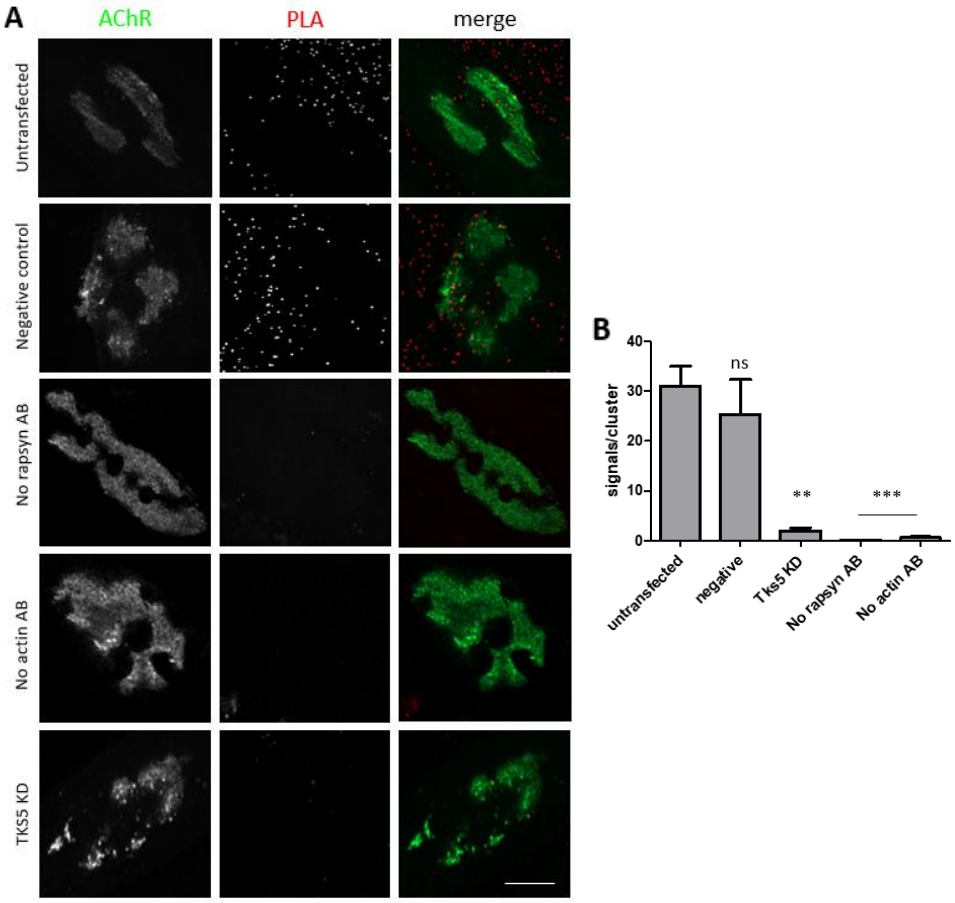


Figure 24: Tks5 is needed for associating postsynaptic machinery with actin. **(A)** *Proximity ligation assay results from C2C12 myotubes. The assay was performed using primary antibodies against rapsyn and actin. Each red puncta denotes interaction between actin and rapsyn. Cells where Tks5 was knocked down had a dramatic decrease in the amount of these interactions. Similarly, no PLA signals were detected when only one primary antibody was used, confirming the specificity of the method.* **(B)** *Quantification of PLA signals. One way ANOVA with Dunnett test (untransfected control used as reference) were used to determine the statistical significance. 20 AChR clusters were used per point in the analysis. Error bars represent SEM. ***P< 0.0001; **P< 0.001. Scale bar= 10 µm.*

Through my investigation of Tks5 protein in cultured muscle cells, I was able to demonstrate that this protein is required for synaptic podosome-driven remodelling of the postsynaptic machinery. Additionally, I showed that Tks5 is also needed for maintaining stability of the AChR clusters as well as receptor turnover. It is possible, that these functions are mediated by Tks5-driven recruitment of actin filaments to the postsynaptic machinery, as there was a significant reduction the number of actin-rapsyn interactions when Tks5 was knocked down.

4.3. Alternative laminin isoforms for culturing of C2C12 myotubes

The C2C12 myotubes provide an excellent model for studying of the postsynaptic machinery as they are able to form clusters of AChRs in the absence of the nerve and Schwann cells. However, they are extremely sensitive to the culturing conditions and even the smallest factors such as agitation or subtle temperature changes can drastically affect their ability to form the postsynaptic machinery. Because there is a need for a reliable way to obtain healthy myotubes that were able to form large amounts of mature AChR clusters, I focused on improving the existing protocols and optimizing the culturing conditions.

4.3.1. Optimal culturing of myoblasts for efficient AChR clustering

From my experience, problems with obtaining high yields of AChR clusters in C2C12 cells arise most often due culturing of myoblasts at inappropriate densities during passaging as well as at the seeding stage. Myoblasts the were cultured too densely enter fusion prematurely, leading to dedifferentiation after a passage and a consequent loss of the myogenic potential that is reflected by a significant reduction in the number of AChR clusters at the later stages of culturing. On the other hand, if the amount of plated cells was too small, myoblasts will grow slowly and form cell clusters as they divide leading to excessive cell-cell contacts and resulting in similar issues as overly dense cultures. As such, I found that the most optimal density during passaging is approximately 1.0 x 10^6 cells/cm^2 (**Figure 25A**). The following figure provides examples of appropriate and inappropriate culturing conditions during passaging as well as when preparing the myoblasts for fusion and differentiation into myotubes (**Figure 25B**).

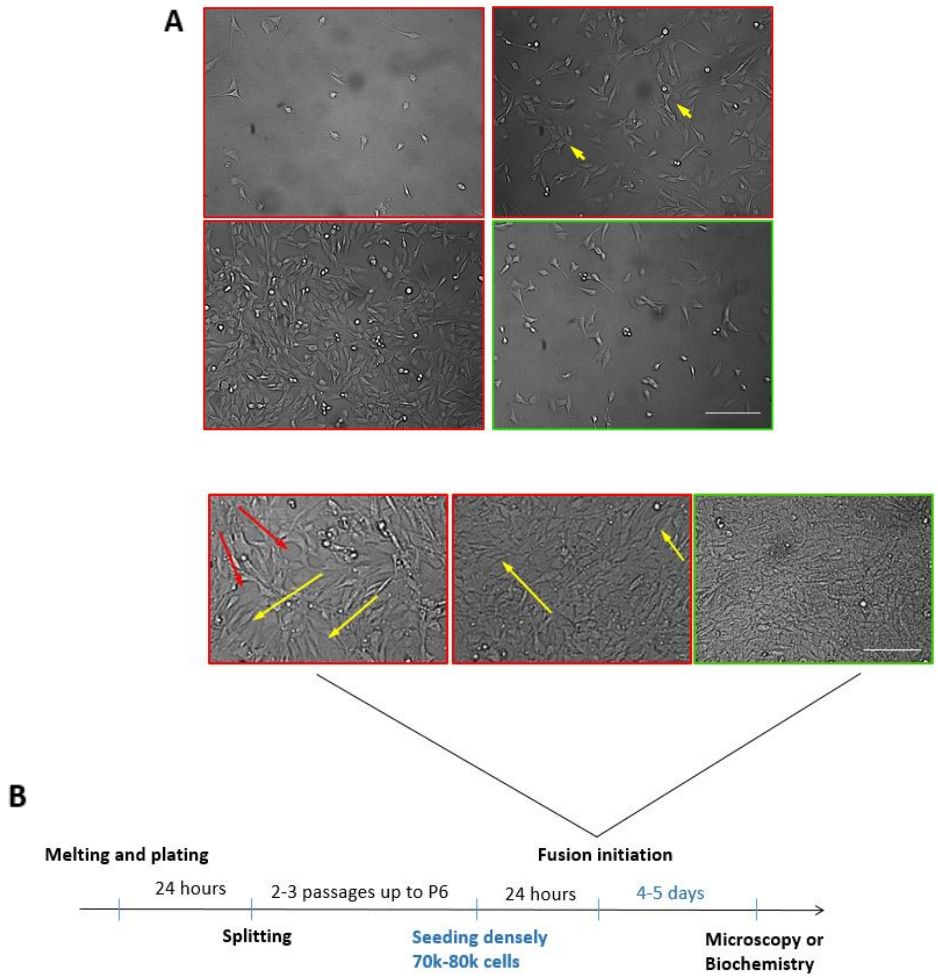


Figure 25: Optimal conditions for C2C12 myoblast culturing. (A) *Examples of appropriate (green box) and inappropriate (red box) cell densities during propagation of the C2C12 culture. The excessive cell-cell contacts are marked by yellow arrowheads. Cells from all boxes were plated at the same time at various densities on 10 cm plates. Cells in the green box were at density 1.0 x* 10^6 *cells/cm*^{2} *density.* **(B)** *Schematic diagram highlighting the key steps of C2C12 culturing for robust AChR clustering. The boxed images show suboptimal (red) and optimal (green) cell densities for initiating the fusion. Yellow arrows point at cells whose morphology can be observed, while red arrows show areas without any cells. Both features should be avoided if the goal is to acquire robust AChR clustering. For obtaining high yields of AChR clusters the cells should form a uniform layer where the morphology of a single cell would be impossible to distinguish (green boxed image). Scale bar= 600 µm in top four panels and scale bar= 400 µm in the bottom three panels.*

4.3.2. AChR clustering on different isoforms of laminin

Myotubes cultured on laminin 111 (α1β1γ1) are recognized to form the most complex assamblies of the postsynaptic machinery. However, there was no study ever done to evaluate the effects of other isoforms of laminins, including the ones that are found at the NMJ *in vivo*. I therefore investigated what is the

most optimal isoform for obtaining robust clustering of AChR. Since properties of these ECM proteins vary depending on their subunits I evaluated 8 different isoforms: 111; 121; 211; 221; 411; 421; 511; 521. Myotubes cultured in the absence of laminin do not form any AChR clusters, and laminin 111 is the isoform that I used as control. The results showed that cultures stimulated with laminin 111 produced significantly fewer cluster of the postsynaptic machinery then the ones where laminins such as 121 or 221 were used (**Figure 26A and B**). Through western blot analysis I was also able to confirm that differences in the number of AChR clusters are not due to the lower expression levels of the receptors (**Figure 26C**). Interestingly, laminins with α4 (411 and 421) subunit produced hardly any clusters, likely due to the absence of integrin binding domain, which is present in other α subunits. However, combining certain laminins had a synergistic effect on AChR cluster yields (**Figure 26D and E**).

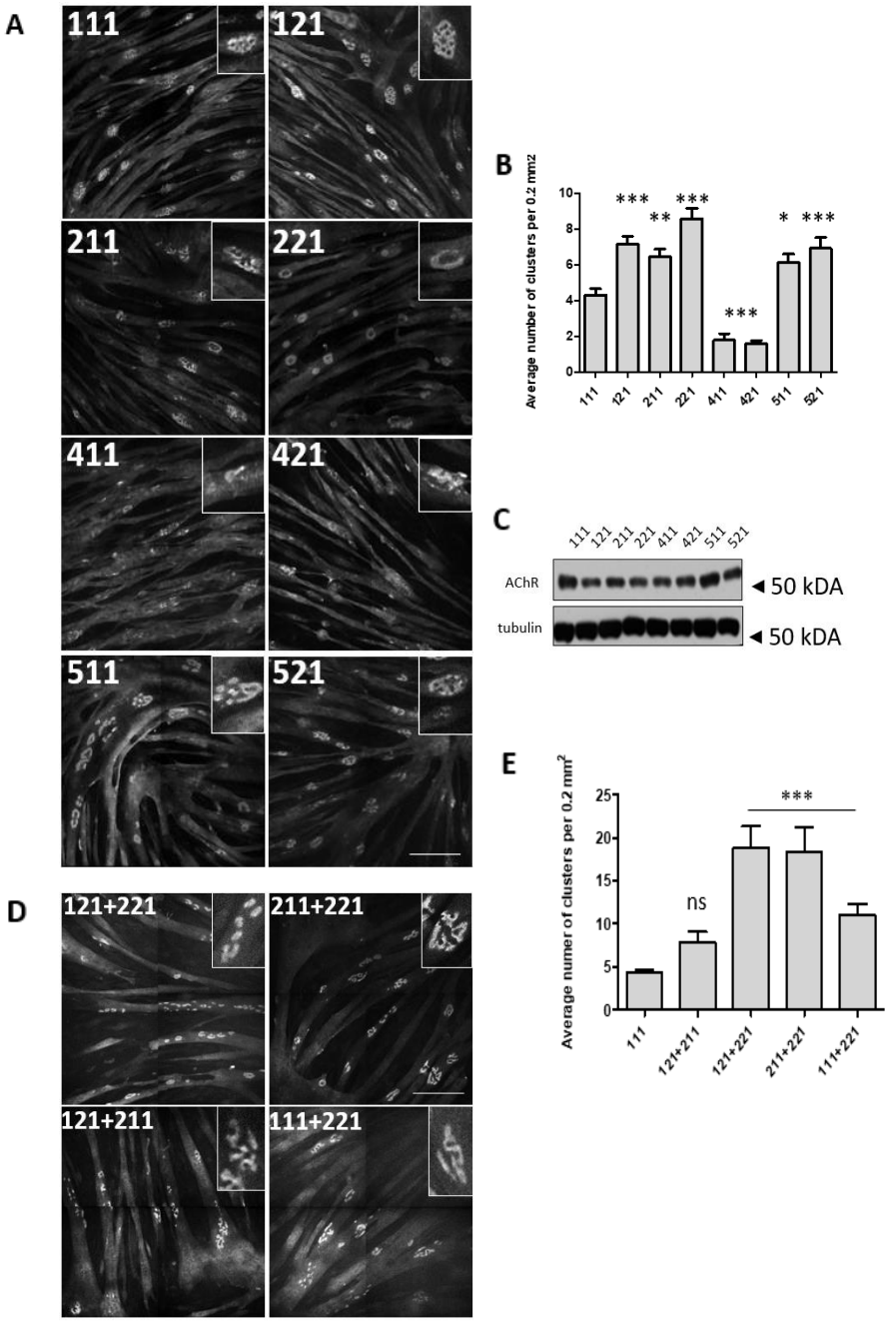


Figure 26: Different laminin isoforms stimulate AChR clustering. (A) *Representative images of myotubes cultured on indicated laminin isoforms. Cells were grown at the same conditions in parallel. Boxes on the right are 4x higher magnifications of example AChR clusters.* **(B)** *Quantification of the number of AChR clusters in myotubes grown on indicated laminins. Total number of AChR cluster used for the analysis was> 300.* **(C)** *Western blot showing the expression level of AChR in myotubes cultures grown on indicated laminin isoforms. Tubulin was used as loading control.* ***(D)*** *Representative images of myotubes cultured on indicated laminin combinations. Cells were grown at the same conditions in parallel. Boxes on the right are higher magnifications of example AChR clusters.* **(E)** *Quantification of the number of AChR clusters in myotubes grown on indicated laminin combinations. The total number of clusters used for the analysis was>70. One way ANOVA with Dunnett test (laminin 111 used as reference) were used to determine the statistical significance. Error bars represent SEM. ***P< 0.0001. Scale bar= 150 µm.*

4.3.3. Differences in AChR clustering are not due to the myotube quality

I have evaluated the diameters of myotubes cultured on the alternative laminin isoforms, since thicker ones (with higher diameter)would be more likely to produce more clusters (**Figure 27A**). However, no significant differences in the quality of myotubes were observed. Additionally, I measured the average size of AChR clusters formed by the myotubes from different laminin cultures and found that only cells that were grown on laminin 511 were induced into production of slightly larger assemblies of the postsynaptic machinery (**Figure 27B**). No significant differences were observed in both parameters across the tested laminin isoforms

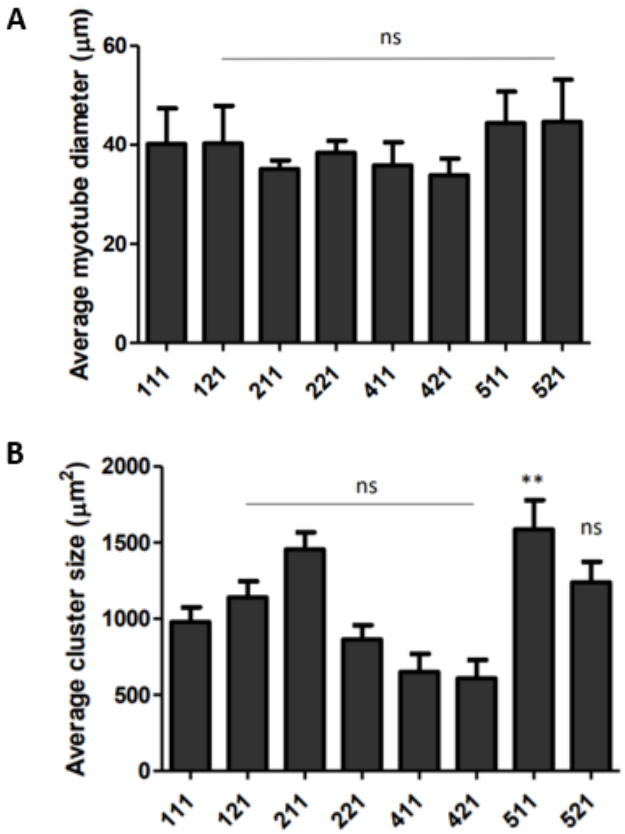


Figure 27: Myotube quality were the same for each laminin. (A) *Diameter of mytubes grown on indicated laminin isoforms.* **(B)** *The average size of postsynaptic machinery clusters in C2C12 myotubes grown on indicated laminin isoform. One way ANOVA with Dunnett test (laminin 111 used as reference) was used to determine the statistical significance. 30 myotubes and 30 AChR clusters were used per point in the analyses. Error bars represent SEM. **P< 0.001*

4.3.4. Myoblast fusion index is affected by the laminin isoform used for culturing

The quality and number of AChR clusters are affected by factors such as effectiveness of myoblast fusion and cell attachment. *In vitro*, myoblast fusion index depends on the attachment of cells to the culturing surface. Importantly, cell attachment can be different for myoblasts cultured on various laminin isoforms. Thus, I measured the fusion index for cells grown on each of the investigated laminin isoforms (**Figure 28**). As a result, the fusion index for cells grown on a particular isoform directly correlated with the amount of AChR clusters produced by myotubes fused on that isoform (**Figure 28A and 28B**). As such, laminin 221 and 121 had the highest fusion index and consequently produced the most clusters of postsynaptic machinery.

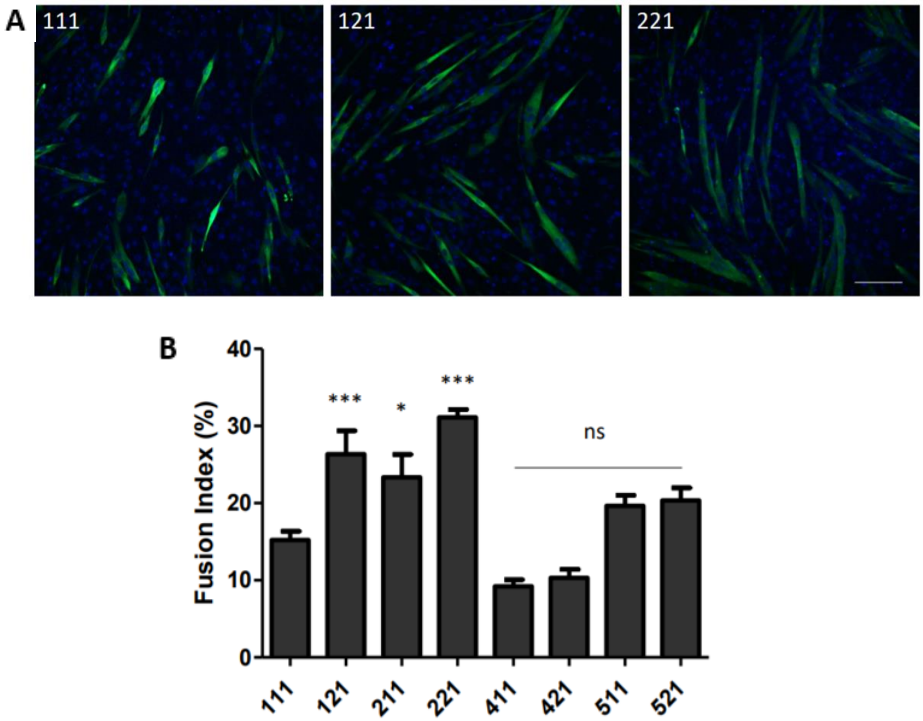


Figure 28: Fusion index of C2C12 myoblasts cultured on different laminin isoforms (A) *Representative images of C2C12 myoblasts cultured on the indicated laminin isoform at day 3 of fusion. Nuclei were visualized with DAPI(blue) and cells were stained with anti-myosin heavy chain (fast) antibody to label differentiated myotubes (green).* **(B)** *Fusion index of C2C12 myoblasts grown on indicated laminin isoforms. One way ANOVA with Dunnett test (laminin 111 used as reference) were used to determine the statistical significance. 5 random areas were used for calculating the fusion index per point. Error bars represent SEM. ***P< 0.0001; *P< 0.01. Scale bar= 100 µm.*

4.3.5. Different laminin isoforms promote various modes of AChR cluster development in C2C12 myotubes

While obtaining high yields of AChR clusters is an important factor when studying the postsynaptic machinery, as it provides the scientist with larger data samples, another important consideration is the topology of said assemblies. Laminin cultures are the preferentially used system for studying the development and remodelling of AChR clusters because these processes closely resemble the maturation of the muscle postsynaptic machinery at the NMJ *in vivo*. In both cases, the remodelling takes place when plaque-shaped, oval AChR clusters begin to acquire perforations that lead to a more complex topology. In case of C2C12 myotubes, two modes of remodelling were observed: removal of large portions of AChR from the centre of the cluster, resulting in C-shaped assemblies; and acquisition of perforations within the cluster caused by synaptic podosomes, that results in a pretzel-like shape of the mature postsynaptic machinery. As such, C2C12 myotubes cultured on different isoforms of laminin displayed differences in the modes of maturation of AChR clusters (**Figure 29**). No single laminin promoted formation of more plaque-shaped clusters than the commonly used laminin 111 (**Figure 29A and B**). Interestingly, cultures on laminins 211 and 121 that stimulated the myotubes into formation of significantly more clusters had significantly fewer plaque-shaped assemblies than those on laminin 111. On the other hand, largest fraction of c-shaped clusters can be attributed to the myotubes cultured on laminin 221, although much like the isoform 511, it induced significantly fewer AChR clusters with podosomal perforations (**Figure 29C,D and E**). However, myotubes grown on laminins 121 and 211 were induced into formation of the most perforated clusters (>70% of the total) from all tested isoforms (**Figure 29E and F**). Interestingly, cells seeded on laminins containing the α4 chain barely produced any assemblies of the postsynaptic machinery. Taken together, this data suggests that various types of laminin lead to differential induction of developmental remodelling of the AChR clusters in C2C12 myotubes, when used for coating of culture surfaces.

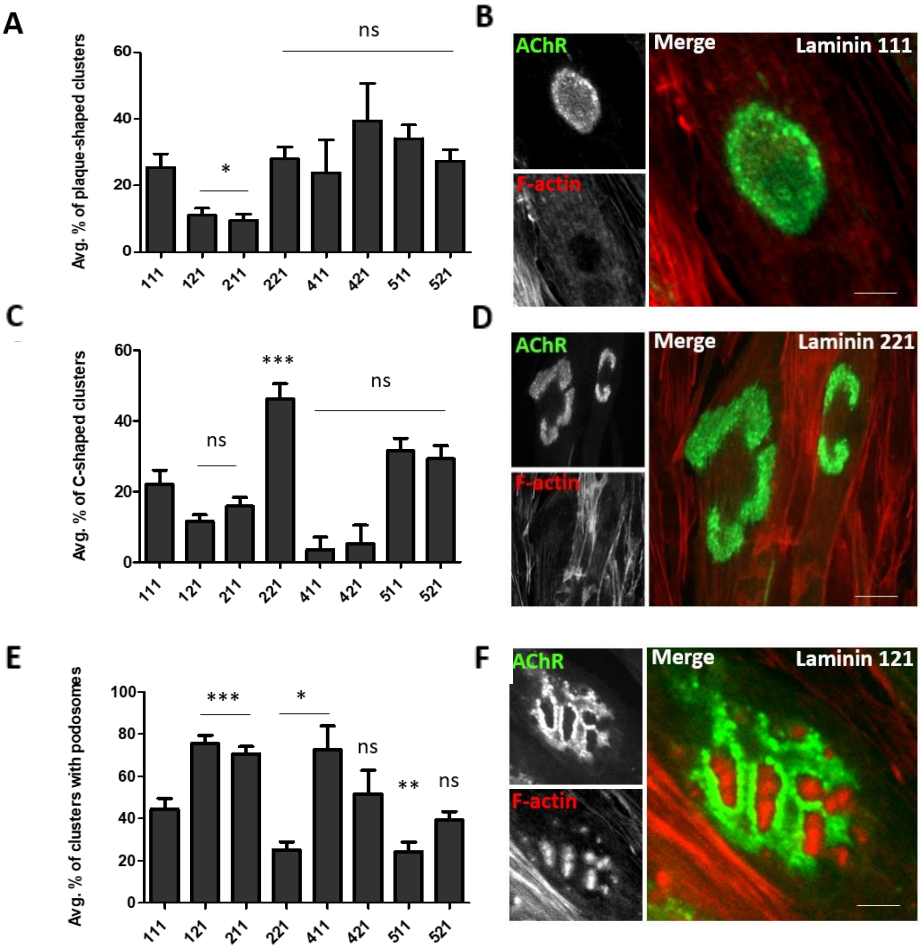


Figure 29: Different AChR cluster remodelling in C2C12 myotubes grown on various laminin isoforms. (A) *Quantification of plaque-shaped clusters in cultures grown on the indicated laminin isoforms, presented as average percentage of total clusters.* **(B)** *Example image of a plaque-shaped cluster.* **(C)** *Quantification of C-shaped clusters in cultures grown on the indicated laminin isoforms, presented as average percentage of total clusters.* **(D)** *Example image of a C-shaped cluster.* **(E)** *Quantification of podosome-containing clusters in cultures grown on the indicated laminin isoforms, presented as average percentage of total clusters.* **(F)** *Example image of a cluster perforated by synaptic podosomes. For all laminins except laminin-111, laminin-411, and laminin-421, the total number of clusters that were used for the analysis was> 300. For laminin-111, 216 clusters were used. For laminin-411, 25 clusters were used. For laminin-421, 30 clusters were used. Clusters were collected from five independent experiments performed on five different days. *p < 0.05, **p < 0.005, ***p < 0.0005. For statistical analysis we used one way ANOVA with Dunnett test; laminin-111 used as a reference. Error bars represent SEM values. AChR (green) was visualized with BTX488. F-actin (red) was visualized with phalloidin. Scale bar= 6 µm.*

4.3.6. Rapsyn expression and localization is not affected by the type of laminin isoform used for culturing

Since most of the analysis that I carried out so far was based on labelling of the AChRs with BTX, I went on to investigate other marker of the postsynaptic machinery- a scaffolding protein rapsyn, in myotubes cultured on various laminin isoforms. The analysis revealed that neither the localization nor the expression level of this protein exhibit variation in any of the cultures (**Figure 30**).

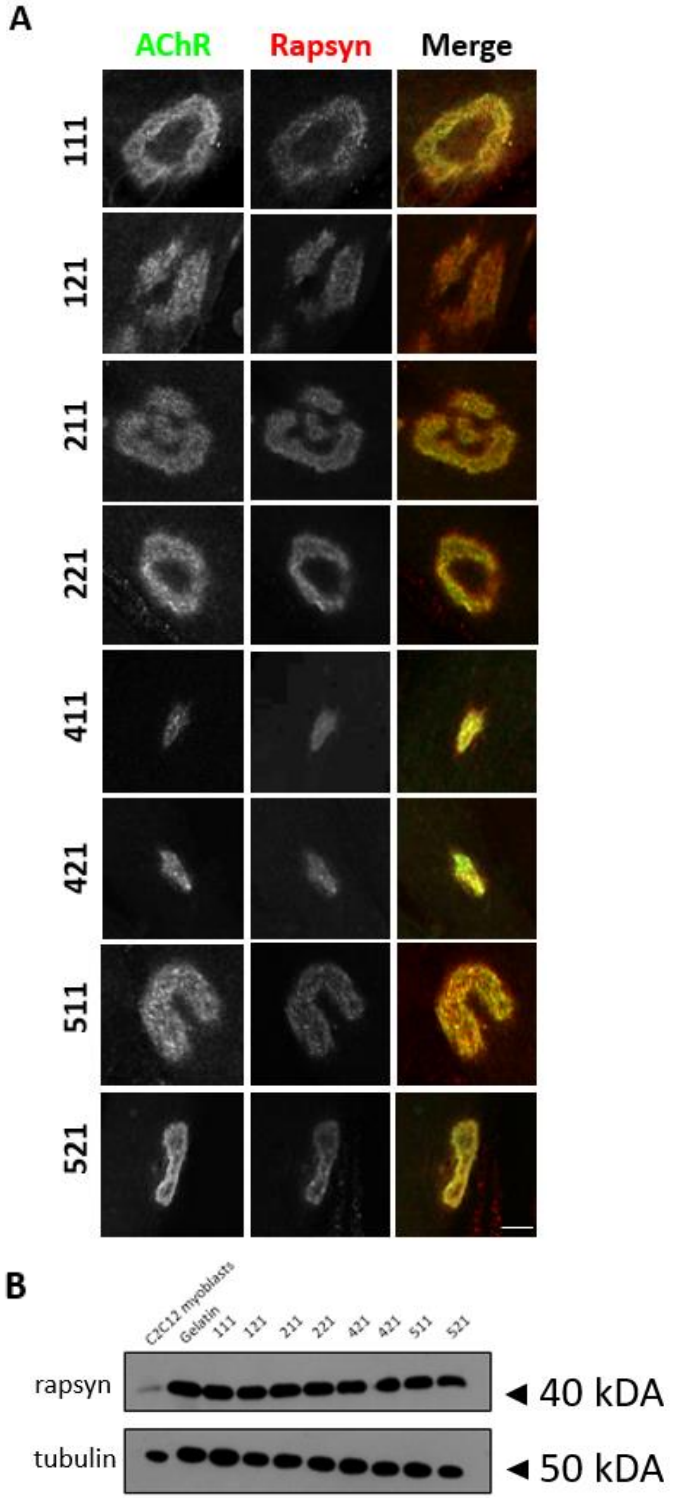


Figure 30: Rapsyn localization and protein level in C2C12myotubes cultured on different laminin isoforms. (A) *Images showing rapsyn (red) localization in the postsynaptic machinery (green) of myotubes cultured on indicated laminin isoform.* **(B)** *Western Blot analysis of rapsyn levels in lysates of C2C12 myotubes cultured on indicated laminin isoform. Tubulin was used as a loading control. Scale bar= 6 µm.*

4.3.7. Human primary myotubes are able to form AChR clusters when cultured on laminin

Because culturing of primary myotubes derived from human patients can provide a useful platform for studying the mechanisms of neuromuscular disorders, as well as screening of drugs that target these diseases, I went on to investigate whether my method can be applied for these cultures. I developed an optimized protocol for fusion of primary human myoblasts that aimed to maximize the amount of AChR clusters formed by these cells (**Figure 31A**).

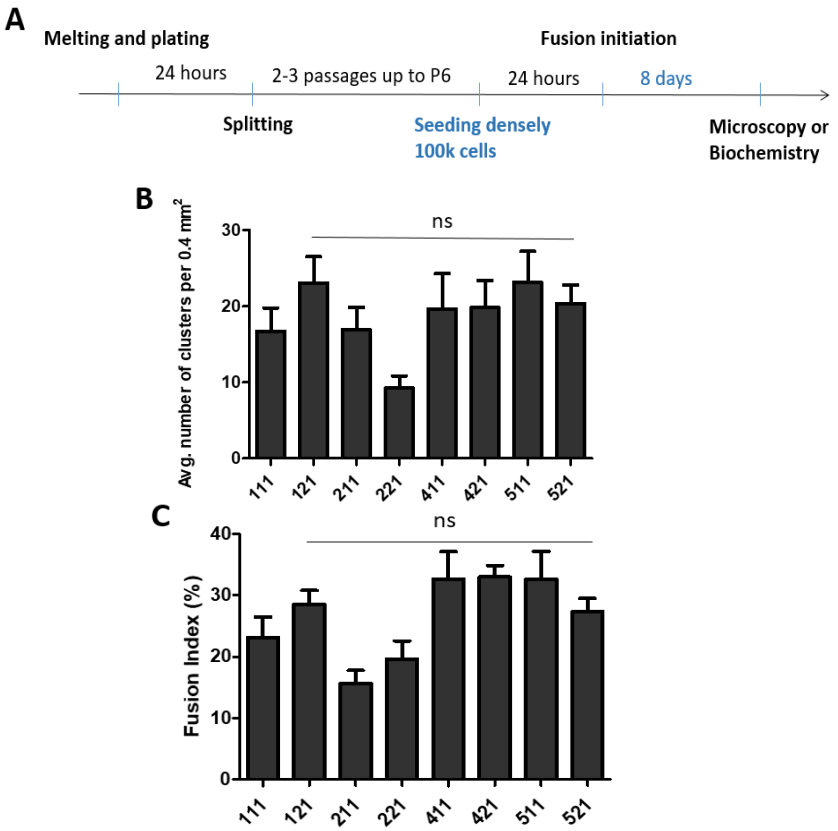


Figure 31: Laminins do not significantly influence AChR cluster formation nor fusion of primary human myotubes. (A) *Schematic diagram highlighting the key steps of primary human myoblast culturing for robust AChR clustering.* **(B)** *Quantification of the number of AChR clusters in primary human myotubes grown on indicated laminins.* **(C)** *Fusion index of primary human myoblasts grown on indicated laminin isoforms. One way ANOVA with Dunnett test (laminin 111 used as reference) were used to determine the statistical significance. 5 random areas were used for calculating the fusion index per point. Error bars represent SEM.*

The key steps are highlighted in blue, as appropriate seeding density and duration of fusion are crucial for obtaining robust AChR clustering. The analysis of human primary myotubes derived from a healthy individual and cultured on various laminin isoforms has shown that while these cells are able to form AChR

clusters, the type of laminin used for coating does not significantly influence the efficiency of their formation nor affect their fusion (**Figure 31B and C**).

4.3.8. Human primary myotubes form AChR clusters that undergo remodelling through synaptic podosomes

The next step was to analyse the morphology of AChR clusters formed by the primary human myotubes. Surprisingly, most of the clusters formed by cells cultured on either of the laminins used in the analysis formed oval, plaque-shaped assemblies (**Figure 32A and B**). The notable exceptions were cells that were cultured on laminins 421 and 511. In these cultures, more than 30% of formed clusters were perforated, reassembling C2C12 clusters that were remodelled by the synaptic podosomes (**Figure 32C**). It would be an important discovery if the postsynaptic machineries of primary human myotubes would be remodelled by the synaptic podosomes, since very little is known about the process of synaptic maturation in humans, I performed immunocytochemical analysis of these cells using known podosomal cortex markers vinculin and LL5β (**Figure 32D and E**). As expected, these proteins localized at the cortex-like domain of perforations within the AChR clusters, circling around the actin-rich cores. As a result, I was able to show that not only did the primary human myotubes form clusters of postsynaptic machinery of complex topology, but also that this process most likely involved synaptic podosomes.

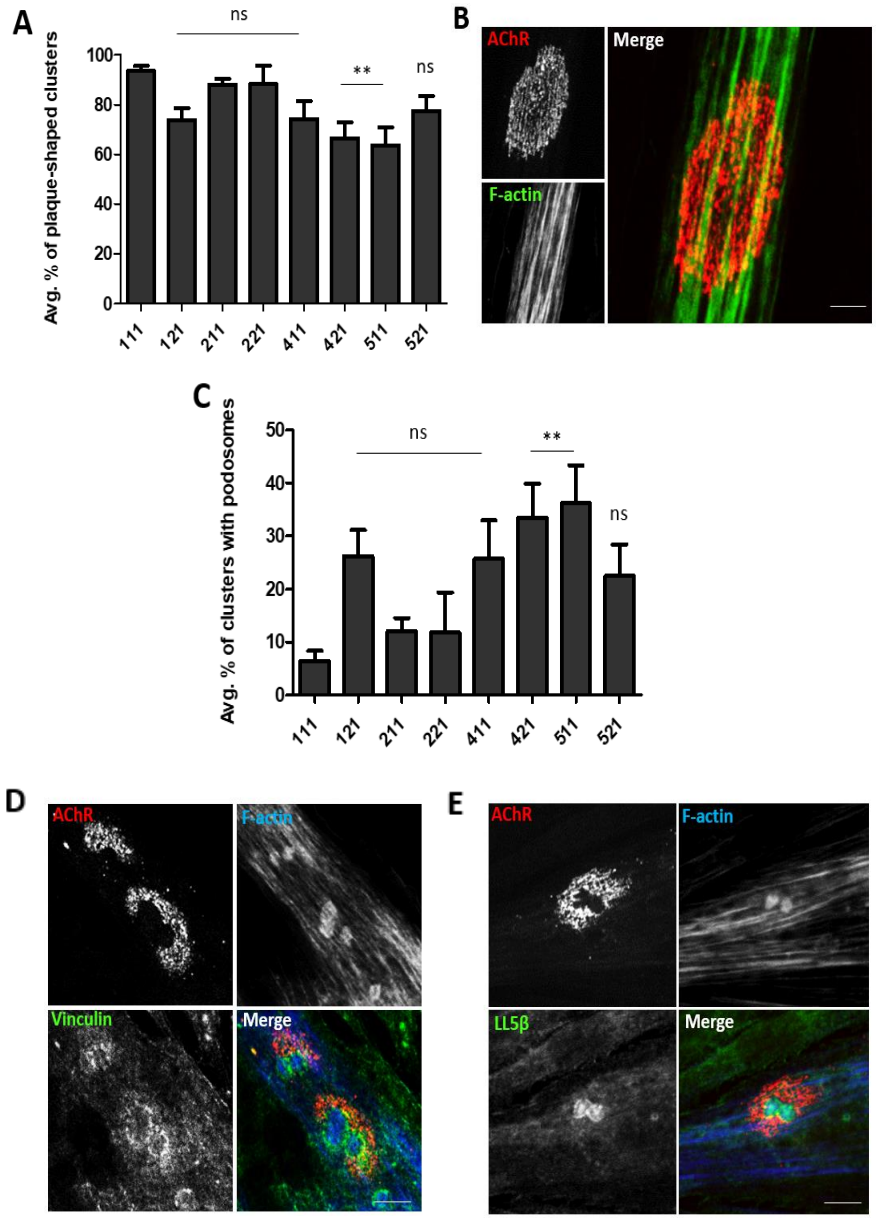


Figure 32: Laminins stimulate formation of plaque-shaped and perforated AChR clusters in primary human myotubes. (A) *Quantification of plaque-shaped clusters in primary cultures grown on the indicated laminin isoforms, presented as average percentage of total clusters. The total number of clusters used in the analysis for at least 200 for each laminin.* **(B)** *Example image of a plaque-shaped cluster formed by primary human myotube..* **(C)** *Quantification of podosome-containing clusters in primary cultures grown on the indicated laminin isoforms, presented as average percentage of total clusters. The total number of clusters used for the analysis for each laminin was at least 250.* ***(D)*** *Primary human myotubes were fixed in 4% PFA, incubated with blocking buffer and labelled with primary antibodies for vinculin (green) flowed by staining with secondary antibody, BTX-555 (red) and acti-stain-647 (blue).* ***(E)*** *Primary human myotubes were fixed in 4% PFA, incubated with blocking buffer and labelled with primary antibodies for LL5β (green) flowed by staining with secondary antibody, BTX-555 (red) and acti-stain-647 (blue). Scale bar= 6 µm.*

I investigated the effects of coating the culturing surfaces with alternative laminin isoforms on their ability to form and remodel the AChR clusters. My data suggested that laminin 221 yields cultures that produce the most postsynaptic machinery assemblies, while isoform 121 was best for obtaining clusters that underwent synaptic podosome-driven remodelling. I have also applied my method to primary human muscle cultures, and showed that these cells not only produce AChR clusters, but also that these specializations also undergo podosome-driven synaptic remodelling. This provides a useful new tool to anyone interested in the biology of the synapse.

5. Discussion

The NMJ is a sophisticated system that is composed of at least three different cell types: muscle, nerve and Schwann cells (Slater 2017). All these components feedback to one another from the earliest moments of prenatal development and throughout adulthood. This is crucial for appropriate formation of the synapse as well as its maintenance. However, not much is known about the specific mechanisms that lead to such cooperation. This poses a great challenge for neuromuscular researchers and clinicians, as we are still lacking elemental insights into the functioning of the NMJ. This is reflected by the fact that more than half of the known neuromuscular disorders have an unknown etiology, despite great efforts of scientists around the globe to solve these ghastly puzzles. Perhaps the overwhelming complexity of the NMJ is a limiting factor in our efforts to understand the synapse. For this reason, I have chosen a minimalistic approach to study this enigmatic structure. In my research, I have focused almost exclusively on the role of muscle and muscle-derived factors that are implicated in establishing the postsynaptic machinery of the NMJ. As a starting point, I identified and investigated the roles of two novel proteins that interact with αDB1: liprin-α-1 and Tks5. αDB1 is an important component of a larger protein complex- the DGC, which has been shown to play important roles in muscle biology (Wagner, Cohen et al. 1993, Grady, Grange et al. 1999). Mutations in some constituents of the DGC (including αDB1) can cause a range of aberrations in the postsynaptic machinery, however as is the case for αDB1, the mechanisms that drive these changes remain largely unknown. As I have successfully determined some of the functions that liprin-α-1 and Tks5 have at the postsynaptic machinery, I went on to improve the method that is commonly used for studying this structure, in hopes that the minimalistic approach of investigating the synapse will become a more valuable tool for identifying novel therapeutic factors targeted at the postsynaptic machinery.

5.1. Liprin-α-1 as a synaptic scaffold

In my thesis I tried to highlight the intricate relationship between the structure of an NMJ and its function. This notion holds true for all the different types of synapses found within an organism, as they very often exhibit similar architecture and undergo analogous processes (Lai and Ip 2003). All chemical synapses such as the NMJ or glutamatergic synapse in the CNS require organization of presynaptic vesicles at the AZs, anchorage of neurotransmitter receptors and ion channels within the postsynaptic membrane and recruitment of other supporting complexes (Lin and McArdle 2021). These can be achieved either by transcriptional regulation in specialized subsynaptic nuclei, or post-translationally when complexes present at synaptic membranes recruit other proteins to dynamically influence the synapse (Simon, Hoppe et al. 1992, Moransard, Borges et al. 2003). These proteins are then assorted into different micro domains to spatially separate functionally diverse complexes. Liprin-α-1 is directed into one of such domains, separated from AChRs and colocalizing with MT-binding LL5β-CLASP complex.

Liprin-α-1 was first described as a crucial component of the neuronal synapses (Spangler and Hoogenraad 2007). It has been shown that a homologue of this scaffold protein, *C. elegans* liprin-α (SYD-2), is required for organizing the presynaptic terminal, as mutant strains of *C. elegans* exhibited significant aberrations in the distribution of AZs and synaptic vesicles (Zhen and Jin 1999, Dai, Taru et al. 2006). This was reflected by changes in the synaptic transmission and linked to alterations in worm behaviour. Studies have also shown that liprin-α-1 binds with microtubule-associated molecular motors, such neuron-specific kinesin 3 (KIF21A), that is involved in delivery of synaptic vesicle components to the presynaptic terminal (van der Vaart, van Riel et al. 2013). This means that liprin-α-1 can post-translationally regulate the synaptic composition by either acting as an adaptor for other complexes or as an anchor that has affinity for molecular motors and thus can direct their cargo to the synaptic site. There were no previous studies that would address whether liprin-α-1 plays any role in the vertebrate NMJ. Therefore given its vast number of functions in the neurons and combined with that fact that my colleagues, Dr Bernadzki and Dr Gawor, have identified it as an interactor of an important postsynaptic protein αDB1, it became an interesting target for investigating its possible functions at

the NMJ. I began by performing an siRNA-mediated knockdown in cells stimulated with agrin glycoprotein (**Figure 13**). In the absence of liprin-α-1 expression, the cells failed to produce hardly any clusters of the receptor. To follow up, I showed that this effect is not caused by reduced expression of AChR (**Figure 14**). Indeed, my functional data from siRNA-mediated knockdown experiments in C2C12 myotubes confirmed that this protein is involved in regulation of the postsynaptic machinery. Similarly to its function in the CNS, I showed that it is required for organization of crucial synaptic components (**Figure 13**). What would be interesting to see is whether localization of other postsynaptic complexes such as Lrp4-MuSK or rapsyn was affected when liprin-α-1 expression was silenced. Since this protein localizes to specialized synaptic domains both at the NMJ and in the CNS, where it can act as a hub for post-translational synaptic regulation one could expect that the AChRs are not the only mislocalized complexes when liprin-α-1 is absent. What remained to be determined is whether the absence of AChR clusters in myotubes transfected with liprin-α-1 siRNA is a result of compromised surface delivery of the receptor, or rather inability of membrane-bound receptors to form high-density clusters. I employed immunohistochemical analysis of C2C12 cells cultured on laminin-coated surfaces after an siRNA-mediated liprin-α-1 knockdown. The cells exhibited the same amount of fluorescence when stained with BTX488 as the cells transfected with scrambled siRNA sequence (negative control) and treated accordingly (**Figure 15**). This suggested that the AChR is not only expressed but also delivered to the cell surface without any problems, leaving me with a hypothesis that the absence of AChR clusters in cells where liprin-α-1 is knocked down is caused by the inability of the receptors to assemble into postsynaptic structures. Interestingly, liprin-α-1 has been shown to be part of the LL5β-CLASP complex, which is involved in recruitment of microtubules to the plasma membrane (Lansbergen, Grigoriev et al. 2006, Astro, Chiaretti et al. 2014). Through immunohistochemical staining of mouse TA muscle, I showed that liprin-α-1 colocalizes with LL5β at the NMJ, suggesting that its function may be mediated through this complex. I went on to see whether MTs are affected by the absence of liprin-α-1 in C2C12 myoblasts and in fused myotubes. In fact, those cells exhibited a dramatic decrease in the number of cortical microtubules, especially within the region of AChR clusters (**Figure 17 and 18**). Thus my data combined with other studies suggests that the function of liprin-α-1 at the postsynaptic machinery of the NMJ may be mediated through the

LL5β-CLASP complex, however it is unknown whether the same mechanism is employed in CNS synapses (Kishi, Kummer et al. 2005, Proszynski and Sanes 2013, Basu, Sladecek et al. 2015).

In order to understand the role of liprin-α-1 in organizing the postsynaptic machinery it is important to discuss the possible functions of microtubule cytoskeleton in assembling functional AChR clusters. Since MTs are involved in vesicular trafficking, the classical view would be that these filaments are solely responsible for mediating delivery of the receptors to the plasma membrane (Connolly 1985). However, several arguments also exist for their involvement in the clustering of AChRs. The first line of evidence for this came from Conolly, who demonstrated that microtubules are dispensable for trafficking of the AChRs to the cell surface (Connolly 1985). This is consistent with my data, which showed that after nocodazole treatment of myotubes, no new receptors are incorporated to the pre-existing clusters, while the amount of AChR delivered to the surface remains unchanged (**Figure 15 and 19**). I also showed that liprin-α-1 is needed for recruitment of microtubules to the plasma membrane in C2C12 cells (**Figure 17 and 18**). However, when liprin-α-1 expression was silenced, the receptors were still delivered without issues, suggesting that presence of microtubules beneath the postsynaptic machinery is again dispensable for receptor trafficking (**Figure 15**). LL5β, a binding partner of liprin-α-1, has been shown previously to regulate addition of new receptors to the pre-existing clusters by capturing microtubules at the postsynaptic machinery (Basu, Sladecek et al. 2015). The fact that MTs are mainly employed for a long-distance trafficking, while most of the synaptic genes at the NMJ are expressed in subsynaptic nuclei located directly below the postsynaptic machinery it is possible that it is the actin cytoskeleton that the cells rely on when delivering synaptic components to the cell surface (Vale 1987, Woehlke and Schliwa 2000). This means that microtubules could either act as signalling components or mechanical scaffolds on which the AChR clusters are formed. Unfortunately, the mechanism for such actions remains to be elucidated. Collectively, these arguments support the view that at the postsynaptic machinery, microtubules are primarily responsible for assembling AChR clusters rather than mediating receptor exocytosis, providing room for exciting new research regarding the nature of microtubule cytoskeleton at the synapse. Interestingly, it is still unknown how new receptors delivered to the surface are assembled into

clusters. However recent data that employed quantum-dot analysis of AChRs has supported a so-called diffusion trapping hypothesis, which states that new receptors move freely within the plasma membrane until they become captured by postsynaptic scaffolds and incorporated into the cluster (Geng, Zhang et al. 2009). The circumferential addition of new receptor to pre-existing clusters that I observed fits into this hypothesis, thus a possibility exists that liprin-α-1 together with LL5β are important components of this AChR "trapping" machinery.

It remains unknown how liprin-α-1 is recruited to the synaptic sites. Since my colleagues have identified this protein as an interactor of the major laminin receptor- the DGC, which is present in muscles but also within the neurons, a possibility exists that liprin-α-1 localization can be regulated by αDB1. Whether liprin-α-1 function requires it to form complexes with both the LL5β and αDB1 or the interaction with the two occurs in an independent manner remains to be elucidated.

5.2. Tks5 regulates integrity and maturation of postsynaptic machinery

Aberrant maturation of the NMJ is a characteristic of many neuromuscular disorders, however the underlying mechanisms for such changes are still not entirely understood (Li, Xiong et al. 2018). The recent discovery that remodeling of the postsynaptic machinery clusters in laminin cultured myotubes is driven by synaptic podosomes - actin-rich structures, able to invade ECM and implicated in cellular adhesion, has opened up doors for investigating actin regulators for their role in postsynaptic maturation (Proszynski, Gingras et al. 2009, Proszynski and Sanes 2013). It was the first time that these structures have been shown to be involved in the biology of a synapse, however a complete evidence for their existence *in vivo* is still lacking. Several known podosomal markers have been identified in the perforations of a mature NMJ, including Amotl2, Arhgef 5, γ-actin and LL5B, which suggests that a similar remodeling mechanism might occur *in vivo* (Proszynski, Gingras et al. 2009, Proszynski and Sanes 2013, Bernadzki, Rojek et al. 2014). My mass spectrometry analysis of αDB1 interactors revealed Tks5 as a protein that interacts with unphosphorylated αDB1, a critical component of the DGC. Tks5 is a scaffold

protein that has been previously shown to be crucial for podosome formation in many cell types, but its role at the synapse has never been extensively studied (Gimona, Buccione et al. 2008). I therefore began from confirming its interaction with αDB1 via co-immunoprecipitation, and I went on to investigate whether Tks5 has a role at the postsynaptic machinery (**Figure 20**). My functional data from siRNA-mediated knockdown of Tks5 in C2C12 myotubes revealed that Tks5, unlike liprin-α-1 is not required for AChR cluster formation. However, when its expression was silenced a number of postsynaptic phenotypes were observed. First, almost complete disappearance of synaptic podosomes (**Figure 21**). Second, compromised integrity of AChR clusters characterized by significant fragmentation of the assembly similar to the one observed in neuromuscular disease models such as mdx mice (**Figures 22 and 9**). Thirdly, when the cells, in which Tks5 was knocked down, were labelled with two colours of BTX in a timespan of 6 hours, it became clear that the new receptors added to the pre-existing clusters were not inserted to the periphery (as they were in control cells), but instead randomly distributed throughout the assembly. Importantly, when I measured the fluorescence signal from the newly added receptors it turned out that the receptor turnover was greatly reduced in the absence of Tks5 (**Figure 23**).

Interestingly, podosomes have only recently been proposed in regulation of postsynaptic machinery remodeling. The mechanisms that drive this process are largely unknown despite its relevance in various pathological states of the NMJ (Li, Xiong et al. 2018). My data suggest that Tks5 is one of the first podosomal regulators involved in remodeling and stabilization of the postsynaptic machinery. Since this protein has been extensively studied in terms of its involvement in formation of podosome-related invadosomes, it would be interesting to see if pathways that mediate this function are also implemented in postsynaptic maturation. The fact that other phenotypes were observed, unrelated to remodeling of cluster topology, indicates that Tks5 has multiple functions in this system. Such aberrations have been previously described for actin organizers that localize to the postsynaptic machinery, thus given that Tks5 is an essential podosomal scaffold, chances are that its function is mediated by the actin cytoskeleton (Cartaud, Stetzkowski-Marden et al. 2011, Bernadzki, Daszczuk et al. 2020). I therefore performed PLA, to check whether association of actin with rapsyn was affected when Tks5 was absent. Indeed, this analysis revealed a dramatic decrease in the amount of PLA puncta when

compared to control, suggesting that the phenotypes of AChR clusters described above are likely due to the reduced number of actin filaments present at these assemblies (**Figure 24**). It would be interesting to see whether coupling of actin to the postsynaptic membrane by Tks5 is regulated by its binding to αDB1. Studies have shown that the three tyrosine phosphorylation sites located on αDB1 are important for maintaining stability of the NMJ (Grady, Akaaboune et al. 2003). Interestingly, the postsynaptic phenotypes observed by Grady and others in mice lacking αDB1 were strikingly similar to the ones that I described in myotubes lacking Tks5. In both cases, AChR turnover was compromised, and the AChR distribution within the clusters appeared to be more diffused (**Figures 22 and 23**) (Grady, Akaaboune et al. 2003). Importantly, αDB1 constructs with mutated posphotyrosine sites were ineffective at restoring the KO phenotypes when electroporated into the muscle (Grady, Akaaboune et al. 2003). This means that the three sites have functional significance in αDB1. Pawlikowski and Maimone (Pawlikowski and Maimone 2009), have demonstrated that αDB1 is required for formation of complex pretzel-like AChR clusters *in vitro*, and that the three tyrosine phosphorylation sites of αDB1 are needed for rescuing the KO phenotype. It is possible that a failure to assemble the complex clusters is due to the absence of Tks5, since Tks5 interacts specifically with unphosphorylated αDB1 p730 site, it may be recruited to αDB1 to mediate actin organization under this condition. A large portion of postsynaptic machinery components is transcribed locally within the perisynaptic nuclei (Vale 1987, Simon, Hoppe et al. 1992). Since actin filaments are more readily used for short-distance cargo delivery, the maintenance of postsynaptic specialization is highly dependent on the interaction with this cytoskeletal component. This may be the reason why I observed aberrations in the AChR turnover once Tks5 was knocked down. Since this protein is clearly needed for recruitment of actin to the postsynaptic machinery, the observed phenotypes may be an indication of compromised delivery pipeline of synaptic components.

The molecular mechanism for Tks5-driven podosome formation has been previously described in other cell types, and chances are that similar processes take place in myotubes (Kudlik, Takacs et al. 2020). Tks5 is initially phosphorylated by c-SRC, and gets recruited to the cell membrane by an adaptor protein growth factor receptor-bound protein 2 (GRB2), which has been also shown to interact with αDB1 in the muscle where it is required for AChR organization (Oikawa, Itoh et al. 2008, Boateng and Huttenlocher 2012,

Gingras, Gawor et al. 2016). Cytoplasmic Tks5 is considered to be inactive, while its recruitment to the membrane leads to its activation. In this state, Tks5 binds to proteins that are essential for podosome formation, including N-WASP, which clusters at the site of adhesion and associates with actin related protein complex 2/3 (ARP2/3) (Oikawa, Itoh et al. 2008, Garcia, Jones et al. 2012). Presence of ARP2/3 allows polymerization of the actin filaments that are then used to protrude the podosome. In other words, Tks5 facilitates podosome formation by recruitment of ARP2/3.

5.3. αdystrobrevin-1- centre for organizing cytoskeleton underneath the postsynaptic machinery.

The purpose of my studies was to elucidate the function that αDB1 has in the postsynaptic machinery organization. From the literature, we know that absence of this protein from the postsynaptic apparatus leads to severe morphological disruptions of the entire NMJ, and that the three phospho-tyrosine sites are crucial for mediating its function (Grady, Akaaboune et al. 2003). Through my work, I have demonstrated that, while both liprin-α-1 and Tks5 bind to αDB1 the former is responsible for organization of MTs, while the latter recruits actin. Liprin-α-1 interacts with αDB1 in a phospho-unspecific manner, meaning that the phosphorylation state of αDB1 is irrelevant for the interaction. Tks5, on the other hand, interacts with αDB1 only when the tyrosine 730 is unphosphorylated (**Figure 33**).

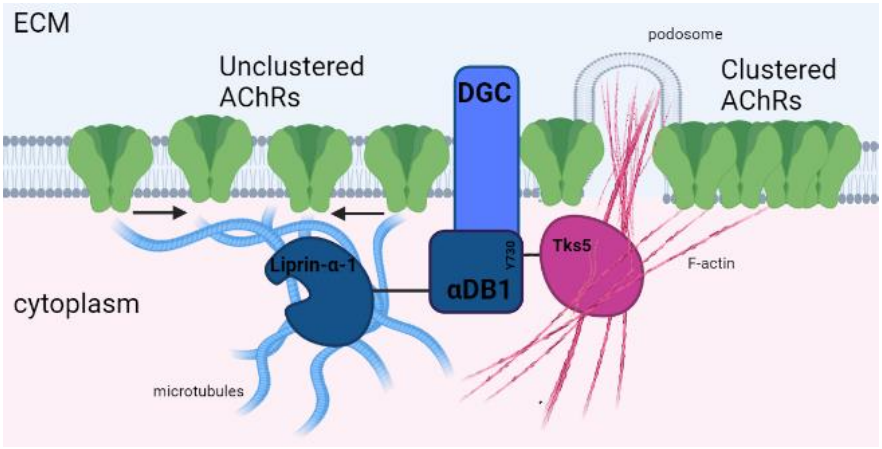


Figure 33: Liprin-α-1 and Tks5 bind to αDB1 and regulate different cytoskeletal filaments. *Despite being part of the same protein complex, liprin-α-1 and Tks5 regulate microtubules (MTs) and actin organization, respectively. The cortical MTs regulated by liprin-α-1 are required for bringing the free-floating AChRs together i.e AChR clustering, while actin filaments recruited by Tks5 are involved in stabilization of clustered AChRs and formation of synaptic podosomes required for postsynaptic machinery maturation. The exact mechanisms by which these two proteins are recruited to αDB1 are yet to be discovered, however it seems that binding of Tks5 to the complex can be affected by the activity of various tyrosine kinases such as Src or Fyn.*

Given the differential mode of binding of these two proteins, as well as their involvement with two different types of cytoskeletal filaments, a question arises what is the master regulator their recruitment to the postsynaptic machinery. It has been shown that αDB1 interacts with a protein kinase Fyn, which binds to the AChRs, while the βDG can additionally recruit Src kinases (Swope and Huganir 1994, Fuhrer and Hall 1996, Gingras, Gawor et al. 2016). It would be curious to see how activation of these kinases affects the functions of liprin-α-1 and Tks5 at the postsynaptic machinery. Since Tks5 is a scaffold protein that only binds to unphosphorylated αDB1, it may itself act as a regulator of the differential αDB1 functions on top of its involvement in actin recruitment. While liprin-α-1 is required from the earliest moments of synaptogenesis, as it regulates the clustering of AChRs, the Tks5 seems to be more important during later developmental stages as it ensures maturation and maintenance of the cluster stability. For this reason it would be interesting to investigate changes in αDB1 phosphorylation states throughout development and how factors involved in such changes affect recruitment of liprin-α-1 and Tks5.

Collectively, αDB1 appears to be a recruitment centre for factors that regulate the dynamics of cytoskeletal filaments beneath the postsynaptic machinery. There is still much to be discovered about the mechanisms through which those regulators are recruited to αDB1, but phosphorylation of its tyrosine residues appears to be of crucial importance.

5.4. Muscle a cells cultured on alternative laminin isoforms produce robust and developmentally mature AChR clusters

The one clear advantage of using laminin-cultured myotubes over other *in vitro* methods for studying of the postsynaptic machinery, is the fact that the AChR clusters in these cultures undergo developmental remodelling, similar to the one observed *in vivo.* I have described a detailed protocol for C2C12 cells as well as primary human myoblasts that is aimed to optimize the culturing conditions and enhance the AChR cluster-producing potential of these cell lines (**Figures 25 and 31**). The most important aspects of the protocol to keep in mind are the low passage number and sufficient cell densities, as both are key factors to obtaining robust clustering of postsynaptic machinery. I also screened eight different laminin isoforms for their ability to stimulate the myotubes into production of AChR clusters and compared the results to laminin 111 as it was the only laminin that researchers have ever used for stimulating AChR clustering. As a result, laminin 221 and 121 turned out to promote assembly of the receptors to the highest extent, while laminins 411 and 421 were the least effective at doing so. Interestingly, the effect of laminins on the AChR cluster number was additive when two isoforms were combined during coating (**Figure 26**). While the size of AChR clusters was the same for all laminins used, the fusion index of cells was directly correlated to the number of AChRs produced, meaning that myotubes cultured on laminins 221 and 121 had also the highest fusion index (**Figure 28**). Thus highlighting the importance of stimulating efficient myoblast fusion for obtaining high yields of AChR clusters. The effect of laminins on the postsynaptic machinery was then evaluated in terms of morphological remodelling. None of the laminin isoforms used was particularly good at inducing developmentally immature, plaque-shaped clusters. However, formation of developmentally mature, C-shaped assemblies was highly

increased when myotubes were fused on laminin 221. In turn, laminin 121 and 211 were extremely efficient at inducing formation of postsynaptic machinery clusters that contained synaptic podosomes, making these two isoforms a prime choice for any studies that investigate remodelling of AChR clusters (**Figure 29**). Curiously, *in vivo* laminin 121 is an extrasynaptic protein, meaning that it is found only in non-synaptic regions of the BL (Patton 2000). The reason why this isoform was so efficient at inducing mature cluster probably comes from the fact that its subunit composition positively affects adhesion of myotubes which is essential for obtaining robust clustering.

I also applied my method to primary human muscle cells obtained from patients biopsies to see whether it can become a clinically relevant model for investigating the mechanisms of neuromuscular disorders. The human cells turned out to be really efficient at producing AChR clusters when cultured on any of the tested laminin isoform, and similarly to C2C12 cells, the number of AChR clusters in these cultures was directly correlated to the fusion index of the myotubes (**Figure 31**). Interestingly, similar number of developmentally immature clusters were formed in each culture, however laminins 421 and 511 promoted formation of significantly more clusters that appeared to be remodelled by synaptic podosomes. It was also interesting to see that human primary myotubes are also able to form clusters of the postsynaptic machinery that undergo podosome-driven developmental remodelling, since these actin-rich structures have not been described at the human postsynaptic machinery before. (**Figure 32**). Such phenomenon indicates that the initial steps of endplate maturation can be similar for rodents and humans. This is especially promising as the data regarding postsynaptic machinery development in humans is still missing, and a reliable *in vitro* model can bring significant insights into the biology of our neuromuscular system.

The laminin- cultured myotubes provide a minimalistic approach for studying of the NMJ, without the context of motor nerves and Schwann cells. While this can be beneficial for those interested in the postsynaptic machinery, it comes with certain limitations. As such both the murine and human myotubes are able to produce postsynaptic machinery clusters that get remodelled by podosomes but these structures have not been yet shown at the NMJs *in vivo.* While some podosomal markers are found in the perforations of the NMJ postsynaptic machinery, their relevance in synaptic biology remains largely unknown.

The major laminin receptor found at the postsynaptic machinery, the DGC, has been shown to play a role in developmental remodelling of the NMJ (Singhal and Martin 2011). However, the role of other important laminin receptors in this process, the integrins has not been discovered (Ross, Webster et al. 2017). Laminin interaction with the surface receptors provides the cells with the stability that they need to establish functional postsynaptic machineries. These extracellular proteins bind to their targets such as the DGC or integrins, through the N'-terminal fragment of α subunits and C'-terminal, respectively (Hohenester, Tisi et al. 1999). The C-terminal binding is mediated by five laminin globular domains (LG1-5) (Colognato and Yurchenco 2000). Different α subunits employ various LG domains to interact with the DGC and thus we observe a difference in their ability to promote AChR clustering and remodelling (Dempsey, Bigotti et al. 2019). Since laminin isoform that contained α4 subunits were ineffective in stimulating C2C12 myotubes into production of AChR clusters, while being the best substrata for human primary myotubes, I hypothesize that formation of these endplates requires different molecular interactions in the two species. Since laminins are also able to form polymers by utilizing their LN domains, a possibility exists that not all isoforms polymerize with equal efficiency (McKee, Aleksandrova et al. 2018). This could lead to differences in the accessibility of receptor-interacting domains, changes to the stiffness of culturing surfaces or even exocytosis of endogenous laminins expressed by the cells. All of the abovementioned factors can lead to altered efficiency of postsynaptic machinery formation and remodelling. For this reason, it would be interesting to measure the relative affinity of each laminin globular domain for the DGC and integrin, which could shed more light into the mechanism by which these interactions promote maturation of AChR clusters.

Since inappropriate maturation of the postsynaptic endplate is often a hallmark of a neuromuscular disorder, and our understanding of this process is still only partial, more new tools are needed to help those affected by such diseases. The benefits of my method include high reproducibility, greatly enhanced AChR cluster formation and ability to promote their specific morphology. These are all great improvements to the previous methods, and may facilitate findings of new molecular regulators of the synapse. This protocol can also be applied to other formats such a multi-well culturing plastics. When combined with the primary human muscle culturing procedure, it can become a tool for screening

of new therapeutic agents and studies that aim to diagnose neuromuscular disorders at an early stage.

6. Summary and conclusions

In my thesis, I aimed to discover new molecular factors involved in formation and maintenance of the NMJ and improve the existing models for studying the postsynaptic machinery. In order to achieve this, I identified and characterized novel interactors of αDB1 and optimized conditions of laminin-based culture systems. The key findings include:

- *Liprin-α-1 is required for assembling the postsynaptic machinery in C2C12 cells, through regulation of cortical microtubules.*
- *Microtubules are not needed for delivery of AChR to the cell surface, but are involved in receptor clustering.*
- *Tks5 interacts with αDB1 and is required for podosome-driven remodelling of postsynaptic machinery and maintenance of its stability.*
- *Actin is recruited to the postsynaptic machinery by Tks5.*
- *Culturing C2C12 myotubes on laminin 221 induces robust AChR clustering.*
- *Human primary myotubes assemble AChR clusters that undergo podosome-driven postsynaptic remodelling.*

In conclusion, liprin-α-1 is a novel postsynaptic machinery regulator that interacts with and may bridge functions of two major complexes the DGC and LL5β-CLASP. Tks5 is one of the first known podosomal regulators that has been shown to have a direct function in maturation of the postsynaptic machinery. Using primary human myotubes cultured on laminin is a promising new model for identifying new molecular regulators of human NMJ as well as screening of therapeutic agents.

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